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SCIENTIFIC OPINION

Scientific Opinion on the risks to public health related to the presence of bisphenol A (BPA) in foodstuffs: PART II - Toxicological assessment and risk characterisation[1]

EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF)[2, 3]

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ASSESSMENT

1. Introduction

Bisphenol A (BPA) is an industrial chemical that is widely used as a monomer or additive for the manufacture of polycarbonate (PC) plastics and epoxy resins and other polymeric materials. It is also used in certain paper products, including thermal paper. The properties conferred by BPA to PC, e.g. rigidity, transparency and resistance, make these plastics particularly suitable for many technical applications, but also to make food and liquid containers, such as tableware (plates and mugs), bottles, microwave ovenware, and reservoirs for water dispensers. BPA-based epoxyphenolic resins are used as protective linings for canned foods and beverages and as a surface-coating on residential drinking water storage tanks. BPA is also used in a number of non food-related applications, including epoxy-resin based paints, poly vinyl chloride (PVC) medical devices, surface coatings, printing inks, carbonless and thermal paper and flame retardants.

BPA was authorised in Europe by the Commission Directive 2002/72/EC[4] of 6 August 2002, to be used as monomer and additive for the manufacture of plastic materials and articles intended to come in contact with foodstuffs together with a specific migration limit of 0.6 mg per kilogram food (SML (T) = 0.6 mg/kg). This Directive was amended by the Commission Directive 2011/8/EU of 28 January 2011[5], placing a temporary ban on the use in the manufacture of polycarbonate infant feeding bottles as from 1 March 2011 and the placing on the market of these feeding bottles as from 1 June 2011. The definition of 'infant' in Directive 2006/141EC[6], namely children under the age of 12 months, applies.

Since May 2011 Directive 2002/72/EC has been replaced by Regulation (EU) No 10/2011[7], which has maintained the ban of BPA in polycarbonate infant feeding bottles and kept the current restriction for BPA as a monomer with a specific migration limit (SML) = 0.6 mg/kg food but removed its authorisation as an additive in plastic food contact materials and articles.

The scientific debate on the risks for public health of BPA focusses on its endocrine-active properties, which might adversely impact physical, neurological and behavioural development. In addition, other perturbations of physiology, both in animals and humans, have been brought in relationship to the endocine-active properties of BPA. Among these are e.g. obesity, modification of insulin-dependent regulation of plasma glucose levels, perturbation of fertility, proliferative changes in the mammary glandpossibly related to the development of breast cancer, immunotoxicity and adverse effects on the cardiovascular system (for an overview see NTP-CERHR, 2007, 2008, EFSA, 2006, 2008, EFSA CEF Panel, 2010, and ANSES, 2011, 2013). Despite the large number of scientific publications and risk assessment reports published on this topic, no scientific consensus has been reached on its risks for human health at the currently estimated levels of exposure, mainly due to qualitative and quantitative divergences in the outcome and interpretation of animal toxicity studies carried out with this compound. Whereas a limited number of large-scale toxicity studies complying with standard/OECD test guidelines have consistently indicated that the oral toxicity of BPA is low, many more small-scale research studies have reported adverse effects of BPA at levels below the current NOAEL of 5 mg/kg bw per day, which was the point of departure for the derivation of the current TDI (for a recent review see Vandenberg et al., 2012). Within the scope of the current evaluation by EFSA the papers addressing these possible associations and effects will be reviewed for inclusion in the hazard identification.

[4] Commission Directive 2002/72/EC of 6 August 2002 relating to plastic materials and articles intended to come into contact with foodstuffs, OJ L 220, 15.8.2002, p.18-58.

[5] Commission Directive 2011/8/EU of 28 January 2011 amending Directive 2002/72/EC as regards the restriction of use of Bisphenol A in plastic infant feeding bottles, OJ L 26, 29.1.2011, p.11-14.

[6] Commission Directive 2006/141/EC of 22 December 2006 on infant formulae and follow-on formulae and amending Directive 1999/21/EC. OJ L 401, 30.12.2006, p.1-33.

[7] Commission Regulation (EU) No 10/2011 of 14 January 2011 on plastic materials and articles intended to come into contact with food. OJ L 12, 15.1.2011, p.1-89.

Assessment of the risks for public health related to the presence of BPA in foodstuffs requires not only identification of its possible health hazards, but also assessment of exposure to BPA from dietary sources and non-dietary sources.

As indicated in the Terms of Reference (see Executive summary), a three-step approach was taken in developing the scientific opinion on BPA. As a first step, the draft exposure part of the opinion was completed and published in July 2013 for public consultation (EFSA CEF Panel, 2013). Secondly, the draft assessment of human health risks which covers hazard asssessment and risk characterisation was endorsed by the CEF Panel in December 2013 and released for public consultation in January 2014. Around 500 comments were received through the two public consultation phases (EFSA CEF Panel, 2014). As a third step, taking into account the public consultation comments, the CEF Panel has then amended the exposure assessment, the hazard assessment and risk characterisation of the opinion and completed its full risk assessment opinion for adoption. The final opinion now consists of 3 separate documents: (i) an executive summary of the whole opinion (including abstract, summary, background, terms of reference, interpretation of the terms of reference, assessment, conclusions and recommendations of the exposure and hazard assessments, and the risk characterisation); (ii) Part I – Exposure assessment and (iii) Part II – Toxicological assessment and risk characterisation.

For the sake of clarity, it should be noted that when the text makes reference to another section (or Appendix) of the opinion, this generally refers to a section included in the same part of the opinion, unless otherwise stated. In this latter case, the specific part of the opinion (i.e. Executive summary, Part I or Part II), to which the mentioned section belongs, is clearly mentioned.

1.1. Previous risk assessments

In the last decade, the available scientific evidence on the risks for public health of BPA has been thoroughly reviewed by a number of risk assessment bodies worldwide (AIST, 2007; 2011; SCF, 2002; EU-RAR 2003; 2008; EFSA, 2006; 2008; EFSA CEF Panel 2010; Health Canada, 2008; NTP-CERHR, 2007, 2008;; US FDA, 2010a, FAO/WHO, 2011; ANSES, 2011, 2013).

European Scientific Committee on Food (SCF)

In 2002, the SCF set a temporary Tolerable Daily intake (TDI) for BPA, of 0.01 mg BPA/kg body weight (bw)/day, by applying an uncertainty factor (UF) of 500 (100 for inter- and intra-species differences, and 5 for uncertainties in the database) to the NOAEL of 5 mg/kg bw per day identified in a comprehensive three-generation study in the rat by Tyl et al. (2002).

European Food Safety Authority (EFSA)

In 2006, the former EFSA Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food (AFC) published a full risk assessment of dietary BPA, encompassing both the setting of a TDI and the estimation of dietary exposure to BPA for various groups of the populations. A full TDI for BPA was set at 50 µg/kg bw per day, by applying a default UF of 100 to the overall NOAEL of 5 mg/kg bw per day from the two multi-generation reproductive toxicity studies in rodents by Tyl, where the critical effects were changes in body and organ weights in adult and offspring rats and liver effects in adult mice, respectively (Tyl et al., 2002, 2008; the latter is the same study as Tyl et al., 2006, cited in EFSA, 2006). For infants, dietary exposure to BPA was estimated to range from 0.2 µg/kg bw per day in 3-month-old breastfed babies to 13 µg/kg bw per day in 6-12-month-old infants, for the worst case scenario (high BPA migration into foodstuffs and high food consumption, taking into account breast feeding, feeding formula using PC bottles as well as consumption of commercial foods and beverages). For young children and adults, worst case exposure estimates to BPA via the diet were 5.3 and 1.5 µg/kg bw per day, respectively, based on high migration levels of BPA from cans as well as on migration data from PC tableware or storage containers, and on high food and drink consumption. The AFC Panel concluded that exposure to BPA through food and drinks was well below the TDI, even for infants and children (EFSA, 2006).

The same TDI value of 50 µg BPA/kg bw per day was reaffirmed by the EFSA AFC Panel in its subsequent scientific opinion (EFSA, 2008).

In 2010, the EFSA CEF Panel carried out a comprehensive evaluation of all the recent toxicological data on BPA and re-confirmed the TDI of 50 µg/kg bw per day (EFSA CEF Panel, 2010). However, the CEF Panel expressed some uncertainties concerning a few BPA-related effects of possible toxicological relevance, such as biochemical changes in brain, immune-modulatory effects and enhanced susceptibility to breast tumours, emerging from recent low-dose studies on developing animals. These studies had several shortcomings and the relevance of these findings for human health could not be assessed. Based on such uncertainties a CEF Panel member expressed a minority opinion, claiming that the current full TDI should become a temporary TDI.

In 2011, the EFSA CEF Panel issued a statement on the report on BPA health effects published by the French ANSES, relating to possible divergences between the conclusions of EFSA in 2010 and those of ANSES in 2011 (ANSES, 2011). In 2011, the ANSES expert group concluded that based on the available scientific literature and by all exposure routes BPA has "proven" effects in animals on female and male reproduction, mammary gland, metabolism and brain, and also has "suspected" effects in humans (reproduction, diabetes and cardiovascular diseases). The CEF Panel overall considered that the information in the ANSES report did not change the views that the Panel expressed in 2010. The CEF Panel however expressed the need to review more in depth some new studies not yet available in 2010, including new data from ongoing low dose studies at NCTR/FDA and at NTP/NIEHS[8] which are currently exploring many of the uncertainties around BPA.

European Chemical Bureau of the European Union

In 2003, the European Chemical Bureau of the European Union published a comprehensive Risk Assessment Report (EU-RAR) for BPA in the context of Council Regulation (EEC) No. 793/93 on the evaluation and control of existing substances. The key health effects of BPA through different exposure routes were considered to be eye and respiratory tract irritation, skin sensitisation, repeated dose toxicity to the respiratory tract, effects on the liver and reproductive toxicity (effects on fertility and on development). Some of these effects are worker-specific (e.g. eye and respiratory irritation, repeated dose toxicity to the respiratory tract) and are not expected to occur in the general population, which is predominantly exposed via food or through environmental sources. With respect to human health risks, a need for further research was identified, to resolve the uncertainties surrounding the potential for BPA to produce adverse effects on neurological and neurobehavioural development at low doses (EU-RAR, 2003).

In 2008, the EU-RAR (EU-RAR, 2008) was updated after evaluation of the two generation reproductive study in mice by Tyl et al. (2008) along with the new data on human exposure and effects of BPA that had become available since 2003. The Rapporteur came to the conclusion that there was no need for further information and/or testing and for risk reduction measures beyond those which were already being applied. However, Denmark, Sweden and Norway considered that the results of four neurodevelopmental studies (Adriani et al., 2003; Carr et al., 2003; Negishi et al., 2004; Ryan and Vandenbergh, 2006) warranted further consideration and expressed a minority view concerning this toxicological endpoint (EU-RAR, 2008).

Japanese Institute of Advanced Industrial Science and Technology (AIST)

In 2005, the Japanese AIST concluded that BPA was unlikely to pose unacceptable risks to human health at current exposure levels. Margins of exposure (MOEs) were calculated as 85,000-1,800,000 based on realistic exposure scenarios, and as >1,000 for adults and children based on worst-case scenarios. For these calculations, the NOAEL or the Benchmark Dose Lower Limit (BMDL) for three

[8] See at: http://ntp.niehs.nih.gov/testing/status/agents/ts-10034-y.html

critical endpoints, namely lower body weight gain, liver and reproductive effects, were in the 5 to 50 mg/kg bw per day range.

AIST updated the Hazard Assessment of BPA in 2011 (AIST, 2011). The oral NOAEL for BPA general toxicity was considered to be 3 mg/kg bw per day based on centrilobular hepatocyte hypertrophy in the two generation study in mice by Tyl et al. (2008). A total uncertainty factor of 25 was applied, consisting of 2.5 for inter-species differences (1 for toxicokinetics, and 2.5 for toxicodynamics), and of 10 for intra-species differences. According to the BPA exposure estimate in Japanese individuals, exposure was highest in 1 to 6 years old children with an estimated 95th percentile (in µg/kg bw per day) of 3.9 (males) - 4.1 (females). In adults, the 95th percentile of BPA intake (estimated from the amount of BPA excreted in 24-hour urine samples) was 0.037-0.064 µg/kg bw per day in men and 0.043-0.075 µg/kg bw per day in women. The relative margins of exposure (MoEs, i.e. ratio between the NOAEL and 95th percentile exposure data) were 730-770 for 1-6 yr old children and 40,000-81,000 for adults. These values were much larger than both the MoE (25) that was considered might possibly result in health effects in humans and the standard (conservative) MoE of 100, and thus the AIST concluded that the risk of BPA with regard to human health was very small.

Health Canada

In its 2008 risk assessment, the Health Canada's Food Directorate did not revise the provisional TDI for BPA of 0.025 mg/kg bw per day set from the lowest NOEL of 25 mg/kg bw per day for general toxicity in a rat 90-day study (NTP, 1982), and concluded that the current dietary exposure to BPA through food packaging uses was not expected to pose a health risk to the general population, including newborns and young children (Health Canada, 2008). Health Canada then estimated the probable daily exposure to BPA to vary from as low as 0.21 µg/kg bw for infants 8-12 months of age to as high as 1.35 µg/kg bw for 0-1 month old infants with the maximum formula intake and the maximum concentration of BPA migrating from epoxy lined infant formula cans.

In 2012, a refined (probabilistic) exposure assessment of Canadians was conducted based on the collective results of a number of recent Canadian surveys, including results from a Total Diet Study (Health Canada, 2012). A mean exposure to BPA of 0.055 µg/kg bw per day was calculated for the general population, which is approximately 3 times lower than the exposure calculated in the risk assessment of 2008. This updated dietary exposure figure generally aligns with exposure estimates that are based on the results of population-based biomonitoring studies. Infants, as an age group, were exposed to the greatest amount of BPA. The probable daily exposure to BPA varied from 0.083 µg/kg bw (0-1 month of age) to 0.164 µg/kg bw (4-7 months old infants). Collectively, also the BPA exposure estimates for infants were, on average, approximately 3-fold lower than those of 2008. Health Canada recommended the application of the general principle of ALARA (as low as reasonably achievable) to limit BPA exposure of newborns and infants, due to uncertainties for low-dose neurodevelopmental and behavioural effects in rodents.

US National Toxicology Program (NTP)

In 2008, the US National Toxicology Program (NTP) released its final report on BPA's potential to cause harm to human reproduction or development (NTP-CERHR, 2008). *Some concern* ("some" is the midpoint on a five-level scale, ranging from "negligible" to "serious") was expressed for effects on development of the prostate gland and brain, and on behaviour in infants and children after pre- and postanatal exposure to BPA at current human exposure levels. The NTP had *minimal concern* for effects of BPA on the mammary gland development and acceleration of puberty in females at current human exposure levels. NTP expressed *negligible concern* that exposure of pregnant women to BPA would result in fetal or neonatal mortality, birth defects, or reduced birth weight and growth in their offspring. NTP also expressed *negligible concern* that exposure to BPA would cause reproductive effects in non-occupationally exposed adults and minimal concern for workers exposed to higher levels in occupational settings.

In the same report, the NTP also provided daily exposure estimates for infants, children and adults based on realistic scenarios. For the general population, the highest estimated daily exposure to BPA was reported to occur for infants and children. Formula-fed infants (0 to 6 months of age) had estimated intakes of 1-11 µg/kg bw per day, 6-12 month-old infants of 1.65-13 µg/kg bw per day, and older children (up to 6 years) of 0.04-14.7 µg/kg bw per day. For the general adult population BPA intake was estimated as 0.008-1.5 µg/kg bw per day.

US Food and Drug Administration (US FDA)

In 2008, the US FDA released a document entitled *Draft Assessment of Bisphenol A for Use in Food Contact Applications*, (US FDA, 2008), which was peer-reviewed during the same year (see report by US FDA Science Board Subcommittee on Bisphenol A, 2008).

Since then, the Center for Food Safety and Applied Nutrition (CFSAN) within FDA has reviewed additional studies of low dose toxicity (US FDA, 2010a).

As of 2013 the US FDA reiterated that at this interim stage it shares the perspective of the National Toxicology Program (NTP-CERHR, 2008) that "recent studies provide reason for some concern about the potential effects of BPA on the brain, behaviour, and prostate gland of fetuses, infants and children." (US FDA, 2013). FDA has also recognized substantial uncertainties with respect to the overall interpretation of these studies and their potential implications for human health effects of BPA exposure and, in cooperation with the NTP, FDA's National Center for Toxicological Research (NCTR), is carrying out in-depth studies to answer key questions and clarify uncertainties about the risks of BPA (US FDA, 2013).

Recent evaluation by the FDA's CFSAN has determined that exposure to dietary BPA for infants, the population of most potential concern, is less than previously estimated (US FDA, 2013). The initial FDA exposure estimates were 0.185 µg/kg bw per day for adults and 2.42 µg/kg bw per day for infants (US FDA, 2008). The new estimate of average dietary exposure, based on increased data collection, is 0.2-0.4 µg/kg bw per day for infants and 0.1-0.2 µg/kg bw per day for children and adults (US FDA, 2010b).

Belgian Superior Health Council

In November 2010 the Belgian Superior Health Council issued a risk assessment that provided the scientific ground for adopting a law banning BPA in materials in contact with food for children aged 0-3 years in 2012. The concern was based on the uncertainties around possible adverse effects of BPA at low doses on brain, immune system, development, and mammary cancer promotion in offspring exposed during pregnancy or lactation. These uncertainties had also been identified by other national or international bodies. This urgent advice mainly consisted of a summary of previous evaluations of BPA made by the French AFSSA, the German BfR, EFSA (EFSA CEF Panel, 2010), the Japanese AIST, Health Canada, the US NTP and FAO/WHO. In this context the evaluation of original data was very limited. The report's recommendations to take risk management measures to protect young children was in line with the application of the precautionary principle.

Food Standard Australia New Zealand (FSANZ)

In 2010 the FSANZ[9] stated that, after thoroughly considering the toxicological database for BPA, it concurred with the hazard assessment previously performed by EFSA, US FDA and Health Canada and the established TDI of 50 µg/kg bw per day. FSANZ undertook a survey of BPA in food and drinks in the Australian market to determine exposure to BPA from packaging materials and came to

[9] http://www.foodstandards.gov.au/science/monitoring/surveillance/documents/BPA%20paper%20October%202010%20FINAL.pdf

the conclusion that Australians of all ages are exposed to extremely low levels (in the range of ng/kg food to µg/kg food) via such packaged foodstuffs.

World Health Organization (WHO)

In 2010 the FAO and WHO jointly held an Expert Meeting on BPA, whose final report was published in 2011. The report identified the sub-population with the highest dietary exposure to BPA as that of infants of 0-6 months being fed liquid formula out of PC bottles: this accounted for 2.4 µg/kg bw per day (mean) and 4.5 µg BPA/kg bw per day (95th percentile). Exposure (in µg BPA/kg bw /day) was estimated not to exceed 0.7 (mean) and 1.9 (max) for children >3 years, and 1.4 (mean) and 4.2 (max) for adults. Based on limited data, for most subgroups BPA exposure from non-food sources was at least one order of magnitude lower than that from food.

As for hazard characterisation, points of departure were considered to be much higher than human exposure for many end-points and thus did not raise health concern. Studies on developmental and reproductive toxicity in which conventional end-points were evaluated showed effects only at high doses, if at all. However, in a few studies some emerging new end-points (sex-specific neurodevelopment, anxiety-like effects, preneoplastic changes in mammary glands and prostate in rats, impaired sperm parameters) showed associations at lower levels, i.e. close to the estimated human exposure, so there would be potential for concern if their toxicological significance were to be confirmed. WHO stated that "*while it would be premature to conclude that these evaluations provide a realistic estimate of the human health risk, given the uncertainties, these findings should drive the direction of future research with the objective of reducing this uncertainty*".

French Agency for Food, Environmental and Occupational Health & Safety (ANSES)

In September 2011, ANSES published a report on BPA, including one part concerning its effects on human health and the other one on its uses (ANSES, 2011). In the hazard identification report "Effets sanitaires du bisphénol A" ANSES classified the effects of BPA on humans and animals as proven, suspected, controversial, or inconclusive (ANSES, 2011). Furthermore, it reached the conclusions that BPA exposure was associated with proven effects in animals and suspected effects in humans, also at levels of exposure below the current regulatory thresholds. These effects were the main focus of the subsequent risk assessment that was completed by ANSES in April 2013. Specifically, the characterisation of human health risks was conducted for the category "pregnant women" (i.e. offspring) considering four toxicological endpoints for which the external threshold doses identified in oral studies in developing animals are reported in brackets: neurobehavioural development (NOAEL: 50 µg/kg bw per day from Xu et al., 2010), female reproductive system (NOAEL: 100 µg/kg bw per day from Rubin et al., 2001), metabolism and obesity (LOAEL: 260 µg/kg bw per day from Miyawaki et al., 2007) and mammary gland proliferation - changes in the structure that make it more susceptible to carcinogens (NOAEL: 25 µg/kg bw per day from Moral et al., 2008). ANSES used a bioavailability factor of 3% to convert the external NOAEL/LOAEL from the experimental data into equivalent internal doses (internal NOAEL/LOAEL), taking into consideration the impact of first-pass metabolism on orally ingested BPA and assuming that only the unconjugated fraction of BPA was responsible for the effects observed. To obtain an internal Derived No Effect Level (DNEL) for each critical endpoint, ANSES applied an overall assessment factor of 300 to the internal NOAELs (or 900 if the starting critical dose was a LOAEL), consisting of a factor 100 to account for inter- and intra-species kinetic and dynamic differences, and an extra factor of 3 to account for uncertainties regarding possible low-dose effects of BPA and non-monotonic dose-response relationships (NMDRs). The resulting internal DNELs that were used in risk characterisation were 0.01, 0.02, 0.0173 and 0.005 µg/kg bw per day for neural-, female reproductive-, metabolic- and mammary gland effects, repectively. The sources of BPA considered for BPA (probabilistic) exposure assessment were air, sedimented dust, food and beverages. Dietary (external) exposure (99^{th} percentile) was estimated to be for children (3-17 yrs) 0.31 µg/kg bw per day, for adolescents (11-17 yrs): 0.12 µg/kg bw per day, and for pregnant women: 0.24 µg/kg bw per day.

The conclusions of the 2013 ANSES risk assessment report that there are risk situations for the fetus, associated with maternal exposure to BPA during pregnancy. In detail, a moderate risk for children of both sexes relates to the mammary gland with particular reference to an increased number of undifferentiated epithelial structures associated with an enhanced susceptibility of the mammary gland to tumour transformation. According to the aggregate exposure estimates (expressed in internal dose as µg/kg bw per day and summed up from all sources and routes), dietary exposure is the main contributor over other sources and routes. Concerning particular exposure scenarios during pregnancy, specific risk situations apply to pregnant women handling thermal paper and consuming water from refillable polycarbonate containers. The scenario for pregnant women handling thermal paper implied, in addition to the effect on the mammary gland, other health risks for the unborn child regarding brain and behaviour, metabolism, obesity and/or the female reproductive system. ANSES did not estimate the risks for other populations, e.g. infants, children and adolescents, due to insufficient data availability (ANSES, 2013).

1.2. Consideration of low-dose effects and non-monotonic dose-response curves in the risk assessment of BPA

In reviewing the toxicological profile of BPA and other endocrine active substances a particularly controversial area has been the reported that not only are there effects at low doses (defined either as doses in the range of human exposure, or doses below the NOAEL of 5 mg/kg bw per day (used in establishing the current TDI of 50 µg BPA/kg bw per day) but there are also non-monotonic dose-response curves (NMDRs) (Teeguarden and Hanson-Drury, 2013). The term "low-dose effects" is not synonymous with or equivalent to NMDR, but there is particular concern about the possibility that non-monotonicity would occur at lower doses than those used in regulatory toxicity studies. The NMDR can be characterised by a change in slope direction along the dose interval studied, contrary to conventional monotonic dose-response, which shows a consistent increase in (adverse) effects along the dose range (Vandenberg et al., 2012). In the papers from Vandenberg and some other authors it is suggested that endocrine mechanisms underlying NMDRs are most important during the development of an organism which is considered as a sensitive "window of exposure". Therefore, it has been recommended that testing of endocrine-active substances should not only span a wide dose range but also encompass the developmental period along with a follow-up later in life to assess potentially latent effects (WHO/UNEP, 2012). The view held by some researchers that low-dose effects and/or NMDRs cannot be sufficiently captured in internationally agreed protocols for toxicological testing challenges the usual practice of setting health-based guidance values. This could particularly apply to endocrine-active substances for which complex sex-, tissue- and species-specific mechanisms of action could be assumed. These mechanisms may interfere with multiple signalling pathways which may influence toxicological outcomes (Watson et al, 2012; Zoeller et al., 2012; Vandenberg et al., 2013b). The biological activity of endocrine-active substances has been extensively reviewed in the scientific literature, most recently by EFSA (EFSA Scientific Committee, 2013), the United Nations Environment Programme (WHO/UNEP, 2012) and the EC Joint Research Centre/Institute for Health and Consumer Protection (JRC, 2013). More specifically, the possibility that endocrine-active substances may display low-dose effects and NMDR has been the subject of several specific reviews (Zoeller et al., 2012; Vandenberg et al., 2012; draft report of US EPA, 2013) and has been debated at a number of dedicated conferences (EFSA, 2012; JRC/NIEHS, 2013). BPA has frequently been cited as an example of a chemical showing such effects, and Vandenberg et al. have recently published extensive reviews of the low-dose effects of BPA, based on in vitro, laboratory animal and epidemiological studies (Vandenberg et al., 2012, 2013a).

Of the recent BPA risk assessments conducted by other authorities, described in section 1.1, non-monotonic (or non-linear) dose-response relationships were specifically discussed in the EU-RAR (2008), NTP-CERHR (2008), US FDA (2010a), FAO/WHO (2011) and ANSES (2013).

The discussion in the EU-RAR (2008) relates to a discrepancy between the results of different studies with *Xenopus laevis* tadpoles, and is therefore not directly relevant to the human health risk assessment. The report notes that there were some drawbacks to the methodology used in the study

suggesting a NMDR, and the apparent effect could be an artefact. Nevertheless the results could not be rejected as invalid and the study was considered as 'valid with restriction'.

NTP-CERHR (2008) noted that at low doses effects may be difficult to statistically distinguish from background variability. Studies of BPA at low doses could be affected by factors that include phytoestrogen content of the animal feed, extent of BPA exposure from caging or water bottles, unintentional contamination from adjacently housed animal receiving higher doses of BPA and the sensitivity of the animal model to estrogens. In contrast, high-dose studies are less susceptible to these types of influences because the toxicologic response should be more robust and less variable. While the NTP-CERHR Panel did not necessarily expect a specific effect to display a monotonic dose-response (e.g. consistently increasing organ size), many members of the panel expected the high-dose studies with BPA to detect some manifestation of toxicity (e.g. altered weight, histopathology) in tissues reported to be affected at low doses even if the study could not replicate the reported low dose effect. Therefore more weight was given to studies that evaluated both low-and high-doses of bisphenol A rather than to low-dose-only studies in cases where the target tissues were comparably assessed. Specific examples of NMDRs were not identified (NTP-CERHR, 2008).

US FDA (2010a) noted that in evaluation of non-linear dose-responses, additional lower dose levels should be included in a protocol and high doses or a large dose range must also be employed to accurately describe the dose-response of the desired endpoint. However they did not specifically comment on whether BPA exhibits NMDRs. FAO/WHO (2011) commented that "depending on the system studied and the dose, BPA may exert pleiotropic cellular and tissue-type specific effects and can exhibit non-monotonic dose–response relationships at cellular and intracellular levels".

ANSES (2013) provided a more lengthy discussion of non-monotonic relationships. It notes that 'before affirming the existence of a non-monotonic dose-response relationship, the statistical and biological plausibility of this non-monotonic relationship must be evaluated' and proposed a decision tree for this purpose. However, overall ANSES concluded that 'Taking into account non-monotonic dose-effect relationships in the quantitative assessment of risks associated with BPA "was not possible due to methodological difficulties". Therefore 'the "standard" approach based on the choice of a starting point associated with a critical effect (NOAEL/LOAEL/BMD)' was used in the risk assessment (ANSES, 2013).

The EFSA Scientific Committee (2013) has also considered the debate over NMDRs, noting that in the assessment of reproducibility of findings that indicate NMDRs it is important to rule out spurious results, and that the arguments of Vandenberg et al. (2012) have been questioned by other scientists.

The CEF Panel noted the conclusions of EFSA Scientific Committee (2013) regarding "the lack of consensus in the scientific community as to the existence and/or relevance of low-dose effects and NMDRs in (eco)toxicology in relation to endocrine disruption, or other endpoints/modes of actions". The responses to the consultation on this CEF opinion further demonstrate this lack of consensus. EFSA Scientific Committee (2013) recommended a follow up activity to clarify NMDRs in the broader context and in further detail. As a result, EFSA has commissioned a project to critically review the scientific peer-reviewed literature published from 2002 onward concerning the evidence for NMDR hypothesis for substances (other than essential nutrients) in the area of food safety. The project was awarded to a consortium of four Organisations (ANSES (leader), AGES, RIKILT and Karolinska Institute) and is expected to end in December 2015.

Whilst it is not in the scope of this risk assessment of BPA to support or to rebut the existence of NMDRs for endocrine active substances in general the CEF Panel in its review of the recent BPA literature and re-evaluation of earlier papers considered a number of papers describing low-dose effects and NMDRs associated with BPA exposure. Considering the limitations of in vitro studies (e.g. cytotoxicity, accumulation of toxic metabolites under usual culture conditions) the CEF Panel decided to focus its evaluation on in vivo studies with a reported NMDR for an adverse BPA-induced effect. For a consistent evaluation of these studies the CEF Panel took into account the considerations of the

EFSA Scientific Committee (2013b) and the outcome of discussions in the 2012 EFSA Scientific Colloquium (EFSA, 2012) on low dose-responses in toxicology and developed the following criteria:

(i) at least two adjacent doses departing from monotonicity or support for the NMDR from a similar study (same species, similar treatments, similar sampling time) on the same effect (this criteria is relevant to reduce the chance for an incidental finding).

(ii) a plausible underlying mode of action/overarching concept;

(iii) the reliability of the study and the relevance of the effect for human health should be considered as medium or high (as expressed in Appendix B and C); the reliability of the study results should also include an appropriate statistical treatment of the reported data

For each BPA-induced toxicological endpoint the studies which reported a NMDR were summarised in Tables 1 and the evidence for a NMDR was assessed according to the above mentioned criteria. In addition for some endpoints a graphical approach was used to get an overview of the possible shapes of the dose-responses (section 4.3.2).

The CEF Panel noted that in two reproductive multi-generations studies covering a broad range of BPA doses including very low doses (i.e. 1 and 3 µg BPA/kg bw per day in the Tyl et al. studies from 2002 and 2008, respectively) and a subchronic study including a prenatal BPA treatment (2.5; 8; 25; 80; 260; 860 and 2 700 µg/kg bw per day) (US FDA/NCTR, 2013/Delclos et al., 2014) only monotonic dose-responses were observed. In contrast, in two reproductive/developmental studies NMDRs were reported for sexual behaviour (De Catanzaro et al., 2013) and reduced fertility (fewer cumulative pups in a forced breeding study; Cabaton et al., 2011). However, the latter three studies were considered too limited to provide sufficient support for the existence of an NMDR event in the respective parameters studied. The CEF Panel noted that none of these studies had two adjacent doses in the low dose range showing a significant effect. Therefore, the CEF Panel concluded that the evidence is not sufficient to support a NMDR in these observations (see Table 1).

Three studies on neurobehavioural effects report non-monotonicity in the data sets (Jones et al., 2011; Jones et al., 2012; Kundakovic et al., 2013).

Jones et al. (2011) investigated sexual behaviour in adult rats perinatally exposed to BPA and reported a non-linear dose–response relationship in males (impaired composite sexual behavior score at 50 µg/kg bw per day). The CEF Panel noted that the low number of dams per group (3 dams/group) implied that littermates were tested. Furthermore that sexual behaviour is in particular sensitive to other study factors than dose; that the endpoints measured have interdependence; that the z-score approach used by the authors implies that statistically significant differences due to other study factors than dose are amplified. The CEF Panel also noted that none of the endpoints looked at had two adjacent doses in the low dose range that showed an significant effect.

Jones et al. (2012) reported non-monotonic dose–response in rats perinatally exposed to BPA as the normal sex differences seen in control rats were eliminated in low dose BPA-exposed adults (5 µg/kg bw per day) but not at the higher doses in an Elevated plus maze. No significant differences across doses of BPA were seen in a Forced Swim test in both males and females, whereas paired comparisons indicated that the sex differences seen in controls appeared to varying degree in the different dose groups. In each of these two tests several endpoints were measured. In addition to the use of few dams per group (2-3), use of littermates for testing, and reporting NMDR based on t-tests and ANOVA results, the CEF Panel noted that none of the endpoints looked at had two adjacent doses in the low dose range which showed an effect.

In the study by Kundakovic et al. (2013) mostly non-monotonic dose-responses were reported on the expression of ERα, ERβ and oestrogen receptor-related receptor gamma and on DNA methyltransferases (DNMT1 and DNMT3A) in different brain regions of mice prenatally exposed to

2, 20 and 200 µg BPA µg/kg bw per day. The CEF Panel noted that in contrast to these sex- and tissue-specific biochemical findings of BPA exposure, the large scatter within the dose-groups for the behavioural endpoints means that while a large number of different dose-response curves can be fitted to the data, it is not possible to conclude on the biological relevance of any of them.

The CEF Panel concluded that the evidence in Jones et al. (2011, 2012) or Kundakovic et al. (2013) is not sufficient to support a NMDR in their observations (see Table 1).

For metabolic effects six studies reported NMDR (Anderson et al., 2013; Angle et al., 2013; Bodin et al., 2013, Marmugi et al., 2012 Wei et al., 2011; Miyawaki et al., 2007), however, according to the above criteria, the evidence provided was not sufficient to confirm NMDR for this endpoint (see Table 1). Two of them are more frequently cited. Wei et al. (2011) reported increased body weights and serum insulin levels in rat offspring after prenatal exposure (oral gavage) only to one dose at 50 µg BPA/kg bw per day but not at higher concentrations (250 and 1250 µg/kg bw per day). Marmugi et al. (2012) reported increased plasma insulin and triglycerides after 28 day- oral treatment of mice with low BPA doses (5-500 µg/kg bw per day) but not at 5000 µg/kg bw per day. No changes in the plasma concentrations of glucose or cholesterol were observed after treatment with various BPA doses. In addition the authors also report on an accumulation of cholesterol esters and of triglycerides in the liver along with induction of hepatic enzymes and transcription factors involved in lipid synthesis with NMDRs. The CEF Panel noted that in contrast to these observations no increases in insulin and triglycerides and no adverse effects on the liver were observed in the corresponding low dose range in the US FDA/NCTR study (2013) and Delclos et al. (2014) or in the Tyl studies (2002, 2008). The CEF Panel concluded that the evidence is not sufficient to support a NMDR in these observations.

In seven studies on proliferative changes in mammary gland, the authors were of the opinion that the results indicated BPA-induced, toxicologically relevant, effects and BPA-induced changes in gene expression with NMDR (Acevedo et al., 2013; Ayyanan et al., 2011; Jenkins et al., 2011; Markey et al., 2001; Murray et al., 2007; US FDA/NCTR, 2013 and Delclos et al., 2014; Vandenberg et al., 2013c). In evaluating the study results in a WoE approach the CEF Panel acknowledged the overall evidence for BPA-induced proliferation as a likely effect. However, the statistically significant proliferative effects are observed in each study for only one BPA dose which makes it difficult to rule out that the results could be due to chance. The CEF Panel concluded that the evidence is not sufficient to support a NMDR in these observations.

In a tumour-prone transgenic mouse strain, a NMDR was reported by Jenkins et al. (2011) for decreased tumour latency and increased tumour multiplicity. Conversely an increase in cell proliferation and apoptosis indexes of mammary gland epithelial cells displayed dose-dependent (monotonic) trends, while the proliferation:apoptosis ratio showed a NMDR with one statistically increased value only. In contrast to the 2011 study, the 2009 Jenkins study in the DMBA mammary tumour rat model did not show a non-monotonic dose-response for any of the parameters tested. The 2009 and 2011 Jenkins studies differed not only in the animal model tested but but also in the period of exposure to BPA (lactational versus during adulthood), and the inconsistency in the results make it difficult to draw any firm conclusions from these studies. The CEF Panel concluded that the evidence is not sufficient to support a NMDR in these observations.

In summary, none of the studies fulfill the criteria for a NMDR established by the CEF Panel. Overall the CEF Panel concluded that the available data do not provide evidence that BPA exhibits a NMDR for the endpoints considered (reproductive and developmental toxicity, neurotoxicity/behavioural effects, metabolic effects, proliferative changes in mammary gland).

Table 1: In vivo-studies on BPA claiming a non-monotonic dose-response relationship for at least one endpoint relevant to reproductive toxicity/development, neurotoxicity/behavioural effects, metabolic effects, or proliferative changes in the mammary gland.

Reprotoxicity/Development					
Author, Year, Title	**Species**	**Dose range/ number of doses (**µg/ kg bw/ d**)**	**Dose(s) with effect according to study report (**µg/ kg bw/ d**)**	**Endpoint**	**Evidence for NMDR according to the criteria of WG**
Cabaton NJ, Wadia PR, Rubin BS, Zalko D, Schaeberle CM, Askenase MH, Gadbois JL, Tharp AP, Whitt GS, Sonnenschein C, Soto AM, 2011. Perinatal exposure to environmentally relevant levels of bisphenol A decreases fertility and fecundity in CD-1 mice.	Female mice	0.025 µg/kg bw per day 0.25 µg/kg bw per day 25 µg/kg bw per day GD8-PND16	0.025 µg/kg bw per day 25 µg/kg bw per day	Cumulative number of pups from forced breeding	This study does not have two adjacent doses in the low dose range showing a significant effect
De Catanzaro D, Berger RG, Guzzo AC, Thorpe JB and Khan A, 2013 Perturbation of male sexual behaviour in mice (*Mus musculus*) with a discrete range of bipshenol-A doses in the context of high- or low- phytoestrogen diet.	Male mice	17.5 µg/day 175 µg/day 1750 µg/day GD10 - PND9	17.5 µg/day 175 µg/day	Intromission at day 85-105: numbers reduced	This study does not have two adjacent doses in the low dose range showing a significant effect for animal given soy-free diet[10].
			17.5 µg/day	Ejaculation at day 85-105: numbers reduced	

[10] Significant effects on two adjacent doses (for a single variable only) were observed in animals fed with high soy-diet with BPA, which was not taken into account because they were confounded by the diet.

Neurotoxicity/Behavioural effects					
Author, Year, Title	**Species**	**Dose**	**Dose(s) with effect**	**Endpoint**	**Evidence for NMDR according to the criteria of WG**
Jones BA, Shimell JJ, Watson NV, 2011. Pre- and postnatal bisphenol A treatment results in persistent deficits in the sexual behavior of male rats, but not female rats, in adulthood	Rats	5 µg/kg bw per day 50 µg/kg bw per day 500 µg/kg bw per day 5000 µg/kg bw per day GD 7 to PND 14	50 µg/kg bw per day (male only)	Consummatory sexual behaviour	This study does not have two adjacent doses in the low dose range showing a significant effect.
Jones BA and Watson NV, 2012 Perinatal BPA exposure demasculinizes males in measures of affect but has no effect on water maze learning in adulthood	Rats	5 µg/kg bw per day 50 µg/kg bw per day 500 µg/kg bw per day 5000 µg/kg bw per day GD 7 to PND 14	5 µg/kg bw per day	Anxiety-like behaviour in the **Elevated plus maze**: -total distance moved, time mobile, per cent open/closed arm entries, nos. of hub entries **Forced swim maze**: -rotations, latency to first immobile episode	This study does not have two adjacent doses in the low dose range showing a significant effect.
Kundakovic M, Gudsnuk K, Franks B, Madrid J, Miller RL, Perera FP, Champagne FA, 2013. Sex-specific epigenetic disruption and behavioral changes following low-dose in utero bisphenol A exposure	Mice	2 µg/kg/d 20 µg/kg/d 200 µg/kg/d GD 1 to GD 19 (delivery)	2 µg/kg/d 200 µg/kg/d	**Home Cage Social Behaviour** -Sniffing and allogroming (male and female **Social approach** -Aggression (decreased at low dose, increased at high dose, male and females)	A number of different dose-response curves can be fitted to the data due to large variations in the outcome of the measurements of behavioural endpoints. It is not possible to conclude on the biological relevance of any of them The data do not provide sufficient evidence for NMDR.

Metabolic effects					
Author, Year, Title	**Species**	**Dose range/number of doses**	**Dose(s) with effect**	**Endpoint**	**Evidence for NMDR according to the criteria of WG**
Anderson OS, Peterson KE, Sanchez BN, Zhang Z, Mancuso P and Dolinoy DC, 2013 Perinatal bisphenol A exposure promotes hyperactivity, lean body composition, and hormonal responses across the murine life course.	Mice 14 days before mating and throughout pregnancy	50 ng BPA/kg diet 50 µg BPA/kg diet 50 mg BPA/kg diet	1. All doses had effects at 3 and 6 months. But: at 9 months only the lowest dose had an effect in females (higher energy expenditure than control) but not in males 2. CO2 production Females had only an effect at 9 months in the highest dose group. Males had an effect at 3 months of age in the middle and the highest dose group. 3. horizontal activity Increase in females in the dose group n50 ng at 3 and 9 months; females exposed to 50 µg or 50 mg had an effect only at 9 months In Males: No effect of the lowest dose; effects of middle and higest dose at 3 months 4. vertical activity	1. energy expenditure (3 , 6 and 9 months) 2. CO2 production (3,6, and 9 months) 3. horizontal activity	Lack of biological plausibility: CO_2 production did not parallel energy expenditure. None of the criteria for NMDR were met by the study

Metabolic effects					
Author, Year, Title	**Species**	**Dose range/number of doses**	**Dose(s) with effect**	**Endpoint**	**Evidence for NMDR according to the criteria of WG**
			microgram-exposed group		
Angle BM, Do RP, Ponzi D, Stahlhut RW, Drury BE, Nagel SC, Welshons WV, Besch-Williford CL, Palanza P, Parmigiani S, Vom Saal FS and Taylor JA, 2013. Metabolic disruption in male mice due to fetal exposure to low but not high doses of bisphenol A (BPA): Evidence for effects on body weight, food intake, adipocytes, leptin, adiponectin, insulin and glucose regulation	Mice GD 9 – GD18	5, 50, 500, 5000, 50,000 µg/kg bw per day BPA,	1. and 2. 5 µg/kg bw per day and 5000 µg/kg bw per day increased glucose AUC vs control 3. 500 µg/kg bw per day increased in renal and gonadal fat 4. 5 µg/kg bw per day and 500 µg/kg bw per day increased in gonadal and 5 µg/kg bw per day and 5000 µg/kg bw per day in renal fat 5. Insulin level higher than control in 5 µg/kg bw per day 6. Leptin level higher than controll in 500 µg/kg bw per day 7. leptin lower than control in 5, 50, 5000 µg/kg bw per day 8. no consistent doses over time different from control 9. 50 µg/kg bw per day and 5000 µg/kg bw per day increased fat weight in the three	1. GTT 2. ITT 3. Fat cell number 4. Fat cell volume 5. insulin 6. leptin 7. adiponectin 8. body weight 9. fat weight of gonadal, renal and total abdominal fat	No consistent dose-responses e.g. for body weight when analysed over time. Effects on food consumption and effects on weight were not correlated Endpoints measured in different animals with the exception of body weight Results of 19 statistical tests were reported but even more were performed because obviously all the endpoints were also compared to the positive control and the doses were compared to each other. In total effects are seen at random. None of the criteria for NMDR were met by the study

Metabolic effects					
Author, Year, Title	**Species**	**Dose range/number of doses**	**Dose(s) with effect**	**Endpoint**	**Evidence for NMDR according to the criteria of WG**
			regions 10. 50 µg/kg bw per day increased weight	10. liver weight	
Bodin J, Bolling AK, Samuelsen M, Becher R, Lovik M and Nygaard UC, 2013 Long-term bisphenol A exposure accelerates insulitis development in diabetes-prone NOD mice.	Mice	1 100 mg/l drinking water corresponding to 3 µg/day/mouse 300 µg/da/mouse	In the pancreas, exposure to both high and low dose BPA gave increased amount of apoptotic cells at week 7 and 12, and reduced number of resident macrophages at week 7. However, the more severe insulitis observed at 12 weeks in female NOD mice was associated with exposure to the lowest BPA dose only, as was the tendency of accelerated diabetes.		Study in NOD mice with questionable relevance for humans. None of the criteria for NMDR were met by the study (2 doses only).

Metabolic effects					
Author, Year, Title	**Species**	**Dose range/number of doses**	**Dose(s) with effect**	**Endpoint**	**Evidence for NMDR according to the criteria of WG**
Marmugi A, Ducheix S, Lasserre F, Polizzi A, Paris A, Priymenko N, Bertrand-Michel J, Pineau T, Guillou H, Martin PG and Mselli Lakhal L, 2012 Low doses of bisphenol A induce gene expression related to lipid synthesis and trigger triglyceride accumulation in adult mouse liver	Mice	5, 50, 500, 5,000 µg/kg/day for 4 week(mice 6 weeks old)	1. No effect 2. No effect 3. 50 increase 4. 5, 50, 500 increase; 5 more than 50 and 500; 50 more than 500 5. no effect 6. elevated TG 500 7. no effect 8. no effect 9. No effect	1. Body weight gain 2. Liver weight 3. pWAT weight 4. Insulin 5. Glucose 6. Triglycerides 7. total cholesterol 8. LDL 9. HDL	Lack of biological plausibility:high insulin along with unchanged glucose levels. Accumulation of cholesterol esters and triglycerides but no adverse effects on liver. The data do not provide sufficient evidence for NMDR.
Miyawaki J, Sakayama K, Kato H, Yamamoto H and Masuno H, 2007 Perinatal and postnatal exposure to bisphenol a increases adipose tissue mass and serum cholesterol level in mice	Mice GD10 to PND 20	1 µg/ml 10 µg/ml No dose given	1. Females: Both doses significant effects (increase) Males effect only at high dose 2.Females: increased only at low dose Males: effects only at high dose 3. Females: Elevated at low dose Males: no effect 4. Females: Low dose elevated TC Males no effect 5. Females: No effect Males: low dose elevated TG	1. Body weight 2. Adipose tissue weight 3. Serum leptin 4. Serum TC 5. Serum TG	None of the criteria for NMDR were met by the study (2 doses only).

Metabolic effects					
Author, Year, Title	**Species**	**Dose range/number of doses**	**Dose(s) with effect**	**Endpoint**	**Evidence for NMDR according to the criteria of WG**
			6. Females: no effect Males: elevated low dose 7.Females no effect Males low serum glucose at low dose	6. non-esterified fatty acids (NEFA) 7. Glucose serum	
Wei J, Lin Y, Li Y, Ying C, Chen J, Song L, Zhou Z, Lv Z, Xia W, Chen X and Xu S, 2011 Perinatal Exposure to Bisphenol A at Reference Dose Predisposes Offspring to Metabolic Syndrome in Adult Rats on a High-Fat Diet	Rat	50, 250 1250 µg/kg / day Group A (without fat) Group B with fat diet	Effects were only seen with 50 µg/kg/d	Increased body weight and insulin levels.	This study does not have two adjacent doses in the low dose range showing a significant effect.

Proliferative changes in mammary gland					
Author, Year, Title	**Species**	**Dose range/number of doses**	**Dose(s) with effect**	**Endpoint**	**Evidence for NMDR according to the criteria of WG**
Acevedo N, Davis B, Schaeberle CM, Sonnenschein C and Soto AM, 2013 Perinatally Administered bisphenol A acts as a mammary gland carcinogen in rats.	Rats	0.25 2.5 25 120 µg/kg bw/d, sc GD9-GD23	no stat. sign. increase	Atypical ductal hyperplasia, tumors	Lack of statistical significant effects compared to control groups

Proliferative changes in mammary gland					
Author, Year, Title	**Species**	**Dose range/number of doses**	**Dose(s) with effect**	**Endpoint**	**Evidence for NMDR according to the criteria of WG**
Ayyanan A, Laribi O, Schuepbach-Mallepell S, Schrick C, Gutierrez, M Tanos, T Lefebvre, G Rougemont, J Yalcin-Ozuysal O and Brisken C, 2011 Perinatal exposure to bisphenol A increases adult mammary gland progesterone response and cell number	Mice	0.6 3 6 12 120 600 1200 µg/ kg bw/ d, Drinking water	3 µg/ kg bw/ d	Increase in the number of TEBs. Progesterone response (at 6 µg BPA) and proliferation (at 3 µg).	This study does not have two adjacent doses in the low dose range showing a significant effect. No supporting study (e.g. Munoz-de-Torro, 2005). Low reliability of the study. None of the criteria for NMDR were met by the study.
Jenkins S, Wang J, Eltoum I, Desmond R and Lamartiniere CA, 2011 Chronic oral exposure to bisphenol A results in a nonmonotonic dose-response in mammary carcinogenesis and metastasis in MMTV-erbB2 mice	Transgenic mice	2.5 25 250 2500 µg/ kg bw/ d, drinking water	25 µg/ kg bw/ d and 2.5 and 25 µg/ kg bw/ d and 25 µg/ kg bw/ d	Proliferation apoptosis ratio and tumors per mouse (tumor multiplicity) and tumor volume, reduced mean time to first tumor onset	MDR for proliferation and apoptosis Priliferation:apoptosis ratio increased at one dose only; some support in Vandenberg et al., 2013c: male mice – but at higher HED. According to the WoE approach: tumor induction is not considered as a likely effect.
Markey et al., 2001 In Utero Exposure to Bisphenol A Alters the Development and Tissue Organization of the Mouse Mammary Gland	Mice	25 250 ng/kg bw/d, sc From GD 9	25 µg/ kg bw/ d	Area of TEBs	TEB increase at one dose only. No concomitant increase in epithelial proliferation. None of the criteria for NMDR were met by the study.
Murray et al., 2007 Induction of mammary gland ductal hyperplasia and carcinoma *in situ* following fetal bisphenol A exposure	Rats	2.5 25 250 1000 µg/ kg bw/ d, sc	2.5 µg/ kg bw/ d at PND 95 (not at PND 50)	Intraductal hyperplasia In addition to hyperplastic ducts cribriform-like structures indicating carcinoma in situ (CIS) at the two highest doses.	Increase in intraductial hyperplasia at one dose only; but CIS at higher doses only. Study reliability is considered low. None of the criteria for NMDR were met by the study.

Proliferative changes in mammary gland					
Author, Year, Title	**Species**	**Dose range/number of doses**	**Dose(s) with effect**	**Endpoint**	**Evidence for NMDR according to the criteria of WG**
US FDA/NCTR, 2013 and Delclos et al., 2014 Evaluation of the toxicity of bisphenol A (BPA) in male and female Sprague-Dawley rats exposed orally from gestation day 6 through postnatal day 90	Rats	2.5 – 300,000 µg/ kg bw/ d, gavage During gestation and up to PND 90	2,700 and 10,0000 µg/ kg bw/ d at PND 21 (but not at PND 90)	Ductal hyperplasia	The departures from MDR in hyperplastic effects (large data variations for each dose level)) could also be interpreted as chance findings. This study does not have two adjacent doses in the low dose range showing a significant effect.
Vandenberg LN, Schaeberle CM, Rubin BS, Sonnenschein C and Soto AM, 2013c. The male mammary gland: a target for the xenoestrogen bisphenol A.	Male mice	0.25 2.5 25 250 µg/ kg bw/ d, sc GD 9 until PND 16	25 µg/ kg bw/ d and 0.25 and 2.5 µg/ kg bw/ d (3-4 months), 2.5 and 25 µg/ kg bw/ d (7-9 months) 25 and 250 µg/ kg bw/ d (12-15 months)	Proliferation (Ki67) and number of branching points and ductal area	Proliferation at one dose only; no other supporting study (e.g. Markey, 2001). MDR for branching and ductal area at latest time point. Study reliability considered as low. The data do not provide sufficient evidence for NMDR.

2. Methodology applied for performing the risk assessment for BPA

The overall methodology to perform hazard identification and characterisation and risk characterisation of BPA is summarised in this introduction and graphically presented in Figs. 1-2. More specific details are given in Appendix A.

Graphical figures showing toxicological data were generated in the statistical computing environment R (R Core Team), 2014) in combination with R using the R lattice package (Sarkar et al., 2008).

The methodology used for BPA exposure assessment is not described here. For such information the reader should refer to Part I- Exposure Assessment of this scientific Opinion.

For hazard identification, studies were retrieved from different sources, as illustrated below, and selected for their relevance for this purpose (Appendix A).

The sources of studies considered for hazard identification and characterisation were:

Study sources
Studies that EFSA (EFSA, 2006; EFSA CEF Panel, 2010) or other risk assessment bodies had previously identified as crucial for BPA toxicological assessment
In vitro and *in vivo* studies on genotoxicity published after the 2006 EFSA opinion
Studies that were present in the list of the retrieved articles for the preparation of the EFSA opinion of 2010 (EFSA CEF Panel, 2010), but were not then evaluated because they did not match the inclusion criteria established at the time, e.g. non oral studies, exposure during adult age, single dose
Studies retrieved via a literature search for the period August 2010-December 2012
Studies included in the report of Réseau Environnement Santé (RES, 2012) on BPA-related risks
Additional studies becoming available after December 2012
Studies cited in the public consultation (ended on 13 March 2014) comments and original raw data submitted in this context (if relevant to the questions addressed by the opinion)

The studies were then grouped according to macro-areas of interest, e.g. toxicokinetics and metabolism, general toxicity, reproductive and developmental effects, and relative study type, i.e. human, animal or in vitro study (see Table 1).

Table 2: Macro-areas by which the relevant studies for BPA hazard identification were grouped and consideration of the studies used for the toxicological evaluation

Study content	**How the study was considered**
1. Toxicokinetics and metabolism (human and animal studies) 2. General toxicity (animal studies)	Appraisal of strengths and weaknesses (see Appendix B)
3. Reproductive and developmental effects (human and animal studies) 4. Neurological, neurodevelopmental and neuroendocrine effects (human and animal studies) 5. Immune effects (human and animal studies) 6. Cardiovascular effects (human and animal studies) 7. Metabolic effects (human and animal studies) 8. Genotoxicity (in vitro and in vivo studies) 9. Carcinogenicity (human and animal studies)	Appraisal of strengths and weaknesses (see Appendix B) and inclusion in the Weight of Evidence (WoE) approach used for hazard identification (see Appendices B and C)
10. Mechanisms of action of BPA (including epigenetics and gene expression studies) 11. In vitro studies	Examination and use as supplementary information for the toxicological evaluation (see Appendix B and Section 3.10)

Then *hazard identification* was performed as follows:

1. The studies belonging to the above macro-areas were assigned for review (see description of individual studies in Appendix B) to two members of the working group on BPA Toxicology

(the rapporteur and co-rapporteur) and further discussed in working group meetings. This led to:

a. Definition of all review questions addressing the association between BPA and the toxicological endpoints for macro-areas 2 to 9 listed in Table 1 and, for each review question, identification of one or several "lines of evidence" addressing different outcomes relevant to the question(s) and grouping of studies relevant to those question(s) by lines of evidence;

b. Appraisal of individual studies against their strengths and weaknesses (see criteria for evaluating human and animal studies in Appendix A and the outcome of the study appraisal in Appendix B) and inclusion in the Weight of Evidence (WoE) approach (Appendix C) used for hazard identification (see below). In vitro and in vivo genotoxicity studies were reviewed according to the EFSA scientific opinion on genotoxicity testing strategy principles (EFSA Scientific Committee, 2011) and submitted to the WoE approach (see Section 3.8). Studies on toxicokinetics and metabolism, and general toxicity were appraised but not considered in the WoE approach: the conclusions from those studies are reported in sections 3.1 and 3.2, respectively. Studies on the mechanisms of action including epigenetics (Section 3.10) and all the in vitro studies belonging to the macro-areas defined above excluding those on genotoxicity) were examined and used as supplementary information for the toxicological evaluation. In vitro studies (not on genotoxicity) using high BPA concentrations (equal or above 100 nM, for the reasons explained in Appendix A) were excluded a priori from the evaluation. Also excluded were reproductive and developmental toxicity studies testing only BPA doses exceeding the (oral) HED of 3.6 mg BPA/kg bw per day (equivalent to the NOAEL of 5 mg BPA/kg bw per day in the rat; see rationale in section 3.3.2.4 and Appendix A) or BPA in mixtures (see list of excluded studies in Appendix B).

2. A WoE approach was used for hazard identification (see Appendix B and Appendix C). The CEF Panel applied a WoE approach to identify the critical toxicological effects ("likely" or "very likely" effects) for BPA. In particular, the CEF Panel assessed the likelihood of the association between BPA exposure and each relevant toxicological endpoint, taking into consideration, for each endpoint, all the lines of evidence (studies in humans and/or experimental animals). The conclusions of earlier assessments of BPA by EFSA in 2006 and/or 2010 were taken as the starting point for the new evaluation. The CEF Panel expressed its conclusions in terms of the likelihood that the answer to the question on the association between BPA exposure and each endpoint was positive (i.e. an effect of BPA on the endpoint could be identified), using the scale of likelihood categories shown in Figure 2 (from "Very unlikely" to "Very likely". Note that, on this scale, "As likely as not" means a level of likelihood between "Unlikely" and "Likely", where it is about equally likely that BPA causes, or does not cause, the effect. For more details, see section 3 of Appendix A.

It is also important to emphasise that the likelihood assessed by the WoE approach refers specifically to hazard identification, i.e. it refers to the likelihood of an association between BPA and the effect under consideration. It does not refer to the likelihood or frequency of the effect actually occurring in humans, which depend on additional factors including the dose-response relationship for the effect (considered in hazard characterisation) and the levels of human exposure to BPA (considered in exposure assessment).

The subsequent step, namely *hazard characterisation* (identification of a dose-response relationship for the effect), was carried out only for those endpoints for which the overall likelihood for the specific effect was considered as "likely" or "very likely" in the WoE approach. The studies supporting "likely" or "very likely" effects were individually weighed and the most reliable studies were used to study dose-response relationships and identify the critical reference point (NOAEL, lowest observed

adverse effect (LOAEL) or BMDLs, depending on the suitability of the dataset) for setting a health-based guidance value.

Risk characterisation was then performed as described in section 5.

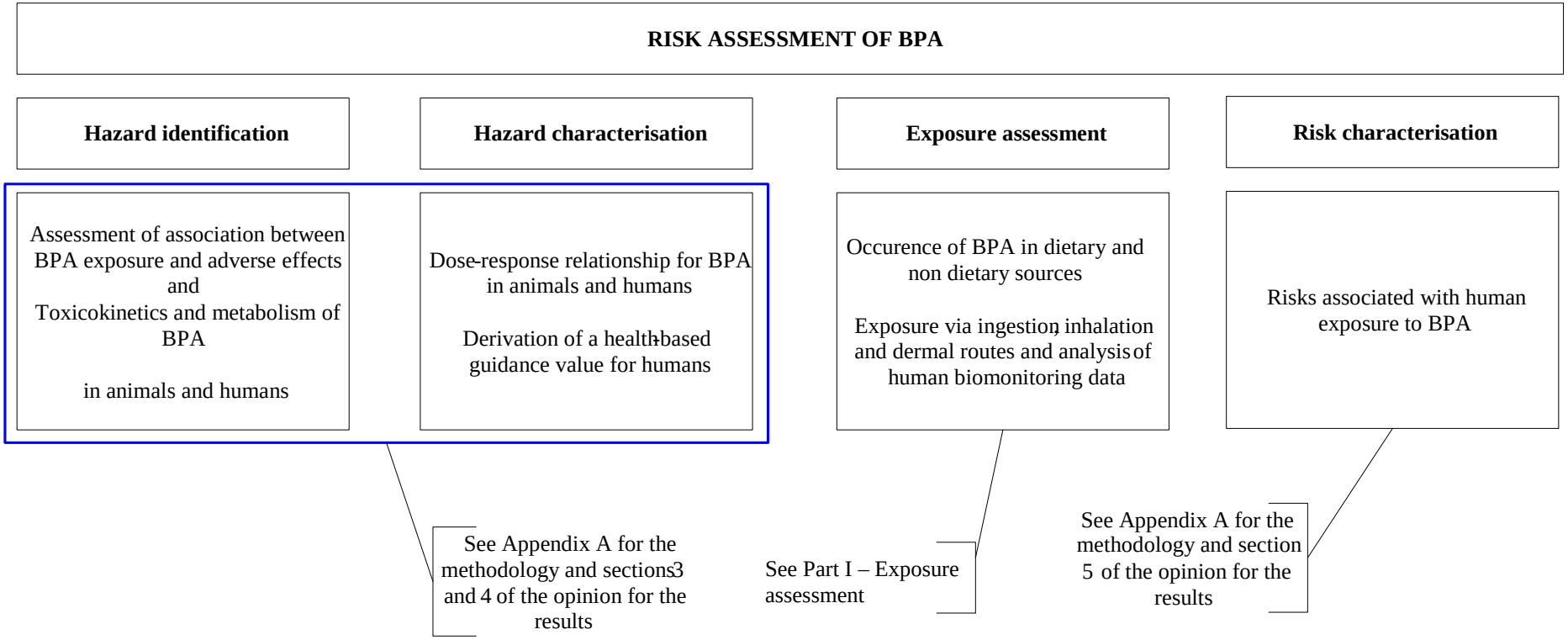


Figure 1: Overview of the steps followed for performing the risk assessment of BPA.

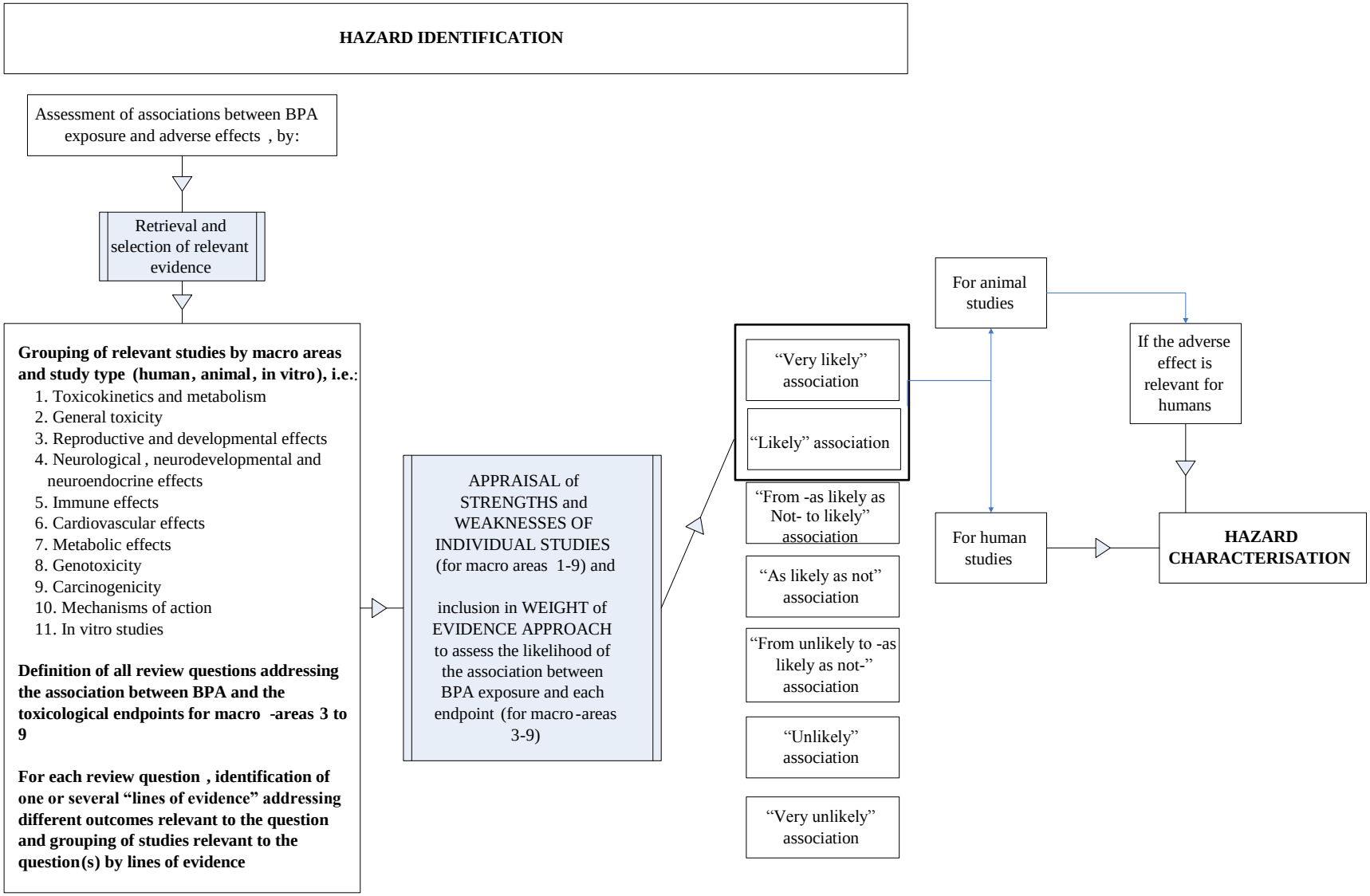


Figure 2: Overview of the steps followed for performing hazard identification and characterisation of BPA.

3. Hazard identification and characterisation

3.1. Toxicokinetics and metabolism

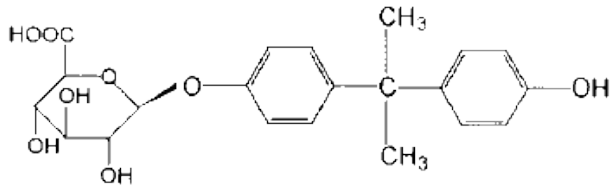
3.1.1. Summary of previous evaluations and introduction of the HED concept

3.1.1.1. Summary of previous evaluations

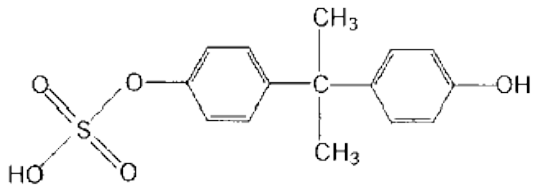
The toxicokinetics of BPA has been reviewed by several risk assessment bodies worldwide (EU-RAR, 2003, 2008; EFSA 2006, 2008; EFSA CEF Panel, 2010; FAO/WHO, 2011; ANSES, 2011, 2013). Concerning the routes of exposure, most information is available from studies with oral administration, whereas only limited information is available on dermal exposure and essentially none on inhalative exposure.

After oral administration, BPA is rapidly absorbed from the gastrointestinal tract. The analysis of total (unconjugated and conjugated) BPA plasma concentration-time profiles after oral and intravenous (IV) administration in terms of the area under the curve (AUC) suggests a high degree of absorption (up to 85-86% in rats and monkeys) from the gastrointestinal (GI) tract. Similarily, human studies have suggested a complete absorption of a relatively low oral BPA dose, based on the urinary recovery of labelled BPA-glucuronide (EU-RAR, 2003, 2008).

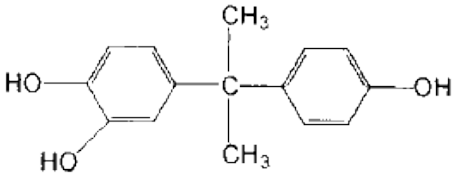
Following oral absorption, BPA is rapidly metabolised by polymorphic UDP-glucuronyltransferases (UGTs) in the gut wall and the liver (first-pass effect) to BPA-glucuronide, which is a biologically inactive form, before reaching the systemic circulation and excreted. In humans, similar to rodents, a sulphate conjugation mediated by sulfotransferases has additionally been observed (EFSA, 2008; EU-RAR, 2003, 2008; ANSES, 2011, 2013; EFSA CEF Panel, 2010). In rodents, the BPA-conjugates are partly eliminated (the remaining being eliminated in the urine) *via* biliary secretion into the intestinal tract, where they are cleaved to release BPA which then undergoes enterohepatic recirculation. In rats, this enterohepatic circulation results in a slow excretion and prolonged low-level systemic availability of unconjugated BPA, which is supported by the observation of urinary excretion of unconjugated BPA as an appreciable fraction (1–4%) of the applied oral dose. Due to biliary secretion and enterohepatic recirculation, the predominant way of elimination of systemically available unconjugated and conjugated BPA in rodents is the fecal excretion of unconjugated BPA. In contrast, humans and monkeys eliminate the systemically available BPA forms primarily *via* urinary excretion of BPA-conjugates.



Bisphenol A-4-p-D-glucuronide



Bisphenol A-4 sulfate



5-Hydroxybisphenol A

In humans there are indications that the metabolic capacity of the UGT forms 2B15 and 1A1 is not yet mature at birth (Allegaert et al., 2008; Gow et al., 2001; Miyagi and Collier, 2011; Zaya et al., 2006), whereas sulfation enzymes are known to be already expressed at the adult level at birth (Pacifici et al., 1993; EFSA CEF Panel, 2010).

Due to the high first-pass effect, peak blood levels of unconjugated BPA in humans after oral exposure to BPA are generally reported to be very low (unconjugated BPA is <0.5% of total serum BPA in humans and monkeys), even after worst-case dietary exposures (see also Section 4.6.3 in Part I – Exposure assessment). In two human studies (Völkel et al., 2002; 2005) unconjugated BPA was below the limit of detection in all urine (LOD of 6 nM) and blood samples (LOD of 10 nM) (equivalent to a ratio of unconjugated BPA to BPA-glucuronide of < 0.5 %). In humans, 75–85% of an oral dose was excreted in urine within five hours post dosing whereas in monkeys 82–85% of the dose was eliminated via the urine within 12 hours after oral administration. Thus, the observation of higher unconjugated BPA levels in urine of rats compared with non-human primates further supports the existence of species differences in blood levels of unconjugated BPA between rodents and primates, with higher AUCs for unconjugated BPA in rats (EFSA, 2006, 2008). A similar conclusion has been reached in the EU-RAR (2008).

The BPA conjugates are considered to have no affinity for oestrogen receptors (EFSA, 2006; EFSA CEF Panel, 2010; ANSES, 2011, 2013). In addition to the conjugation pathways, in vivo and in vitro studies suggest that in the rat, BPA may be subject to oxidation to bisphenol O-quinone by cytochrome P450 and to 5-hydroxy-BPA (EU-RAR, 2003, 2008) to a small extent. EFSA (2006) reported oxidative BPA metabolites to also occur in mice.

Previous assessments concluded that BPA is rapidly distributed in all tissues and has no clear affinity for one particular organ. Analysis of the fetal compartment shows that BPA is mainly present as its conjugates and that only a minor fraction is present in its unconjugated form (EFSA, 2006; ANSES, 2011, 2013). In rats, fetal exposure to unconjugated BPA changes over the duration of pregnancy, based on the apparent development of Phase II metabolic capacity in the fetus: in early pregnancy the concentration of unconjugated BPA in fetal tissue is up to three times higher than in the dams, whereas later the concentration is about the same (EFSA CEF Panel, 2010).

In neonatal rats orally administered 1 or 10 mg ^{14}C-BPA/kg bw at postnatal days (PND) 4, 7, or 21, the serum levels of both total and unconjugated BPA were considerably higher in younger neonates compared to older neonates (Domoradzki et al., 2004). BPA was metabolised to BPA-glucuronide at all three ages, although an age dependency in the concentration of glucuronidated BPA was observed, consistent with the ontogeny of UGTs (Domoradzki et al., 2004). Differences in the plasma concentration-time profiles of unconjugated and glucuronidated BPA between neonatal and adult rats additionally suggested a decreased biliary excretion and/or enterohepatic recirculation in neonatal rats, which is consistent with a developmental immaturity of hepatic excretory function. Overall, these findings indicate a reduced metabolic capacity in early neonatal life, which is however sufficient to efficiently metabolise BPA to non-oestrogenic conjugates in rats (EFSA, 2006; EU-RAR, 2003, 2008). The age-related changes described above after oral administration were not observed after subcutaneous (SC) injection, indicating that even in early postnatal pups, which possess lower conjugation activity/capacity, the first-pass effect is relevant (EFSA CEF Panel, 2010; FAO/WHO, 2011).

Data from an experimental study in rats suggest limited excretion of BPA in the milk. However, the data do not allow a reliable quantitative determination to be made (EFSA, 2006; EU-RAR, 2003, 2008). The CEF Panel estimated that in rats the exposure to total BPA (the major constituent being the glucuronide) through lactation is very low. For dams receiving 410 mg/kg bw per day, the estimated dose delivered to pups lactationally was approximately 350 µg/kg bw per day (EFSA CEF Panel, 2010). This is 1 171-fold lower than the dose administered to the dams (EFSA CEF Panel, 2010).

In monkeys, similar to the rat, the systemic availability of unconjugated BPA is very low after oral administration. In adult monkeys, the contribution of unconjugated BPA to the total plasma BPA level was higher following intravenous (IV) administration than after oral administration of the same dose (Doerge et al., 2010b). Following the same oral dose of BPA (100 µg/kg bw) to adult rats and monkeys, unconjugated BPA plasma concentrations in both species were below 1 nM. The only notable difference was the longer elimination half-life in rats versus monkeys (3.5 hours versus 0.39 hours), due to the enterohepatic recirculation in the rat. Comparing newborn animals, PND 3 rats have longer elimination half-life and approximately 10 times higher plasma levels of unconjugated BPA than PND 5 monkeys, when treated with the same oral BPA dose. These data provide evidence for a different developmental profile of hepatic and intestinal conjugation of BPA in rats and monkeys, consistent with literature data describing a higher degree of metabolic immaturity of rats at birth as compared to primates (Doerge et al., 2010a,b; Doerge et al., 2011b; EFSA CEF Panel, 2010; FAO/WHO, 2011).

Concerning dermal absorption of BPA, the EU-RAR (2008) mentioned an *in vitro* dermal absorption study using human skin that found limited absorption of BPA at millimolar concentrations with the extent of absorption being in the region of 10% of the applied dose. Dermal absorption of BPA is discussed further in section 3.1.7..

3.1.1.2. Introduction of the human equivalent dose (HED) concept

An understanding of the toxicokinetics and metabolism of BPA is of major importance for its risk assessment as it enables quantification of the toxicokinetic relationships between the critical exposures in animal experiments and the corresponding (equivalent) exposures in humans. Information on how these relationships are modified by factors such as age, gender and pregnancy is also essential. Approaches to performing this animal-to-human extrapolation include, among others, the *internal dose* concept recently used by ANSES (2013) and the *human equivalent dose* (HED) concept recommended by the US EPA (2011).

By making assumptions about route-dependent bioavailability factors, the ANSES approach translates external exposures *via* different routes and sources into internal doses which are then combined to give an estimate of the total internal exposure in humans. This internal dose estimate is finally compared to internal toxicological benchmarks which are derived from animal experiments and adjusted for bioavailability and uncertainties. The US EPA (US EPA, 2011) endorses a hierarchy of approaches to derive oral HEDs from data from laboratory animals *via* factors accounting for the toxicokinetic portion of the interspecies differences. This hierarchical framework comprises (i) physiologically-based pharmaco-toxicokinetic (PBPK) modelling as the optimal approach, (ii) the use of chemical-specific information (e.g. use of internal dosimetrics such as the maximum serum concentration, C_{max}, or the area under the curve, AUC) as an intermediate approach when the available data do not permit PBPK modelling, and (iii) allometric scaling of dose with body weight as the default lower-tier approach (see Section 3.1.5 for further details).

The CEF Panel noted that new data have recently become available from toxicokinetic studies in various laboratory animal species (Doerge et al., 2010a,b; Doerge et al., 2011a,b; Doerge et al., 2012). These studies provide internal dose metrics for neonatal-to-adult stages and for different routes of exposure. Moreover, physiologically-based pharmaco-toxicokinetic (PBPK) models have been developed to simulate and predict the internal exposures in laboratory animals and humans in a route-specific manner. Specifically, a PBPK model has been developed to enable estimation of internal dose metrics for the aggregated oral and dermal exposure in humans (Mielke et al., 2011). Overall, this body of information permits extrapolation to humans based on the application of human equivalent dose factors (HEDFs). The CEF Panel therefore decided to apply the HED concept and to provide HEDs for reference points derived from critical animal data.

The following sections describe the new body of evidence and the PBPK modelling that lead to the determination of HEDFs.

3.1.2. New information on toxicokinetics (animal and human studies)

Since the last EFSA evaluation, a consistent body of toxicokinetic information has become available for mice, rats and rhesus monkeys at different developmental stages ranging from neonatal to adult stages; the routes of administration comprised oral dosing (gavage) as well as intravenous (IV) or subcutaneous (SC) injections. The main contribution came from Doerge's group (Doerge et al., 2010a,b; Doerge et al., 2011a,b; Doerge et al., 2012), who used a consistent methodology with identical experimental protocols in all species studied. This methodology included (i) the administration of stable isotope-labelled (deuterated) BPA to avoid issues related to contamination of samples with BPA from laboratory materials and other sources, (ii) the application of a dose of 100 µg/kg bw which enables the quantification of unconjugated and conjugated BPA forms in serum and (iii) the use of a specific and sensitive analytical method based on LC/MS/MS, having a method detection limit of 0.2 nM (= 45.6 ng/l). The chosen dose of 100 µg/kg bw was demonstrated to enable linear pharmacokinetics. The methodology additionally included a consistent toxicokinetic analysis of serum concentration-time profiles to estimate the maximum serum concentration, C_{max} (mean value $\pm$s.d.), the area under the curve (AUC) from time zero to infinity, and the elimination half-life ($t_{1/2}$). In the present opinion, the AUC for unconjugated BPA in serum was chosen to derive HEDFs for the animal-to-human extrapolation.

The main body of evidence is summarised in the following sections, starting with animal data on adults and neonates and then continuing with human data. This is then followed by in vitro data. A detailed description and evaluation of each study are provided separately in Appendix B.

3.1.2.1. Data in adult animals

The group of Doerge published several studies in adult CD-1 mice (Doerge et al., 2011b; Doerge et al., 2012), SD rats (Doerge et al., 2010a), and rhesus monkeys (Doerge et al., 2010b), using either oral administration or IV injection of 100 µg/kg bw d6-BPA (Figure 3). To provide a measure of variability, the data on C_{max} and AUC given below are expressed as mean ± standard deviation if available.

Following the oral administration of BPA by gavage to mice of both sexes (n = 12) serum concentrations of unconjugated BPA were below the LOD (0.2 nM) in the majority of samples at all time points (Doerge et al., 2011b). Levels of unconjugated BPA that were above the LOD were observed only at the earliest three time points (at 0.25, 0.5 and 1.0 hours after dosing), and only in one, two or three samples out of the twelve determinations at each time. By choosing a lower-bound approach (i.e. setting all non-detectable observations to zero) and calculating the mean and the standard deviation from the few detectable observations and the remaining set-to-zero non-detects, a C_{max} of 0.18±0.31 nM and an AUC of 0.10 nM×h was obtained (Figure 3A, Doerge et al., 2011b). The CEF Panel noted the large standard deviation around the calculated $C_{max,}$ which results from setting the non-detects to zero. The CEF Panel further noted that, due to the way by which the non-detects were handled, the AUC value of 0.10 nM×h needs to be regarded as a lower-bound estimate of the true AUC for adult mice with oral administration. Based on additional information and considerations, which are detailed in Section 3.1.6.1, the CEF Panel recalculated the AUC to be 0.244 nM×h.

Under the same dosing conditions as above, female rats (n = 5) showed low but detectable serum levels of unconjugated BPA with a C_{max} of 0.39±0.19 nM and an AUC of 2.6±2.1 nM×h (Figure 3B, Doerge et al., 2010a); the levels of unconjugated and total BPA changed only slightly during the time interval from 0.5 to 8 h and elimination occurred mainly between 8 and 24 h, both reflecting the effect of enterohepatic recirculation (Doerge et al., 2010a). In female rhesus monkeys (n = 4), the serum levels of unconjugated BPA were only detectable within the first 90 min after oral dosing; the C_{max} was 0.84±0.46 nM and the AUC was 1.5±1.1 nM×h (Figure 3C, Doerge et al., 2010b); the rapid decrease in levels of unconjugated BPA was reflected by the short elimination $t_{1/2}$ of 0.39±0.24 h which contrasted with the delayed decrease and the longer elimination $t_{1/2}$ of 3.0±3.7 h in rats. Overall, the internal dose metrics (C_{max}, AUC) for unconjugated and total serum BPA revealed a very

low proportion of unconjugated BPA in the total BPA serum concentration (<1%) which indicates the presence of extensive first-pass metabolism in adult mice, rats, and rhesus monkeys.

The IV injection in female mice (n = 6) resulted in an initial rapid distribution of unconjugated BPA into tissues, followed by a rapid terminal elimination phase with an elimination $t_{1/2}$ of 0.8 h (Figure 3D, Doerge et al., 2012). An oral bioavailability of 0.45 % for unconjugated BPA could be derived based on the ratio of the oral AUC (= 0.244 nM×h, recalculated value, see Section 3.1.6.1) to that of 54 nM×h for IV injection. Enterohepatic recirculation was suggested by the presence of an apparent "re-entry peak" at 2 h for total, but not for unconjugated BPA (Figure 3D, Doerge et al., 2012). After IV injection in female rats (n = 7) a rapid elimination of unconjugated BPA from the serum was observed with an elimination $t_{1/2}$ of 0.66±0.04 h (Figure 3E, Doerge et al., 2010a); in contrast to mice, the serum concentration-time data for unconjugated BPA did not indicate a separate distribution phase. There was evidence for enterohepatic recirculation being responsible for the extended time course of total BPA concentration. In rats, a systemic oral bioavailability of 2.8±3.1 % for unconjugated BPA could be derived based on the ratio of the oral AUC (= 2.6 nM×h) to that of 95±8.8 nM×h for IV injection. In female rhesus monkeys (n = 4), the IV dosing led to a rapid distribution of unconjugated BPA from the serum, followed by a slower terminal elimination phase ($t_{1/2}$ = 3.6±1.3 h). A systemic oral bioavailability of 0.94±0.54 % for unconjugated BPA was obtained by dividing the oral AUC (= 1.5 nM×h) by the AUC of 180±76 nM×h for IV injection. Overall, the experiments with oral and IV administration revealed an oral bioavailability for unconjugated BPA ranging from 0.45 % in mice, 0.9% in monkeys to 2.8 % in rats.

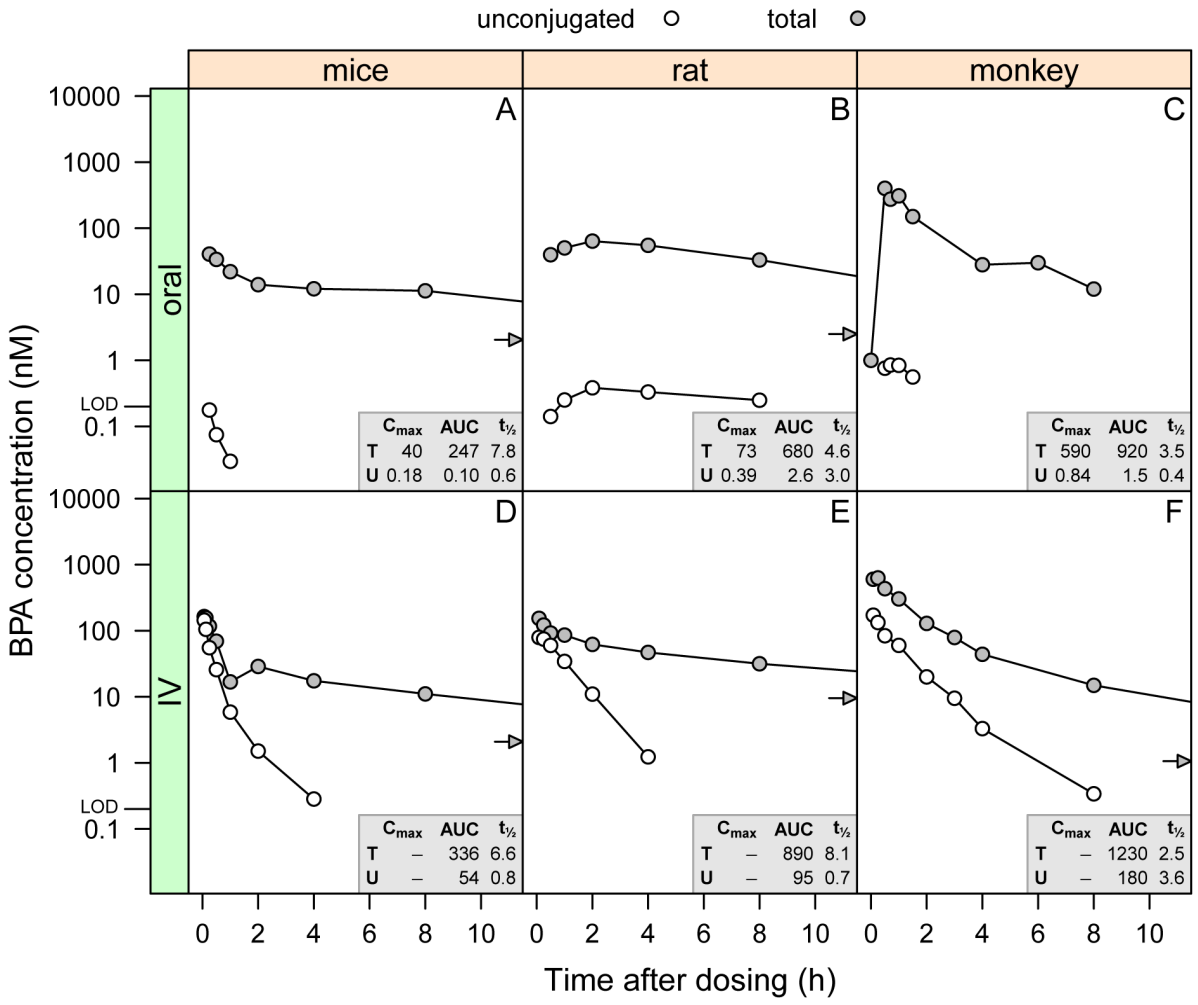


Figure 3: Time course of serum levels of unconjugated and total BPA in adult mice, rats, and rhesus monkeys following oral administration or IV injection of a single dose of

100 µg/kg bw per day of isotope-labelled (deuterated) BPA. Each symbol represents the mean concentration of unconjugated BPA (open circles) and total BPA (filled circles) at a given time point. Horizontal arrows indicate the serum concentrations after 24 h. The LOD was 0.2 nM in all experiments. Additionally given are the pharmacokinetic parameters for unconjugated (U) and total (T) BPA, comprising the maximum serum concentration Cmax (nM), the area under the curve AUC (nM×h) from time zero to infinity, and the elimination half-life t1/2 (h). The data shown were taken from Doerge et al (2011b), Doerge et al (2010a), Doerge et al (2010b) and Doerge et al (2012).

Additional toxicokinetic information on the tissue/serum concentration ratios for unconjugated and conjugated BPA and on placental transfer of both BPA forms in rodents and monkeys, available from the studies of Doerge et al., are briefly summarised below, together with information from studies by other groups.

In the above mentioned study in adult female CD-1 mice with IV injection, Doerge et al. (2012) additionally measured the concentration of unconjugated BPA in adipose tissue and reported a fat-to-plasma AUC ratio of about 2.2. The levels of unconjugated BPA in adipose tissue rapidly reached a maximal level (0.25 h) that did not exceed the plasma C_{max} at the initial sampling time (0.08 h). The terminal elimination $t_{1/2}$ of 7.0 h for unconjugated BPA in adipose tissue was similar to that for conjugated BPA in serum ($t_{1/2}$ = 6.6 h), and <0.01% of the administered dose remained in adipose tissue after 24 h.

A study in pregnant rats (after dosing with 100 µg/kg bw deuterated BPA) (Doerge et al., 2011a) showed no kinetic differences to non-pregnant rats. After oral administration, unconjugated BPA was not detected in the fetal tissue, and the maternal serum levels were close to the LOD (= 0.2 nM). This study also showed that BPA crosses the placental barrier, since the BPA conjugates were measurable in the fetal tissue of every age. However, no selective affinity of either yolk sac/placenta or embryo/fetus for unconjugated and conjugated BPA relative to maternal plasma or tissues was observed. After IV injection, in contrast, the concentration of unconjugated BPA at gestational day (GD) 12 in the fetal tissue was threefold higher than in the maternal serum, at GD 16 equal to the maternal serum, and at GD 20 half the concentration in the maternal serum. The fetal brain at GD 20 had a 4-fold higher concentration of unconjugated BPA compared to the maternal serum and an 11-fold higher concentration compared to the fetal serum. At GD 21 after IV administration the amniotic fluid contained both unconjugated and unconjugated BPA; the concentrations were 0.35-fold and 0.2-fold, respectively, of the maternal serum concentrations. The ratios of the amniotic fluid/fetal serum concentration at GD 21 were 0.8 for unconjugated BPA and 0.05 for BPA-conjugates (Doerge et al., 2011a).

The above mentioned study with pregnant rats additionally measured the distribution of unconjugated and conjugated BPA into different tissues in adult female rats after IV injection (Doerge et al., 2011a). The tissue/serum concentration ratios for unconjugated BPA were 5 for adipose tissue, 4 for mammary gland, 2.8 for brain, 2.7 for muscle, 2.6 for ovary, 1.5 for uterus and 0.73 for liver. For conjugated BPA, tissue/serum concentration ratios were below 0.1 for all tissues except the ovary (0.2), the uterus (0.3) and the liver (5.4).

Nishikawa et al. (2010) studied the transfer of glucuronidated BPA across the placenta by uterine perfusion in pregnant SD rats (GD 18.5). The uterus was perfused with a solution containing 2 µM glucuronidated BPA for 20 min, followed by a 70-min perfusion without glucuronidated BPA, after which the perfusate as well as the fetus, amnion, amniotic fluid, placenta, and uterus were collected. HPLC and LC/TOF-MS were used for the determination of glucuronidated and unconjugated BPA. Almost all of the glucuronidated BPA (113 nmol) was detected in the perfusate leaving the uterine vein. Glucuronidated BPA was also detected in the fetus in a small amount (109 pmol, ~0.09% of the total amount) but not in the amnion, amniotic fluid, placenta, and uterus. Moreover, unconjugated BPA was detected to a small amount in the amniotic fluid (31 pmol) and in the fetus (6 pmol). The

findings are in line with results of a toxicokinetic study in pregnant rats (Doerge et al., 2011a) (see above).

In the study of Mita et al. (2012), pregnant adult Balb-C mice were exposed daily to two different doses of BPA by subcutaneous injection (100 and 1000 µg/kg bw per day) beginning on GD 1 through the seventh day after delivery. The dams were sacrificed on day 21 (14 days after the last dose) and the offspring at 3 months after delivery. Liver, muscles, hindbrain and forebrain were dissected and processed using HPLC with UV and fluorescence detection to measure BPA. The authors reported measurable unconjugated BPA levels in the tissues of all animals exposed to BPA. The results are, however, questionable due to the analytical limitations known for UV and fluorescence detection (the author did not give results for the analytical quality) and implausible since >99.9% of BPA is eliminated from mice within 24 h, even in adipose tissue (Doerge et al., 2012).

In lactating Sprague–Dawley dams, treated by daily gavage with 100 µg/kg bw d6-BPA starting at the day of delivery, unconjugated BPA and total BPA was detected in all dam serum (0.55 nM and 126 nM respectively) and milk (0.87 nM and 7.6 nM respectively) samples (Doerge et al., 2010c). In pup serum the concentrations of unconjugated BPA were below the level of detection (<0.2 nM). The dose of total BPA delivered to pups lactationally, estimated from milk concentrations and body weights, was 0.32±0.12 µg/kg bw per day. This is 300-fold lower than the dose administered to the dams. Similarly, serum concentrations of total BPA in pups were at least 300-fold lower than those in their dams (Doerge et al., 2010c).

Taylor et al. (2011) measured unconjugated and conjugated BPA levels in serum from adult female rhesus monkeys (n = 11) using isotope-dilution LC-MS (LOQ: 0.2 ng/ml) after oral administration of deuterated d6-BPA. The rhesus monkeys received 400 µg/kg bw of d6-BPA (in fruits) per day for 7 days; the C_{max} of unconjugated BPA of 4 ng/ml (= 17.5 nM) was reached 1 hr after feeding and declined to low levels by 24 hr (see Figure 9 in Part I – Exposure assessment) with no significant bioaccumulation after seven daily doses. In a second experiment with adult female CD-1 mice (n = 5–7 per blood-sampling time point), the authors used (i) LC with ^{3}H-scintillation counting for a 400 µg/kg bw dose of ^{3}H-BPA and (ii) LC with electrochemical detection for a 100,000 µg/kg bw dose of BPA. The sensitivity of the ^{3}H-scintillation-counting assay was 0.28 ng/ml, which was calculated as two-fold above the background counts per minute. The LOD of the LC-ED assay was 9 ng/ml. The doses were dissolved in corn oil and were administered into the animal's mouth via a micropipetter (oral bolus dosing). The serum-concentration time profiles for unconjugated BPA were subjected to a toxicokinetic analysis. The experiment with 400 µg/kg bw dosing yielded a C_{max} of 3.28 ng/ml, an AUC of 38.72 ng×h/ml, and a terminal elimination half-life ($t_{1/2}$) of 33.6 h. The experiment with 100,000 µg/kg bw dosing showed a C_{max} of 949 ng/ml, an AUC of 2991 ng×h/ml, and a $t_{1/2}$ of 4.9 h. The C_{max} values indicated linear kinetics over the dose range of 100 to 100,000 µg/kg bw per day.

To compare the toxicokinetic results of Taylor et al. (2011) for adult mice with oral bolus dosing of ^{3}H-BPA and BPA in corn oil with the results of Doerge et al. (2011b) for adult mice with gavage dosing of d6-BPA in aqueous solution (see Figure 4D–F), the doses administered were scaled to the common dose of 100 µg/kg bw by multiplying the serum concentrations and the concentration-related pharmacokinetic parameters by a factor of 0.25 (= 100/400) and 0.001 (= 100/100.000), respectively. In addition, the serum concentrations and the concentration-related pharmacokinetic parameters were converted into molar concentration-based values. Compared to the results of Doerge et al. (2011b), the dose-adjusted pharmacokinetic parameters of the experiment of Taylor et al. (2011) with 400 µg/kg bw dosing showed a 20-fold higher C_{max} (3.6 *vs*. 0.18 nM), a 4200-fold higher AUC (42 vs. 0.1 nM×h), and a 57-fold longer terminal half-life $t_{1/2}$. The CEF Panel noted that the use of a corn-oil dosing vehicle, which is known to influence the kinetics and extent of absorption of chemicals versus aqueous dosing solutions (Gallo et al., 1993), and which very likely prolonged the time to reach C_{max} (Figure 4E), cannot alone explain these large-scale differences. The fact that the C_{max} and AUC of Doerge et al. (2011b) are lower-bound estimates contributes to the discrepancy but only to a minor extent. The pharmacokinetic analysis of Taylor et al. (2011) showed that the AUC (i.e. the $AUC_{0-\infty}$)

was 2.3-fold higher than the $AUC_{0–24h}$, which suggests an analytical problem with the last data point at 24 h leading to unreliable estimates for $t_{1/2}$ and AUC (Figure 4E). Also, the use of the corn-oil vehicle makes it difficult to deconvolute the kinetics of absorption, distribution, and elimination processes. By additionally taking the results on C_{max} of the toxicokinetic study of Sieli et al. (2011) in mice (see below) into account, the CEF Panel assigned a low reliability to the pharmacokinetic parameters of Taylor et al. (2011).

Sieli et al. (2011) measured unconjugated and conjugated BPA levels in serum from adult female C57Bl/6J mice (n = 8 per blood-sampling time point) using isotope-dilution HPLC-MS/MS (LOD: 0.1 ng/ml) after oral administration of deuterated d6-BPA. Two different oral administrations were used. The first group of animals received a dose of 20,000 µg/kg bw per day which was dissolved in corn oil and administered into the animal's mouth via a micropipetter (oral bolus dosing). The second group was exposed to a dose of ~13,000 µg/kg bw *via* the diet containing 100 mg d6-BPA/kg feed. In the experiment with oral bolus dosing, the C_{max} for unconjugated BPA in serum of 21.0 ng/ml was observed at the first sampling time at 1 h after dosing. The AUC (i.e. the $AUC_{0–\infty}$) was 210 ng×h/ml, and the terminal elimination half-life ($t_{1/2}$) was 6.4 h. Scaling the administered dose to the common dose of 100 µg/kg bw yielded a dose-adjusted C_{max} of 0.45 nM (Figure 4G) which was in the range of C_{max} values of 0.1–1.1 nM for adult and PND21 mice with orogastric administration (Doerge et al., 2011b) but considerably lower than the dose-adjusted C_{max} value of 3.6 nM in the study of Taylor et al. (2011). The differences in C_{max} between Sieli et al. (2011) and Doerge et al. (2011b) can at least partly be explained by the different vehicles (corn oil *vs.* aqueous solution) and the type of administration (oral bolus dosing *vs.* gavage). These methodical differences also very likely explain the apparently higher $t_{1/2}$ of 6.4 h in Sieli et al. (2011) compared to the lower $t_{1/2}$ values of 0.2–0.6 h in PND21 and adult mice of the study of Doerge et al. (2011b). In the experiment with diet-exposed mice, the shape of the serum concentration-time profile for conjugated and unconjugated BPA changed in a significant manner as reflected by the delayed time to C_{max} of 6 h (Figure 4H). The CEF Panel noted that, despite of the change of the serum concentration-time profile due to the diet-related exposure to BPA, delaying gastrointestinal absorption processes, the pharmacokinetic parameters C_{max} and AUC were comparable to those observed in the oral-bolus dosing experiment (Figure 4G/H).

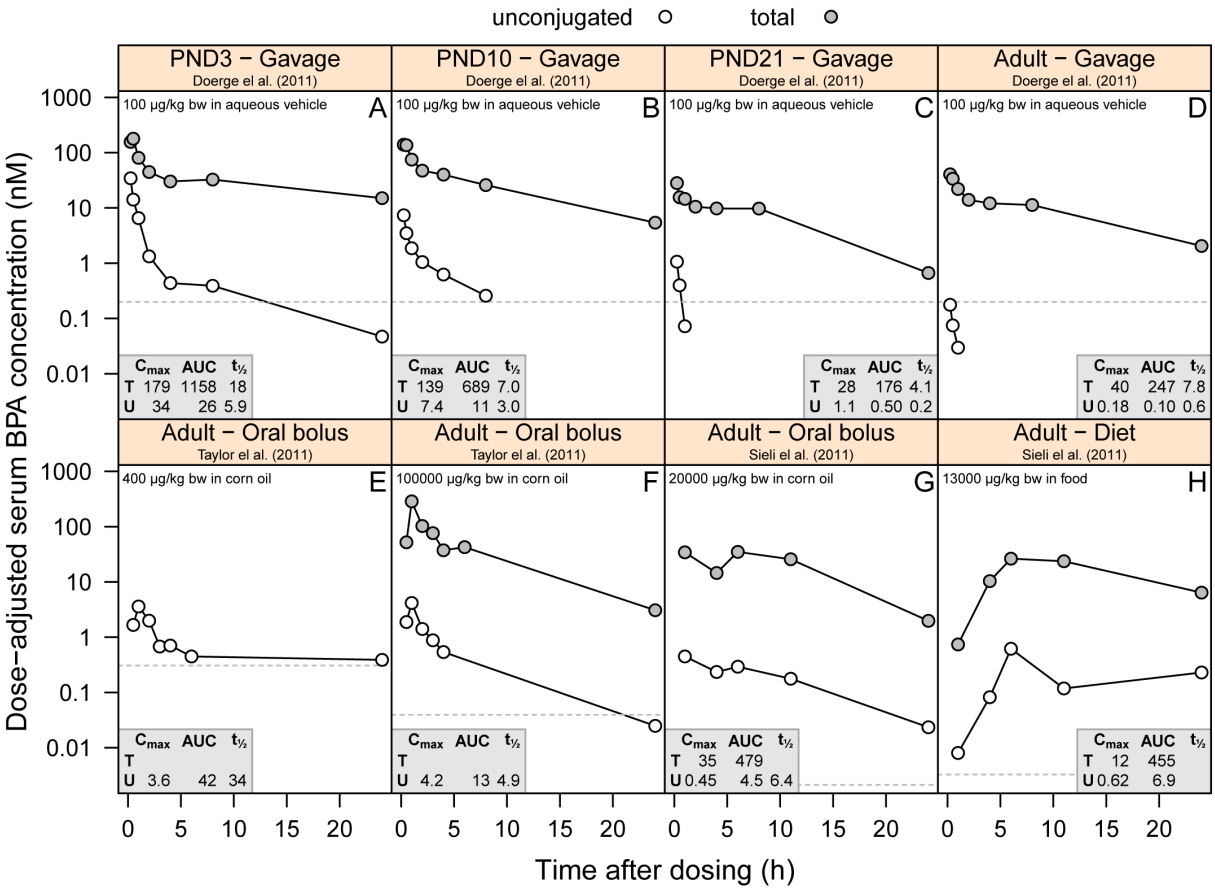


Figure 4: Time course of serum levels of unconjugated and total BPA in newborn (PND3), juvenile (PND10, PND21) and adult mice following oral administration. BPA was administered by orogastric gavage, oral bolus, or via diet. All serum concentration profiles and the pharmacokinetic parameters for the maximum serum concentration C_{max} (nM) and the area under the curve AUC (nM×h) from time zero to infinity were scaled to a common dose of 100 µg/kg bw per day. Additionally given is the elimination half-life $t_{½}$ (h). Note the effect of the administration procedure and the BPA vehicle (aqueous solution, corn oil, food). The data shown were taken from Doerge et al (2011b), Taylor et al (2011) and Sieli et al (2011).

In a study by Tharp et al. (2012) in pregnant rhesus monkeys, an oral dose of 400 µg/kg bw per day of deuterated BPA (in fruits) was administered daily during gestational days 100–165. Maternal serum samples were taken near the time of spontaneous birth, ≈4 h after oral dosing. The serum samples were analyzed for conjugated and unconjugated BPA (LOD: 0.2 ng/ml) as described in Taylor et al. (2011). The observed serum concentrations of 0.68 ± 0.312 ng/ml (mean ± SEM, n=3 animals) for unconjugated BPA at 4 hours after ingestion agreed with the serum unconjugated BPA concentration of 0.6 ng/ml which was measured by Taylor et al. (2011) in adult female rhesus monkeys at 4 hours after oral administration of 400 µg/kg bw.

In the study of Patterson et al. (2013) 100 µg/kg deuterated BPA was given daily to monkeys by the intravenous or the oral route to two groups of dams during late pregnancy (days 121–139 of gestation). The animals were pre-medicated with glycopyrrolate, buprenorphine or fentanyl prior to induction of anesthesia, then sedated with ketamine for intubation, and finally anesthetized with a gas mixture of isoflurane and oxygen during the experiment. Concentrations of unconjugated and conjugated BPA were measured at 0 min (predose) and after approximately 5, 15, 30, 60, 120, 180, 240, 480 and 1440 min in the plasma of rhesus monkey dams. Concentrations were similarly measured in the plasma of the fetus at 0 min (predose) and approximately 5, 15, 30, 60, 120, 180, 240, and 480 min. Furthermore, concentrations of unconjugated and conjugated BPA was also determined

in the amniotic fluid and in the placenta. The authors used a validated LC/MS/MS methods for their measurements. The kinetics in the dams after the intravenous administration were similar to findings in non-pregnant monkeys from a previous study (Doerge et al. 2010b). Plasma concentrations of unconjugated BPA were several fold lower in the fetuses than in the dams, and internal exposure as measured by AUC was 0.43-fold of the exposure in dams given BPA by the intravenous route. Concentrations of unconjugated BPA in the dams were approximately 45.600 ng/ml 5 minutes after IV dosing and declined to below 0.0228 ng/ml 24 hours thereafter.

In this study fetal blood samples were collected from the fetal femoral vein or umbilical cord artery or vein at 0 min (predose) and approximately 5, 15, 30, 60, 120, 180, 240 and 480 min following maternal IV administration of BPA to the dams. After the 480-min post-dose blood collection, the fetuses were extracted from the uterus and euthanized via a pentobarbital overdose. The concentration of 0.0228 ng/ml unconjugated BPA in fetal plasma was reached already 8 hours after dosing. In the amniotic fluid, concentrations of unconjugated BPA were detectable (less than 0.0228 ng/ml) but 10 to 100-fold lower than the conjugated BPA. The concentrations of both conjugated and unconjugated BPA were two orders of magnitude lower in the amniotic fluid compared to the fetal plasma. In the placenta, unconjugated BPA concentration was 2.7 fold higher than in the serum of the dams and the tissue-to-serum ratio for conjugated BPA was 4.5. The data show that the fetus is exposed to unconjugated BPA, but to a lower extent than the dams. In the fetus, the ratio of the concentrations of conjugated to unconjugated BPA in serum was approximately 10 in the first half hour and increased with time to a ratio of 300. This is due to the fact that the unconjugated BPA concentration declined with a half life of roughly 5 hours whereas the concentrations of conjugated BPA remained constant within the observation period, indicating fetal metabolism. Levels of unconjugated BPA in brain were analysed in three fetuses, but only one of three brains contained a measurable level of 1.3 pmol/g above the LOD of 0.4 pmol/g (0.1 ng/g).

In the study of vom Saal et al. (2014), isotope-labelled BPA (d6-BPA) and its conjugated metabolites were measured in serum in non-pregnant and pregnant female rhesus monkeys, their fetuses and amniotic fluid by a reliable isotope-dilution LC/MS analysis. d6-BPA was administered daily by a single oral dose injected into a piece of fruit (400 µg/kg bw per day) or by continuous administration by SC implantation of silastic capsules. A concentration time profile was obtained in the animals orally exposed whereas only one concentration was measured every other day for 12 days in the animals with continuous administration. Concentration time profiles and single concentrations were measured at different time points in pregnancy. The average serum unconjugated d6-BPA concentrations were below 1 ng/ml. Using the AUC of the IV route reported previously in rhesus monkeys (Doerge et al., 2010b) for an IV BPA dose of 100 µg/kg bw, the authors estimated the absolute bioavailability of d6-BPA by the oral route to be about 7.3% in the non-pregnant animals and about 5% for pregnant animals at GD 50. At GD 100, when pregnancy was terminated by hysterotomy in a subgroup, the observed serum concentrations of unconjugated dBPA in pregnant females were lower relative to pre-pregnancy values. Lower concentrations in fetal serum were measured than in maternal serum, 1 hour after oral administration, and levels in fetal serum were below LOQ (0.2 ng/ml) at 3 hours after oral administration. No parent BPA was detected in the amniotic fluid whereas conjugated BPA was present although in much lower concentration than in the fetus. In the maternal deciduas and in the fetal placenta d6-BPA was measured 1 hour after oral administration, and levels of parent compound were below LOQ (0.2 ng/ml) in tissues from one animal and at a similar concentration in the tissues form the second animal at 3 hours after oral administration. The ratio of conjugated BPA-AUC/unconjugated BPA-AUC in serum was several fold higher after oral administration (between 35 and 62) than the ratio for the continuous SC administration (between 1 and 4). This is in line with high first-pass metabolism. The observed differences in pharmacokinetics of d6-BPA, that were evident between pre-pregnancy, early and late pregnancy, reflect changes in maternal physiology during pregnancy with a lower absorption rate and higher renal clearance.

The CEF Panel considered the study of vom Saal et al. (2014) a well performed study that adds to the toxicokinetic knowledge on BPA. It demonstrates that BPA concentration in fetus is low (lower than the maternal concentration) and is not retained in the fetal tissue as well as in placenta. Furthermore, there is only a very low concentration of unchanged BPA in the amniotic fluid which renders study results in humans doubtful which has measured high concentrations of unchanged BPA in amniotic fluid. It confirms that the fetus is able to conjugate BPA (GD 50 and older) and excrete the conjugate into the amniotic fluid. The oral administration was by way of a fruit which was hoarded in the cheek. This would explain the higher bioavailability in this study (7.3% in non-pregnant and 5% in pregnant animals) compared to the findings of the Doerge et al. (2010b) (0.94%) as by hoarding the contact time of a high-concentration BPA point source with the buccal mucosa is prolonged and for the absorbed amount first pass is circumvented.

Gayrard et al. (2013a) performed a toxicokinetic study with sublingual exposure in dogs. They reported that application of concentrated solutions of BPA (50 mg/ml in 40–100% ethanol for a 5 mg/kg bw dose, and 0.5 mg/ml in 1% ethanol in water for a 0.05 mg/kg bw dose) under the tongues of anaesthesized Beagle dogs led to concentrations of unconjugated and conjugated BPA in the venous blood draining the oral cavity (i.e. jugular vein) similar to those produced by intravenous injection of identical doses. The bioavailability values were reported by the authors to be similar for intravenous and sublingual administration. The CEF Panel noted however that the choice of jugular blood sampling after sublingual administration compromised an accurate evaluation of systemic exposure because AUC determination assumes complete mixing of the administered chemical in the blood compartment. The authors noted that when blood was sampled from the cephalic vein in the leg, a site better reflecting systemic exposure, blood concentrations of unconjugated BPA were lower and less variable than those from jugular sampling. Similar to BPA toxicokinetic investigations involving bolus gavage in animals (e.g., Doerge et al., 2010ab) or gelatin capsule administration in humans (Völkel et al., 2002), Gayrard et al. (2013a) also reported that the absolute bioavailability for unconjugated BPA in blood was below 1% after orogastric dosing in dogs. The authors also concluded that "Currently, the results of Teeguarden et al. (2011) do not support sublingual absorption as a major contributor of dietary BPA to a much higher than expected human internal exposure".

In response to a commentary by Teeguarden et al. (2013) addressing the possible implications for human exposure of the toxicokinetic study in dogs with sublingual BPA administration, Gayrard et al. (2013b) clarified that they did not report that "nanograms-per-milliliter serum concentrations of BPA resulting from sublingual absorption are plausible in humans." To further substantiate their statement, Gayrard et al. (2013b) applied the elementary pharmakokinetic (PK) computation of Teeguarden et al. (2013) to the subpopulation of children 6–11 years of age. By using the 95th percentile of the aggregate daily BPA exposure of 0.481 µg/kg bw per day from the 2005–2006 NHANES database for the US population (Lakind and Naiman, 2011), and taking into account their own data from dogs on the maximum systemic plasma concentration (C_{max}) of 64 ng/ml following bolus IV administration of 50 µg/kg bw, their scaling-by-dose approach yielded a maximum initial plasma concentration of 0.6 ng/ml. The CEF Panel noted that if the elementary PK computation had been based on the maximum mixed-systemic plasma concentration (C_{max}) of 20 ng/ml (blood sampling from the cephalic vein in the leg) following sublingual administration of 50 µg/kg bw, the scaling approach would have yielded a maximum plasma concentration of only 0.2 ng/ml. That the average BPA concentration in food of <0.1 mg/kg food (see Section 4.3.2 in Part I - Exposure assessment) is more than 3 magnitudes lower than the concentration of 500 mg/L of the sublingually applied solution is a further argument against nanograms-per-milliliter serum concentrations in the human population. Finally, the CEF Panel further noted that it is hard to imagine a common, chronic exposure scenario in which BPA, which is normally and mainly taken up via food, is administered separate from the food in concentrated solution to the oral cavity to enable substantial sublingual absorption.

3.1.2.2. Data in newborn and immature animals

The group of Doerge published several studies in neonatal CD-1 mice (Doerge et al., 2011b), SD rats (Doerge et al., 2010a), and rhesus monkeys (Doerge et al., 2010b) using an oral administration, IV injection, or SC injection of 100 µg/kg bw d6-BPA (Figure 5).

The oral administration by gavage in mice of postnatal day (PND) 3 (n = 12) resulted in detectable serum concentrations for unconjugated BPA with a C_{max} of 34±25 nM and an AUC of 26 nM×h (Figure 5A, Doerge et al. 2011b). These concentrations were many fold higher than those in adult mice, in which the unconjugated BPA levels were essentially below the limit of detection (= 0.2 nM). With increasing developmental age from PND 3 to PND 21, the unconjugated BPA levels declined progressively to approach adult levels. Under the same dosing conditions, rats of PND 3 (n = 4) had serum concentrations for unconjugated BPA with a C_{max} of 29±16 nM and an AUC of 56 nM×h (Figure 5B, Doerge et al., 2010a) which were several fold higher compared to adult rats. C_{max}, AUC and the elimination $t_{1/2}$ decreased with increasing postnatal age to approach adult levels at PND 21, again indicating the age-dependent maturation of the metabolic capacity. In rhesus monkeys of PND 5 (n = 6), the serum levels of unconjugated BPA were within an order of magnitude of the LOD; the C_{max} of 2.0±2.4 nM and the AUC of 5.7±4.8 nM×h (Figure 5C, Doerge et al., 2010b) were similar to those determined for adult monkeys. Overall, the internal dosimetrics (C_{max}, AUC) for unconjugated and total serum BPA revealed noticeable species-dependent proportions of unconjugated BPA in the total BPA serum concentration. The C_{max}-derived values for the unconjugated form expressed as a percentage of total BPA ranged from 23±17 % (mice) *via* 6.6 % (rat) to 2.9 % (monkey) and the AUC-derived values ranged from 2.2 % (mice) *via* 1.4 % (rats) to 0.1 % (monkeys). These noticeable proportions of unconjugated BPA in neonatal animals contrast with the consistently low proportion of <1% in adult animals.The SC injection in mice of PND 3 (n = 12) revealed a concentration-time profile for unconjugated BPA which in its initial part was similar to that observed under oral dosing (Figure 5D, Doerge et al., 2011b). The similarity was also reflected by SC/oral ratios for C_{max} and AUC of 1.2 and 1.0, respectively. This apparent similarity in the toxicokinetics in newborn mice following SC or oral administration was mice-specific and was explained by the metabolic immaturity, rapid oral absorption, and rapid distribution of unconjugated BPA (Doerge et al., 2011b). The typical differences in toxicokinetics between the SC route and the oral route developed, however, with advancing postnatal age (PND 10 and 21), indicating the maturation of metabolic and elimination processes. An oral bioavailability of 100 % for unconjugated BPA in PND 3 mice was derived based on the ratio of the oral AUC (= 26 nM×h) to that of 26 nM×h for SC injection.

In contrast to the toxicokinetic immaturity of neonatal mice, neonatal rats of PND 3 (n = 12) showed substantially larger SC/oral ratios for C_{max} and AUC of 34 and 17, respectively, indicating the presence of a first-pass metabolism in these early postnatal pups (despite some evidence for diminished Phase II metabolic capacity) (Figure 5E, Doerge et al., 2010a). Compared to oral dosing, there was no statistically significant, postnatal age-related decrease in the unconjugated-BPA fraction of the C_{max} values from SC administration, which further emphasized the importance of first-pass metabolism in neonatal rats following oral administration. An oral bioavailability of 6.0 % for unconjugated BPA in PND 3 rats was derived based on the ratio of the oral AUC (= 56 nM×h) to that of 930 nM×h for SC injection.

Compared to newborn animals, in rhesus monkeys of PND 77 (n = 5), the IV dosing revealed a rapid elimination of unconjugated BPA (Figure 5F, Doerge et al., 2010b) similar to that in adults. Based on the AUC values for orally dosed monkeys of PND 70 and for IV injected monkeys of PND 77, an oral bioavailability of 1.9±1.8 % for unconjugated BPA was obtained. Overall, the data for oral administration and SC/IV injection show an age-dependent maturation of metabolic capacity of different magnitude and rats and mice, and a metabolic capacity in neonatal monkeys that already approached adult levels.

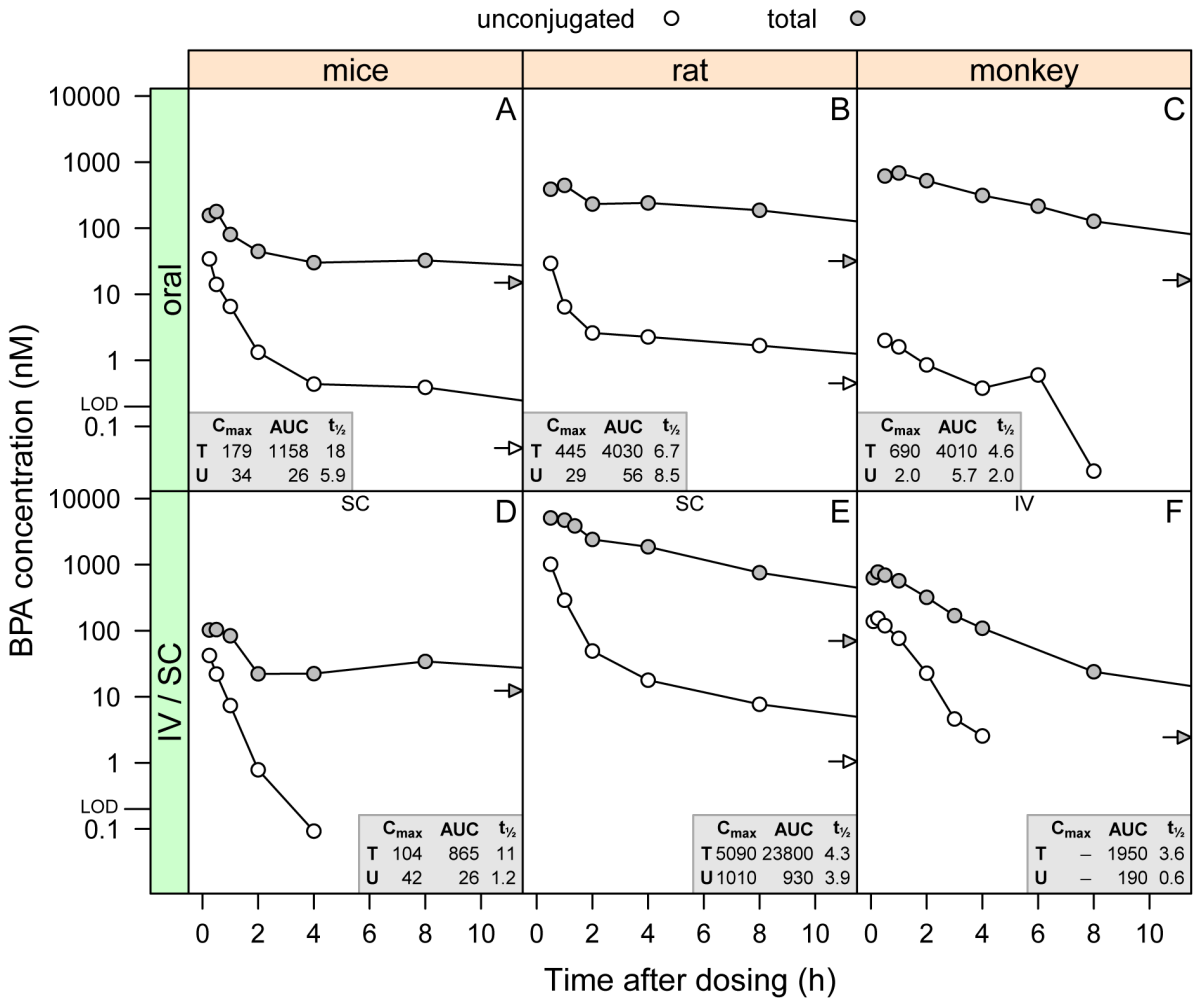


Figure 5: Time course of serum levels of unconjugated and total BPA in newborn mice (PND3), rats (PND3), and rhesus monkeys (PND70, PND77) following oral administration, IV injection, or SC injection of a single dose of 100 µg/kg bw of isotope-labelled (deuterated) d6-BPA. Horizontal arrows indicate the serum concentration after 24 h. The LOD was 0.2 nM in all experiments. Additionally given are the pharmacokinetic parameters for unconjugated (U) and total (T) BPA, comprising the maximum serum concentration Cmax (nM), the area under the curve AUC (nM×h) from time zero to infinity, and the elimination half-life t½ (h). The data shown were taken from Doerge et al (2011b), Doerge et al (2010a) and Doerge et al (2010b).

Prins et al. (2011) measured unconjugated and total BPA levels in serum from PND3 male SD rats using isotope-dilution HPLC-MS-MS (LOD: 0.05 ng/ml) following oral administration and SC injection of 10 µg/kg bw of BPA. The doses were dissolved in corn oil and were either administered through gentle feeding with a pipette tip (oral bolus dosing) or by SC injection of a ~8–10 µl depot in the nape of the neck. Pups were killed by decapitation at 0.5, 1 and 2 h after dosing and blood was collected for preparation of serum. Sera from 8–10 pups at each time point and route of exposure were pooled, and 3–5 separate sample pools at each time point for both oral and injection exposure routes were used for BPA quantitation. The recovery of the internal (deuterated) BPA standard was 58%±11%, and the mean recovery of native BPA standard spiked to selected sample matrices and passed through the entire analytical procedure was 84% (range: 62–114%). In the experiment with SC injection, serum C_{max} levels seen at 0.5 h were 1.77±0.63 (mean ± s.e.) and 2.00±1.00 ng/ml for unconjugated and total BPA, respectively, suggesting 88% of total BPA being in the free bioavailable form at this early time point. In the experiment with oral administration, C_{max} values for unconjugated and total BPA of 0.26±0.04 and 1.02±0.30 ng/ml were observed at 0.5 h and 1 h, respectively; the

proportions of unconjugated BPA at these two time points were 29% and 21%, respectively. The authors additionally calculated the area under the curve from time zero to 2 h ($AUC_{0–2}$) for unconjugated and total BPA, which was found to be 4.1-fold and 1.8-fold greater, respectively, for SC injection compared to oral administration.

The CEF Panel compared the toxicokinetic results of Prins et al. (2011) for PND3 rats with oral bolus dosing and SC injection of BPA in corn oil with the results of Doerge et al. (2010a) for PND3 rats with gavage dosing and SC injection of d6-BPA in 10% aqueous EtOH/DMSO solution. Scaling the administered dose used by Prins et al. (2011) to the common dose of 100 µg/kg bw, and translating the serum levels and AUC values to molar-based values, yielded dose-adjusted C_{max} and $AUC_{0–2}$ values for unconjugated BPA of 11 nM and 18 nM×h, respectively, which agreed well with the respective values (C_{max} = 29 nM, $AUC_{0–2}$ = ~21 nM×h) derived from Doerge et al. (2010a). The somewhat larger difference in the C_{max} values could be discussed in the context of different types of oral administration and the use of the different vehicles (see, e.g., Gallo et al., 1993). Given the good correspondence between the $AUC_{0–2}$ values for unconjugated BPA, it was unexpected to find a large discrepancy in the $AUC_{0–2}$ values for total BPA. The data of Doerge et al. (2010a) showed an $AUC_{0–2}$ of ~644 nM×h, whereas an approximately 10-fold lower $AUC_{0–2}$ of 70 nM×h was obtained for Prins et al. (2011). Given that Prins et al. (2011) did not report quality control measures to check the efficiency of enzymatic deconjugation (e.g., use of serum containing conjugated BPA, acid hydrolysis control), an insufficient deconjugation of the conjugated serum BPA cannot be excluded. The data of the SC injection experiment could support this explanation, since Prins et al. (2011) reported 88% of the total BPA in serum being still in the unconjugated form after 0.5 h. Again, this high proportion is an unexpected result when considering that at 0.5 h the levels of unconjugated BPA are already strongly decreasing whereas total BPA has already reached more or less a plateau level. Extrapolation of the serum concentration time profiles for unconjugated and total BPA to time points somewhat earlier than 0.5 h would lead to the impossibility of unconjugated BPA levels exceeding those of total BPA. The CEF Panel noted, however, that alternative explanations for the relatively high proportions of unconjugated BPA such as sample contamination and/or inadequate control of de-conjugation during sample collection and clean-up can also not be excluded, because critical quality control measures (such as used in Doerge et al., 2010a) were not reported, and serum concentration levels at time zero (i.e. taken from non-exposed control animals) and at time points >2 h were not available. Moreover, the fact that $AUC_{0–2}$ and C_{max} values for unconjugated BPA following SC injection were 10–13-fold lower than in Doerge et al. (2010a) could indicate an additional problem, the reduced systemic absorption of BPA from the injected corn-oil depot. The CEF Panel therefore assigned a low reliability to the pharmacokinetic data of Prins et al. (2011).

3.1.2.3. Summary of BPA Toxicokinetics and Metabolism in animals

New information compared with previous risk assessments came from several toxicokinetic studies using specific and sensitive methods and dosing with deuterated BPA. Data obtained following oral versus subcutaneous exposure in rodents indicate low first-pass metabolism in neonates. The results indicate maturating metabolic capacity in rodents with age. In monkeys the metabolic capacity was similar between adults, juvenile and newborn animals. A study in monkeys and in mice of a second research group (Taylor et al., 2011) confirmed the linear kinetics of BPA in mice. However, it also showed maximum plasma unconjugated BPA concentrations (normalised by the dose) in mice and monkeys that were approximately 5 to 20 fold higher than the levels reported by the group of Doerge. Overall the animal data indicate that the systemic availability of unconjugated BPA by the oral route varies between the species, being 0.45% of the dose in mice, 0.9% in monkeys and 2.8% in rats (Doerge et al., 2010a,b, 2011a,b, 2012; Fisher et al., 2011).

Studies in pregnant rats indicate that unconjugated BPA does cross the placenta and its glucuronide is formed in the fetal compartment. Data in rats indicate that in early pregnancy exposure to the fetus is greater compared to later pregnancy based on serum concentrations at GD 20. This finding is apparently the result of fetal Phase-II metabolic capacity that increases throughout gestation in rats (Doerge et al., 2011a). The concentration in the fetal brain was 4-fold of the concentration in the

maternal serum at GD 20. However, also in adult rats the brain concentration is roughly 3-fold of the concentration in serum, which reflects the high fat contents of this tissue. Both unconjugated BPA and BPA-conjugates can be measured in the amniotic fluid of rats and rhesus monkeys at concentrations lower than those in maternal serum (Doerge et al., 2011a; Patterson et al., 2013). However, the levels of conjugates consistently exceed those of unconjugated BPA in amniotic fluid from both rats and monkeys.

Unconjugated and conjugated BPA is found in milk of rat dams orally dosed daily with 100 µg/kg bw per day (Doerge et al., 2010a). The amount delivered to the pups is so small that the concentrations in pup serum are below 0.2 nM (45.6 pg/ml), and therefore pup exposure via lactation is therefore extremely low (1/300 of the maternal dose). BPA does not accumulate in the body even though the concentration of unconjugated BPA in fat is 5-fold higher than the concentration in serum in rats 2 h after injection (Doerge et al., 2011a) and 6.9-fold higher than the serum concentration in mice 1 h after injection (Doerge et al., 2012).

3.1.2.4. Human studies

BPA in urine and serum in the general population after oral exposure

A controlled dietary exposure study in 20 healthy volunteers exposed to BPA via food by eating three defined meals gives important insight into the internal exposure over a 24-hours period (Teeguarden et al., 2011). Although the BPA content of the food was not measured, the exposure to BPA was reliably estimated based on total collection of urine over the whole study period. The average daily exposures amounted to 0.27 µg/kg bw (range, 0.03-0.86 µg/kg bw). Unconjugated BPA concentrations in serum and urine were consistently below the LOD which was 1.3 nM (0.3 ng/ml) in the serum and 1.8 nM (0.4 ng/ml) in the urine. Serum samples containing detectable levels of total BPA were also analysed independently in a second laboratory in which the LOD varied within the range of 0.2-0.7 nM. A serum concentration-time course of total BPA was observable only in six individuals with exposures being 1.3 - 3.9 times higher than the 95th percentile of aggregate US exposure. The highest C_{max} values for total BPA were always below 1.3 nM. The peak serum concentrations for total BPA in serum occurred about one hour earlier than that in urine, which occurred at 2.75 hours (range, 0.75-5.75 hours) after a meal. The study results are considered reliable as the analytical measurements were performed in two independent laboratories using a validated up to date method (on-line HPLC-isotope dilution tandem mass spectrometry) and extensive measures were taken to avoid contamination and to identify possibly contaminated samples.

BPA in urine in a controlled exposure study to BPA in thermal paper

Ehrlich et al. (2014) performed a pilot study involving the simulation of dermal exposure of human volunteers to thermal paper in a cross-over design. In the first simulation experiment, participants printed and handled receipts continuously for 2 hours without gloves. After a washout period of at least 1 week, a second simulation experiment was conducted in which the participants repeated the handling of receipts while wearing nitrile gloves. The participants provided a spot urine sample immediately before handling of receipts and 4 hours later. The urine was collected polypropylene specimen cups and stored in polypropylene cryogenic vials. The urinary concentration of total BPA was measured at the US CDC, and the concentrations of BPA were adjusted for specific gravity to account for urine dilution. In the group handling thermal paper without gloves (n=23 participants), the geometric mean (GM) concentration of urinary (total) BPA was 1.8 µg/L (95% CI: 1.3-2.4 µg/L) before simulation and 5.8 µg/L (95% CI: 4.0-8.4 µg/L) post-simulation; the increase was statistically significant ($p<0.05$). 12 of the 23 participants provided sequential urine samples following the receipt handling; the GM concentrations were 2.1 µg/L at baseline, 6.0 µg/L at 6 h, 11.1 µg/L at 8 h, 10.5 µg/L at 12 h, and 4.7 µg/L at 24 h. In the group handling thermal paper with gloves (n=12 participants), no significant increase in urinary BPA after the simulation was observed.

The CEF Panel noted as a weaknesses that the BPA content in thermal paper and the urinary output (i.e. volume) were not measured. Nevertheless, the study results make it possible to obtain a rough estimate of the dermal exposure to BPA in thermal paper. From the experiment with sequential urine sampling over 24 h, a time-averaged mean concentration for total BPA of 7.7 µg/L can be calculated. This level is 5.6 µg/L above the baseline. Assuming a standard urinary output of 1.4 L per day and a body weight of 70 kg yields an internal exposure to total BPA of 0,112 µg/kg bw per day resulting from the dermal contactto BPA in thermal paper. Although this estimate is representative for occupational exposure to BPA in thermal paper, it provides and upper bound or worst case from the consumer perspective.

BPA concentrations in mothers and fetuses in different stages of pregnancy

Total BPA in serum and umbilical cord blood: In the study of Kosarac et al. (2012) total BPA concentrations in human maternal serum were measured at mid-pregnancy and at delivery and ranged from <0.026 ng/ml to 10.425 ng/ml (median 0.548 ng/ml, n=12) and <0.026 ng/ml to 3.048 ng/ml (median 1.461 ng/ml, n=12), respectively. Matching umbilical cord serum concentrations of total BPA were in the range of <0.026-2.569 ng/ml (median 1.823 ng/ml; n=12). The CEF Panel considered that although the analytical methodology used was sound, the study had some shortcomings. The CEF Panel considered that the biomonitoring data reported have low credibility due to limited reporting, in particular with respect to sample collection and handling, and discrepancies with other studies, in particular with that of Teegarden et al. (2011). In the latter study no unconjugated BPA could be measured (LOD 0.3 ng/ml) and total BPA was measurable only in 6 out of 20 subjects which had peak concentrations of 2.6 - 5.7 nM (corresponding to 0.6 - 1.3 ng/ml) (Teeguarden et al., 2011).

Gerona et al. (2013) used isotope-dilution LC/MS/MS to directly measure concentrations of unconjugated, glucuronidated and sulfated BPA in umbilical cord serum collected from 85 study participants during elective second trimester pregnancy terminations. The LODs were 0.05 ng/ml (unconjugated and glucuronidated BPA) and 0.025 ng/ml (sulfated BPA), the LOQ was 0.1 ng/ml for all three analytes. d16-BPA was used as (surrogate) internal standard for all analytes. Procedural blanks and quality control materials at low and high concentrations (0.5 and 20 ng/ml) were included. To reduce the likelihood of contamination, the authors used glass/polypropylene containers for sample collection, processing, and storage containers. Umbilical cord blood was drained directly into collection tubes during the procedure to avoid contact with medical devices and the environment. The authors additionally performed a field blank testing by simulating the sample collection, extraction, and analytical run using double-charcoal stripped serum and synthetic human serum. No BPA was detected ≥ LOD in any of the sample collection and extraction materials or in any of the instruments used in the analysis. The possible contamination from the IV equipment (IV bag, needles and tubing) and medical equipment (gloves, laminaria) used during the termination procedure was also evaluated. The analysis of the umbilical cord serumsamples revealed detection levels of 47%, 76%, and 96% for unconjugated, glucuronidated and sulfated BPA. The GM concentrations were 0.16 ng/ml for unconjugated BPA (range: <LOD–52.26 ng/mL), 0.14 ng/ml for glucuronidated BPA (range: <LOD–5.41 ng/mL), and 0.32 ng/ml for sulfated BPA (range: <LOD–12.65 ng/mL). The concentration ratio of unconjugated BPA/conjugated BPA ranged from 1:100 to >400:1. The CEF Panel noted that extreme concentration ratios such as 400:1 between the unconjugated and conjugated BPA forms are not in line with the evidence from toxicokinetic studies in pregnant monkeys (vom Saal et al., 2014) and questions the attempts of the authors to have the sample contamination and the exposure to BPAfrom medical devices fully under control. The CEF Panel also noted the specific exposure situation of pregnant women and fetuses during the termination procedure, which makes an extrapolation of the results to pregnant women in general (i.e. outside a hospital setting) questionable.

Transplacental transfer rate: The transplacental transfer rate in human placentas was measured in *ex vivo* experiments in a multi-centre study (Mose et al., 2012). Based on their results the authors concluded that unconjugated BPA has a transplacental transfer rate of 1 (concentration at the fetal

site/concentration of the maternal site =1) explained by passive diffusion. The result of the study is comparable to a study published earlier (Balakrishnan et al., 2010) also reporting a factor of 1. Thus, in late pregnancy the concentration in the fetal blood is unlikely to be higher than in the blood of the mother.

BPA concentrations in amniotic fluid and fetal liver samples: measurements of amniotic concentrations of unconjugated BPA and BPA-conjugates by Edlow et al. (2012) were performed according to current standards. Unconjugated BPA was detected in 9/20 second trimester samples; levels ranged from 0.31 to 0.43 ng/ml (median 0.38 ng/ml) and in 1 out of the 20 samples in the third trimester. When detected, unconjugated BPA comprised 83% and 91% of total BPA in second and third trimester amniotic fluid, respectively, whilst in experimental human studies less than 10% of total BPA in serum is considered to be unconjugated BPA. In addition, it has previously been reported that in humans, concentrations of unconjugated BPA after meals with canned food were below the level of detection (<0.3 ng/ml) (Teeguarden et al., 2011) and the placental transfer rate in humans is 1 (Mose et al., 2012; Balakrishnanet al., 2010). In rats, the concentration of BPA in amniotic fluid is 0.35-fold of the maternal concentration and levels of conjugates consistently exceeded those of unconjugated BPA in both rats and monkeys (Doerge et al., 2011a; Patterson et al., 2013). Thus it is improbable that high proportions of unconjugated BPA in amniotic fluid, as reported by Edlow et al. (2012) can be due to excretion by the fetus. The CEF Panel considered that the observed results might be explained by deconjugation of BPA-conjugates excreted in the amniotic fluid or false positive responses near the LOQ.

In a study of Nahar et al. (2013) in 50 first- and second-trimester human fetal liver samples, the internal levels of unconjugated BPA and conjugated BPA were measured and gene expression of biotransformation enzymes specific for BPA metabolism was evaluated. Both unconjugated BPA and conjugated BPA concentrations in the fetal livers varied widely, with unconjugated BPA (GM: 2.26 ng/g tissue) exhibiting three times higher concentrations than conjugated BPA concentrations (GM: 0.65 ng/g tissue). As compared to gender-matched adult liver controls, UDP-glucuronyltransferases, sulfotransferases and steroid sulfatase genes exhibited reduced expression whereas β-glucuronidase mRNA expression remained unchanged in the fetal tissues.The CEF Panel considered that shortcomings in the study description with regard to liver sample isolation, procedure of surgery and surgical instrument used to avoid contamination and deconjugation of BPA during sample handling, hamper the usefulness of the study results.

<u>BPA concentrations in early life</u>

Nachman et al. (2013) measured the content of unconjugated BPA and BPA-glucuronide in the urine of newborns and young infants (see also Section 4.6.2 in Part I - Exposure assessment)). The study population consisted of 11 healthy neonates plus 1 young infant (median age 17 days) born to healthy non-smoking mothers. Urine samples were collected using BPA-free pediatric urine collection bags during the neonates' regular well-child care visits. After voiding the urine was transferred on ice to the laboratory, transferred to a pre-cleaned glass vial which was stored at -70°C until analysis. The average concentration of BPA glucuronide, as measured in all of the duplicate urine samples, was 0.87± 0.51 ng/ml (median: 0.66 ng/ml). Unconjugated BPA was not found in any of the urine samples with the exception of one sample whose replicate sample was a non-detect. With the exception of one fully breastfed baby, all babies received infant formula. The study demonstrates that neonates and infants are capable of conjugating BPA to the BPA-glucuronide.

The study of Christensen et al. (2012) evaluated the urinary excretion of conjugated BPA in five volunteers during a course of a two days fasting (0-48 hrs). In four of the five volunteers the amount of conjugated BPA excreted in the urine declined during the fasting period to 5% of the amount on day 1 by the second day. In one of the volunteers the urinary excretion increased between 32 and 42 hours without a defined exposure. According to the authors, the study shows that even after the oral exposure to BPA by meals ceases, BPA is still excreted from the body indicating (a) non-food exposure to BPA or (b) excretion of BPA from store tissue such as lipid tissues. However, the CEF

Panel noted that the conclusion under (b) was inconsistent with the results of well-controlled animal studies showing that there is no toxicologically relevant accumulation in fat tissue.

Other information:

There are several other studies on BPA (Krotz et al., 2012; Cao et al., 2012a; Geens et al., 2012; Genuis et al., 2012) which are not relevant or appropriate to be taken into consideration, as explained in Appendix B.

- In reproductive age women undergoing infertility treatments there is little transfer or accumulation of BPA into the microenvironment of the human preovulatory oocyte as reported by Krotz et al. (2012). However, the low number of subjects in the study (n=5) preclude generalization of the results.
- A high ratio of unconjugated BPA/total BPA in placenta samples and in samples of fetal liver is also given in the study of Cao et al. (2012a). As reported by Doerge et al. (2011a) tissue samples should be handled deep frozen to avoid that ß-glucuronidase present in the tissue can release unconjugated BPA from conjugated BPA. Uncertainties about the handling of samples preclude conclusions from the study of Cao et al. (2012a)
- The study of Geens et al. (2012) used human material obtained by autopsies in deceased patients, aged 9-62 years, and measured BPA in brain, liver and fat. They did not find metabolites in the liver, in which tissue in animal studies the highest concentrations of the metabolites have been measured, explained by the high levels of the conjugating enzymes in this tissue. This may point at post-mortem changes.
- Genuis et al. (2012) reported on concentrations of total BPA in serum, urine and sweat. Their results are highly improbable as high concentrations in sweat were reported in subjects without a measurable concentration in serum, which is impossible because sweat in humans is produced as an ultrafiltrate of the blood.

3.1.2.5. Summary of BPA Toxicokinetics and Metabolism in humans

The new data published since 2010 confirm that after oral exposure to BPA the concentrations of the unconjugated BPA in plasma and urine of humans are so low that they can only be detected/quantified with analytical methods with a LOD of < 1.3 nM (0.3 ng/ml). The transplacental transfer ratio of BPA in humans is reported to be 1. Therefore, in late pregnancy the concentrations in the fetal blood are expected to be similar to the blood of the mother. Levels of unconjugated BPA were found in the livers of first and second trimester fetuses which were 3-fold higher than the concentrations of BPA conjugates. The specimens were from induced abortion. As the surgical procedures, the sort of surgical instruments used and the liver sample isolation from fetal tissues are not described, it remains open whether the results are due to contamination by hospital processing of the samples. In the urine of healthy newborn and young infants only conjugated BPA was found. Several other studies showing high concentrations of unconjugated BPA in biological fluids have several methodological shortcomings, e.g. not avoiding contamination by medical instruments, storage of samples over years without confirmation that the samples would be stable, in particular ensuring that no deconjugation of BPA conjugates may occur during storage and during thawing the samples (Liao et al., 2012, Krotz et al., 2012, Cao et al., 2012a, Geens et al., 2012, Genuis et al., 2012.

3.1.2.6. In vitro studies

Native microsomes were used from rat and from human liver and intestine to study the enzyme kinetics of glucuronidation of BPA (Mazur et al., 2010). BPA glucuronidation in liver microsomes was sex dependent. Female rat and female human liver microsomes had a higher V_{max} values than those from males. K_m for glucuronidation was much higher in female rats than in humans and male rats. The dissimilar K_m measured for female rat microsomes together with inhibition studies suggests that different UDP-glucuronosyltransferase (UGT) enzyme(s) are involved in BPA glucuronidation in rats, UGT2B7 and UGT2B15 being candidates. Human intestinal microsomes (mixed gender) showed little BPA glucuronidation activity compared with those from male rat intestine, which in the presence

of alamethicin, a membrane-disrupting agent, exhibited a V_{max} that was nearly 30-fold higher than that for mixed human microsomes.

In a further study by Mazur et al. (2012), BPA and its major metabolite BPA-glucuronide (BPA-G) showed significant interspecies differences in kinetics in vitro. ATP-Binding Cassette (ABC) transporter enzymes were considered to play important roles in the physiological processes underlying the kinetics. P-glycoprotein (MDR1), multidrug resistance-associated proteins (MRPs), and breast cancer-resistant protein (BCRP) were investigated in rat and human tissues.The results reported suggest that BPA is likely a substrate for rat mdr1b but not for human MDR1 or rat mdr1a whereas BPA is a potential substrate for rat mrp2 and human MRP2, BCRP, and MRP3. BPA-G had the highest apparent substrate binding affinity for rat mrp2 and human MRP3. It was not active or even a potential inhibitor for human MRP2, MDR1 and BCRP and for rat mdr1a, mdr1b, and bcrp. Theauthors suggested that substrate specificity of ABC transporters might be explained by differences in amino acid sequences of putative substrate-binding sites and that apical efflux transporters would transport unconjugated BPA into the bile and/or into the intestinal lumen, while BPA-glucuronide would undergo a similar transport pathway in rat. In humans, due to the basolateral location of the MRP3 transporter, BPA-glucuronide would likely enter the hepatic blood.

The study of Trdan Lušin et al. (2012) aimed to gain insight into intestine, kidney, liver, and lung glucuronidation of BPA, human microsomes of all tested organs were used. Human lung microsomes did not show glucuronidation activity towards BPA. While the liver intrinsic clearance was very high (857 ml min^{-1} (kg body weight)$^{-1}$), the tissue intrinsic clearances for the kidney and intestine were 8.0 and 2.1 ml min^{-1} (kg body weight)$^{-1}$. Since BPA is a UGT1A1 substrate, the authors postulated that the common UGT1A1*28 polymorphism influences BPA glucuronidation, and consequently, BPA detoxification. Hepatic tissue intrinsic clearances for UGT1A1*1/*1, UGT1A1*1/*28, and UGT1A1*28/*28 microsomes were 1113, 1075, and 284 ml min^{-1} (kg body weight)$^{-1}$, respectively. These in vitro results show that the liver is the main site of BPA glucuronidation (K_m 8.9 µM, V_{max} 8.5 nmol min^{-1} mg^{-1}) and BPA metabolism may be significantly influenced by a person's genotype (K_m 10.0–13.1 µM, V_{max} 3.4–16.2 nmol min^{-1} mg^{-1}).

3.1.2.7. Summary of in vitro studies relevant to the toxicokinetics of BPA

From the in vitro studies of Mazur et al. (2010) and Trdan Lušin et al. (2012) it can be concluded that local BPA metabolism in the lung does not play a role. Hence, following this route of exposure, no first-pass metabolism has to be taken into consideration. The intrinsic clearance in the intestine is low (2.1 ml min^{-1} (kg body weight)$^{-1}$) compared to the intrinsic clearance of the liver (857 ml min^{-1} (kg body weight)$^{-1}$). Hence, the presystemic elimination (first pass) equals the hepatic clearance. Glucuronidation of BPA is catalysed by multiple UGT isoforms in the order of catalyic efficiency of 2B15 > 1A9 > 2B7 > 1A8 > 1A1 > 1A3. Some of the UGTs are polymorphically expressed and are expressed at birth at a lower expression level than in the adult (Miyagi and Collier, 2011). Transporters may influence absorption in the rat and humans (Mazur et al, 2012).

The CEF Panel concluded that the in vitro studies contribute to the understanding of the mechanisms by which BPA is metabolized in humans.

3.1.3. Physiologically based pharmacokinetic (PBPK) modelling in humans

No toxicokinetic study in humans is currently available to inform about the time course of unconjugated BPA concentrations in plasma (serum). Experimentally derived internal dosimetrics (e.g., AUC) for unconjugated BPA in humans are therefore lacking to support the human-equivalent dose (HED) approach (see Sections 3.1.3.4 and 3.1.5). However, several physiologically based pharmacokinetic (PBPK) models for oral exposure in humans have been developed to enable predictions of serum concentration-time profiles and estimations of internal dose metrics for a given oral dose. In the following, an overview on existing PBPK models for humans is given, continued by

a description of model predictions on serum BPA levels for adults and newborns, and followed by the derivation of PBPK model-based internal dosimetrics for the HED approach.

3.1.3.1. Overview on PBPK models in humans

PBPK models for the oral exposure in humans have been developed by Teeguarden et al. (2005), Mielke and Gundert-Remy (2009), Edginton and Ritter (2009), and Fisher et al. (2011). Yang et al. (2013) developed a PBPK model for neonatal and adult rats with implications for the extrapolation of toxicity studies from neonatal rats to neonatal monkeys or infant humans. In addition, Yang et al. (2013) used the monkey-based model of Fisher et al. (2011) to predict internal dosimetrics for humans with oral exposure. All PBPK models are based on the same general model structure (see e.g. Figure 6) consisting of a group of tissue compartments.

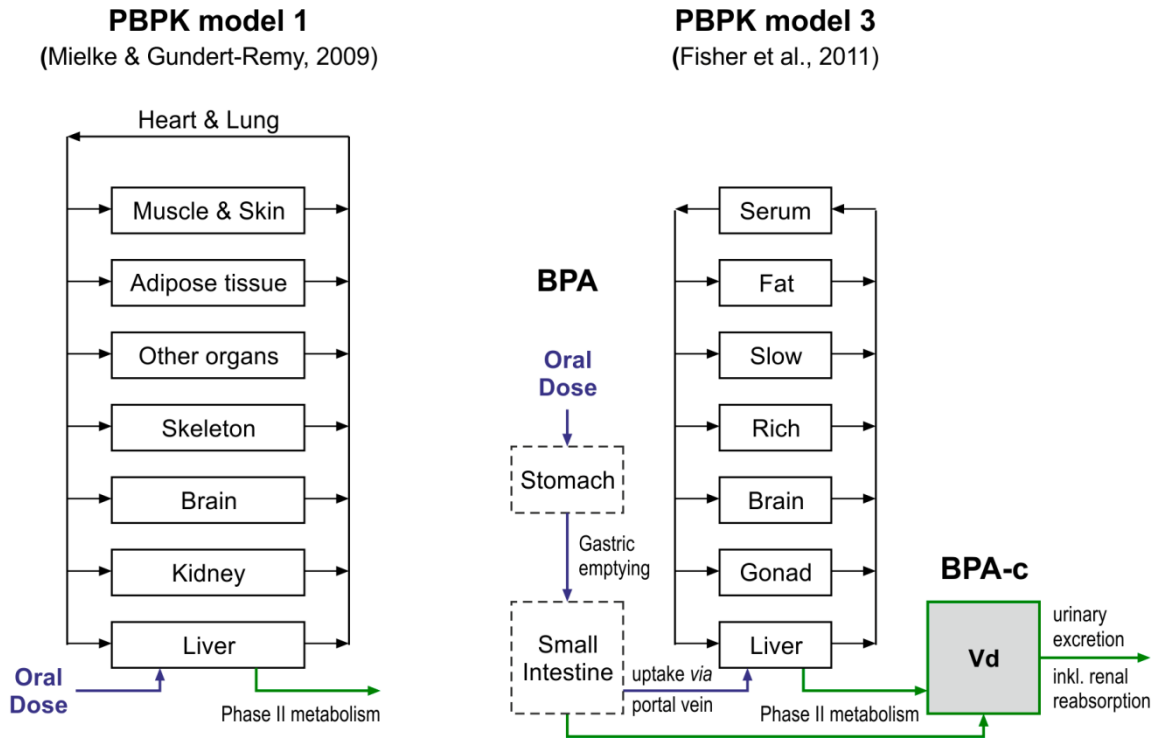


Figure 6: Structure of two example PBPK models for oral exposure in humans. Left: Human-based PBPK model for unconjugated BPA (Mielke and Gundert-Remy, 2009). Right: Monkey-based PBPK model which was used for the extrapolation to humans (Fisher et al., 2011). The compartments "Slow" and "Rich" refer to the slowly and richly perfused tissues.

These compartments are defined by their volume, blood flow, and tissue-blood partition coefficients, and a perfusion-rate-limited kinetics is assumed for describing the distribution of the chemical between the blood and the tissues (Andersen, 1981). The PBPK models differ in the number and kind of the tissue compartments for unconjugated BPA, in respect to the incorporated ADME processes (e.g., consideration of intestinal first-pass metabolism in addition to the hepatic first pass), and in the detail of describing the fate of conjugated BPA. Also, the PBPK models originate from either animal-based or human-based concepts. Common to all PBPK models is the calibration/evaluation against toxicokinetic data (i.e. the serum-concentration time course for conjugated BPA and the information that the serum-concentration time course for unconjugated BPA is below the LOD of 10 nM) for human adults with low-dose oral administration (5 mg d16-BPA, i.e. 54–90 µg/kg bw) (Völkel et al., 2002).

Teeguarden et al. (2005) developed a PBPK model consisting of a five-compartment submodel (gastrointestinal [GI] tract, liver, blood, uterus, body of remaining tissues) for BPA, a two-

compartment submodel (volume of distribution, GI tract) for glucuronidated BPA, oral and intravenous inputs, and outputs *via* urinary and fecal excretions of glucuronidated BPA. The model was initially developed for rats and incorporated rat-specific toxicokinetic processes such as biliary secretion, enterohepatic recirculation (EHR), and predominant fecal elimination of conjugated BPA; it was later extended to humans. Hepatic glucuronidation of BPA was assumed to be the only metabolic process in the rat and human PBPK models since the experimental data available were insufficient to include intestinal first-pass metabolism. However, the authors concluded from rat pharmacokinetic data that the ~7-fold greater metabolism V_{max} observed for the oral route vs. intravenous was due to additional first-pass metabolism in the GI tract. A specific model feature was the consideration of protein binding in plasma and the oestrogen receptor binding in uterine tissue. Unknown/uncertain parameter values for gastrointestinal absorption, metabolism, excretion, and EHR in rats were estimated by fitting to oral gavage data in rats. Other parameter values such as the volume of distribution for the conjugated BPA were fitted to human toxicokinetic data and the Michaelis constant for glucuronidation was taken from *in vitro* studies with rat liver microsomes/hepatocytes. Extending the model to humans required (apart from adjusting the physiological parameters) to scale the parameters for metabolism and elimination to human toxicokinetic data (Völkel et al., 2002). In rats, the predicted oral-route blood kinetics were well-described for BPA but less exactly for glucuronidated BPA. The human PBPK model accurately simulated the plasma concentration for conjugated BPA until 12 hours after dosing but under-predicted the concentration afterwards (24–48 h post-exposure). For the plasma concentration of unconjugated BPA, which was not detectable in the Völkel et al. (2002) study because of the LOD of 10 nM, the human PBPK model provided an upper-bound estimate for the concentration-time profile which approached the LOD.

Mielke and Gundert-Remy (2009) developed a human-based PBPK model for unconjugated BPA consisting of eight tissue/organ compartments (including blood), an oral input to the liver *via* a dosing compartment, and an output from the liver as a consequence of the phase II metabolism (Figure 6). The authors acknowledged the first-pass effect in the gastrointestinal tract but regarded its contribution to the hepatic first pass as low; the first-pass metabolism in the gastrointestinal tract was therefore not taken into account in the model. Absorption was set as 90% of the oral dose corresponding to the recovery of total BPA in urine in human volunteers (Völkel et al., 2002). The liver metabolism included glucuronidation and sulfation pathways, and the metabolic parameters were based on *in vitro* data from human liver cells (Kuester and Sipes, 2007). Parameter sets for different age groups ranging from newborns to adults (males) were provided based on age-specific human physiological parameters and a set of experimentally determined tissue:blood partition coefficients for rats. The glucuronidation activity (assumed to be exerted by the isoform UGT 2B15) in a newborn was set at 5% of the activity in adults. The sulfation capacity (assumed to be mediated by the enzyme isoform SULT 1A1) in adult humans was assumed to be 15% of the glucuronidation capacity, and the expression of sulfation enzymes at birth was considered to be at the same level as in adults. The absorption half-life was set based on published peak concentrations of (total) BPA in urine and of conjugated BPA in plasma (Völkel et al., 2002). The evaluation of the PBPK model was based on the prediction of the blood concentration-time profile for unconjugated BPA after a single oral dose of 5 mg to an adult, which simulated the exposure scenario used in the Völkel et al. (2002) study. The predicted concentration-time profile exceeded only slightly the level of 10 nM (Figure 7A), which was consistent with the findings of Völkel et al. (2002) who did not detect unconjugated BPA above the LOD of 10 nM in blood taken 40 min after dosing. Accordingly, the predicted serum concentration profile for unconjugated BPA is to be regarded as an upper-bound estimate.

Edgington and Ritter (2009) built a human-based PBPK model for adults and children < 2 years which consisted of structurally identical multi-compartment submodels for unconjugated and conjugated BPA. Each submodel was comprised of 15 organ compartments and 3 blood compartments. The two submodels were coupled by the hepatic glucuronidation of BPA. The input of BPA into the portal vein was implemented by a physiologically based model for gastrointestinal transit and absorption, and the output was by renal clearance of glucuronidated BPA. 100% of the applied BPA dose was modelled as absorbed to the portal vein (i.e. intestinal first-pass metabolism

was not considered). The PBPK model additionally included protein binding in plasma as well as sub-compartments for red blood cells, plasma, interstitial and cellular spaces. The authors considered the UDP-glucuronosyltransferase isoform UGT 2B7 as the enzyme responsible for BPA glucuronidation, and they used the enzyme ontogeny of this isoform to scale the intrinsic hepatic clearance of BPA to glucuronidated BPA from adults to children. In concrete terms, the UGT 2B7 activity in a term newborn was assumed to be 5% of the activity in adults. The model was parametrized using anatomical and physiological data for adults and children and by using an algorithm for partition coefficient estimation based on physicochemical properties. Unknown or uncertain parameter values for the clearances, the lipophilicity of glucuronidated BPA (required for calculation of partition coefficients), and the intestinal permeability of BPA were estimated for the adult model by fitting the predicted plasma concentrations to the toxicokinetic data of Völkel et al. (2002). Specifically, the intrinsic hepatic clearance of BPA was set to the lowest integer that maintained the plasma concentrations of unconjugated BPA at time points ≥ 51 min below the 10-nM LOD of the Völkel et al. (2002) study (Figure 7B). The thus optimized adult model was then scaled to children by means of allometric scaling functions for ADME processes.

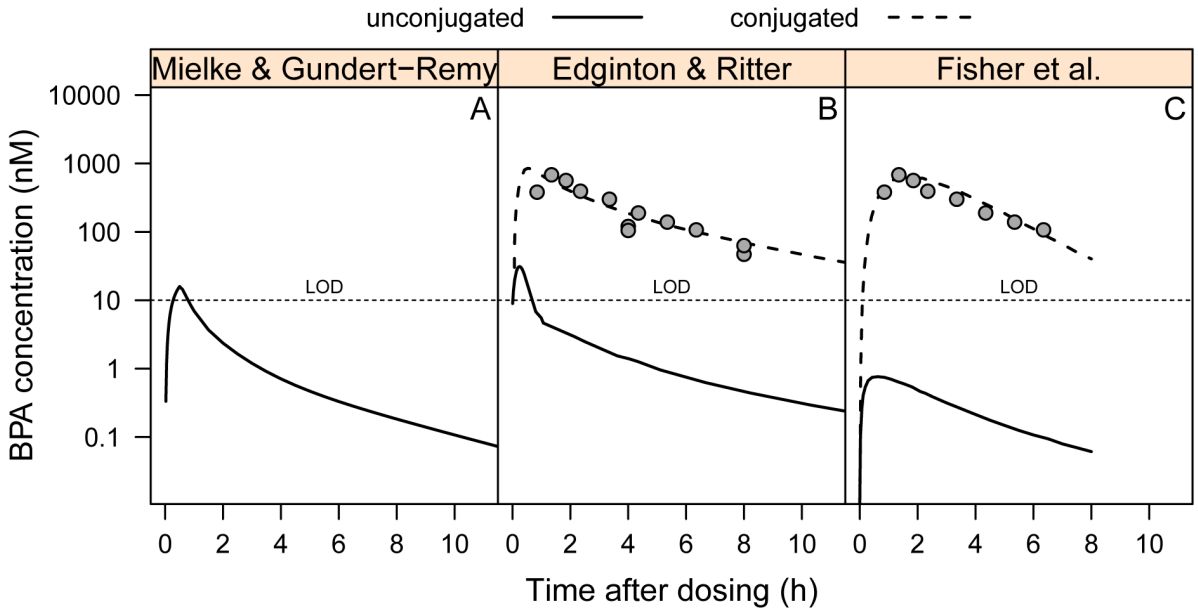


Figure 7: Predictions of three PBPK models on the plasma concentrations of unconjugated (solid lines) and conjugated BPA (dashed lines) for an adult human male after oral dosing of 5 mg BPA (corresponding to 63 µg/kg bw). The observed concentrations of glucuronidated BPA (filled symbols) were taken from the toxikokinetic study of Völkel et al. (2002), which could not detect unconjugated BPA above the LOD of 10 nM in blood taken 40 min after dosing. (A) Prediction of a human-based PBPK model for unconjugated BPA with hepatic glucuronidation and sulfation (Mielke and Gundert-Remy, 2009). (B) Predictions of a human-based PBPK model with liver glucuronidation (Edginton and Ritter, 2009). (C) The predictions of a monkey-based PBPK model with gastrointestinal and liver metabolism, which was extrapolated to humans (Fisher et al., 2011).

Fisher et al. (2011) developed a PBPK model for the prediction of route-dependent dosimetry of BPA in adult and infant rhesus monkeys with extrapolation to humans. The oral-route model consisted of a 7-compartment submodel for BPA, a one-compartment submodel (volume of distribution) for conjugated BPA (i.e. glucuronide + sulphate), an oral dosing compartment (stomach), and two compartments (small-intestine) for unconjugated and conjugated BPA (Figure 6). Intestinal first-pass metabolism and hepatic metabolism (including first pass) of BPA was incorporated. Additionally included was a term for renal reabsorption of conjugated BPA to account for the "lingering residual" conjugated BPA in monkey serum. The PBPK monkey model was calibrated against toxicokinetic

data for adult and neonatal monkeys with intravenous and oral-bolus administration (Doerge et al., 2010) to estimate the parameters for gastrointestinal absorption, intestinal and hepatic metabolism, the volume of distribution, and renal elimination and reabsorption. The evaluation of the calibrated adult-monkey model against published kinetic studies in monkeys revealed deviations between the predicted and observed data, which were related to study differences in analytical methodology, the monkey strains, and the vehicles used for oral administration of BPA. The calibrated adult-monkey model was then applied to extrapolate to adult humans using the Völkel et al. (2002) data for model evaluation (Figure 7C). For parameterization, human physiological parameters (including a human gastric emptying rate) were used together with BPA-specific model parameters derived from the adult monkey model. Compared to the predictions of the human PBPK models of Mielke and Gundert-Remy (2009), and Edginton and Ritter (2009), the calibrated PBPK model by Fisher et al. (2011) predicted 10–50-fold lower unconjugated BPA levels in human serum (Figure 7C). A replacement of the calibrated model parameters by a set of revised model parameters, which were based on the monkey BPA kinetic data of Taylor et al. (2011), increased the unconjugated BPA levels in human serum but the concentration-time profile did not exceed the level of 10 nM representing the LOD for unconjugated BPA in the Völkel et al. (2002) study.

Yang et al. (2013) developed a PBPK model for neonatal and adult rats to quantitatively evaluate age-dependent pharmacokinetics of BPA and its phase II metabolites and to enable a route-dependent dosimetry of BPA in rats at different life stages. The PBPK model was calibrated in adult rats using studies on BPA metabolism and excretion in the liver and gastrointestinal tract, and pharmacokinetic data with BPA in adult rats. For immature rats the hepatic and gastrointestinal metabolism of BPA was inferred from studies on the maturation of phase II enzymes coupled with serum time course data in pups. The calibrated model predicted the measured serum concentrations of unconjugated BPA and BPA conjugates (glucuronide + sulphate) after administration of 100 µg/kg of d6-BPA in adult rats (oral gavage and intravenous administration) at postnatal days 3, 10, and 21 pups (oral gavage). The observed age-dependent BPA serum concentrations were partially attributed to the immature metabolic capacity of pups. A comparison of the dosimetry of BPA across immature rats and monkeys suggests that dose adjustments would be necessary to extrapolate toxicity studies from neonatal rats to neonatal monkeys or infant humans.

3.1.3.2. Summary of the PBPK modelling

To summarise, four PBPK models have been developed for the oral exposure in humans. The models originated either from animal-based concepts (which were then extended to humans) or from human-based concepts. The animal-based concepts offered the opportunity to calibrate the model against a training set of animal toxicokinetic data and to then evaluate the calibrated model against a (new) test set of data. To parametrize the models for humans required an estimation or optimization of ADME parameters, in general by fitting the predicted plasma concentrations to the toxicokinetic data of Völkel et al. (2002), specifically to the observed plasma concentration of conjugated BPA and to the 10-nM LOD for unconjugated BPA. The predicted plasma concentration profiles for unconjugated BPA of Teeguarden et al. (2005) and of Edgington and Ritter (2009) have to be regarded as upper-bound estimates, because ADME parameters were adjusted to allow the profile to approach the 10-nM LOD. The predictions of the PBPK model of Mielke and Gundert-Remy (2009), which derived the metabolic parameter values from in vitro studies and optimized the absorption parameter against "observable" human toxicokinetic data (i.e. urinary BPA excretion), also need to be regarded as an upper-bound estimate, even though the metabolic liver parameters were not adjusted to reach a certain target level in the serum concentration of unconjugated BPA. Arguments in favour of the Fisher et al. (2011) PBPK model are (i) the inclusion of pre-systemic metabolism in the GI tract (i.e. intestinal first pass) into the model structure for oral BPA, and (ii) the explicit use of the serum concentration-time course data for unconjugated and total BPA in model validation. The CEF Panel therefore decided to use the PBPK model of Fisher et al. (2011) to derive internal dosimetrics for oral BPA, as done by Yang et al. (2013), for the HED approach. The CEF Panel noted however that due to uncertainty around the assumptions used in the different models, there will be uncertainties about the outcome of the PBPK modelling.

3.1.3.3. Further PBPK model predictions on serum BPA levels in human adults and newborns

The study of Mielke and Gundert-Remy (2009) further explored the influence of the dosing schedule mimicking the age-specific patterns of meals per day (i.e. considering that newborns are usually fed more frequently than older children and adults). For an oral exposure of 50 µg/kg bw per day, the PBPK model predicted a three times higher steady-state blood concentration of unconjugated BPA for newborns in comparison to adults (0.44 versus 0.13µg/l). Edginton and Ritter (2009) predicted a children/adult ratio of the steady-state plasma concentration of unconjugated BPA of 2–11 (depending on different ages), whereas Yang et al. (2013) predicted a children/adult ratio of the AUC for the serum unconjugated BPA concentration of 0.85. The difference in the children/adult ratio of the internal dose metrics between the three studies may be explained by the pattern of exposure. The simulation by Mielke and Gundert-Remy suggests that the well-expressed sulfation activity in the newborn can counteract at least partly a lower glucuronidation activity in neonates associated with UGT 2B7 ontogeny, as already highlighted in the EFSA opinion of 2008 (EFSA, 2008). Mielke and Gundert-Remy (2009) also calculated in the adult a steady-state concentration of unconjugated BPA of 0.0014–0.0026 µg/l, resulting from a daily intake of 0.905 µg/kg bw per day. The value compares well with the steady-state concentration of unconjugated BPA of 0.004 µg/l following the oral exposure to 1 µg/kg bw per day reported by Edington and Ritter (2009). The derived estimated C_{max} value was 2–3 orders of magnitude lower than measured mean values reported by some authors. This underlines the need for cautious interpretation of data on extremely high concentrations of unconjugated BPA, due to the possible background contamination affecting the analytical detection (EFSA CEF Panel, 2010).

Teeguarden et al. (2005) used their PBPK model to simulate an exposure scenario consisting of a daily dietary uptake of 1 µg BPA/kg bw per day separated into three meals. The peak concentrations of unconjugated (not bound to plasma proteins) BPA in blood were predicted as 0.003 nM in a one-year-old child and as 0.0037 nM in 50-year-old adults. The PBPK model also permitted the prediction of oestrogen receptor binding in uterine tissue. Normalised for the oestrogenic activity of endogenous 17-ß-oestradiol, the highest increase in the oestrogenic activity induced under the described exposure scenario was calculated to be 0.22% for 11-year-old boys (lowest circulating 17-β-oestradiol levels).

Computational modelling of the possible effects of plasma protein binding of oestradiol and BPA, incorporating affinities of oestradiol and BPA to different binding proteins and physiological concentrations of these proteins in rodents and in male and female humans, predicts that unless very high concentrations (> 100 nM) of BPA are reached in blood, oestradiol binding to the receptor will always dominate. Therefore, under realistic blood concentrations expected in humans from oral exposure to BPA from diet in the range of up to 0.05 nM, only a very small fraction of the oestrogen receptor will be occupied by BPA (Teeguarden et al., 2005).

Occupancy of the oestrogen receptor by BPA is predicted to be further decreased when the rapid elimination of BPA is incorporated into the modelling (EFSA 2006, EU-RAR, 2003/2008).

Edgington and Ritter (2009) used their PBPK model to simulate a repeated daily oral dosing of 1 µg/kg bw (given once per day) in adults and young children (0–2 years of age). The average steady-state plasma concentration of unconjugated BPA in newborns and 3 months-old infants were 11 and 2 times greater than in adults, because the authors included a much less efficient BPA conjugation in newborns and children of very young age. For breast-fed newborn exposure, unconjugated BPA average plasma concentration at steady state in newborns and in breastfed 3 month-old infants were estimated to be 1.8 and 0.26-fold that in adults (0.004 µg/L), while in formula-fed 3 and 6 month-old infants the modelled plasma concentrations were approximately 5 times greater than those in adults.

The CEF Panel noted that the PBPK models of Edgington and Ritter (2009) and Mielke and Gundert-Remy (2009) assumed an intestinal absorption of 100% and 90%, respectively, BPA in humans, irrespective of age, but no presystemic (first-pass) metabolism of BPA in the GI tract. The assumption of (almost) complete absorption is consistent with the quantitative excretion of total BPA in urine

(Völkel et el al., 2002; Teeguarden et al., 2011); however, the assumption that no pre-systemic metabolism occurs in the GI tract is overly conservative, although direct experimental data to estimate the magnitude of this gastro-intestinal first-pass effect in humans are not currently available. The results from the human-based PBPK models underline the importance of taking into account both metabolic pathways (i.e. glucuronidation by multiple UGT isoforms and sulfation) for dietary BPA in both liver and the GI tract at different ages. Simulations taking into account both age-dependent metabolic differences and specific pattern of exposure predict for newborns a 3-fold greater steady-state blood concentration of unconjugated BPA as compared with the adult (0.44 µg/L versus 0.13 µg/L) after exposure to the same quantity of 50 µg BPA per kg bw (Mielke and Gundert-Remy, 2009). The two new PBPK models are based on lifestage-specific kinetic data (monkey and rat), which are scaled up to the human situation (Fisher et al., 2011; Yang et al., 2013). The extrapolation to adult and neonatal humans would suggest 10 to 50-fold lower concentrations for unconjugated BPA in blood than as predicted by Mielke and Gundert-Remy (2009) and Edginton and Ritter (2009), and leads to the conclusion that the neonatal rat has an impaired metabolism for BPA compared with the adult rat whereas in the neonatal primate (i.e. monkey), the metabolism seems more similar to the adult primate.

3.1.3.4. Derivation of PBPK model-based internal dosimetrics for the HED approach

In order to perform a comparison of BPA dosimetry across species including humans, Yang et al. (2013) applied the monkey-based PBPK model of Fisher et al. (2011) for the prediction of internal dosimetrics in human newborns and adults, based on a repeated daily oral bolus administration of 50 µg/kg bw over a period of 5–14 days to ensure periodicity (steady state) of the serum concentrations of unconjugated BPA. The authors predicted AUC values of 1.53 and 1.80 nM×h for human newborns and adults, respectively. The corresponding C_{max} values were 0.23 nM and 0.51 nM, respectively. The HED approach, as applied in this opinion, is based on a common oral dose of 100 µg/kg bw per day, which was used throughout all toxicokinetic key studies in neonetal and adult mice, rats, and monkeys. The above mentioned AUC values had therefore to be multiplied by a factor of 2 (to adjust for moving from 50 to 100 µg/kg bw) to obtain equivalent-dose AUCs of 3.0 and 3.6 nM×h for human newborns and adults, respectively (see table 3 and 4 in Section 3.1.5).

3.1.4. Role of polymorphisms in the kinetics of BPA

3.1.4.1. Summary of previous evaluations

The following text is taken from EFSA (EFSA CEF Panel, 2010) with some minor modifications (e.g. deletion of references).

"*The enzymes which are involved in BPA conjugation are UDP-glucuronyl-transferases (UGT) and sulfotransferases (SULT). In both monkeys and rats, the predominant pathway is glucuronidation, with the sulfation reaction representing <20% for monkeys and <5% for rat. Both enzyme families consist of different isoforms, which can have different affinities for and capacities to metabolise BPA. The various isoforms also demonstrate different ontogenetic patterns. In addition, they show genetic polymorphisms. Information on which isoform is involved at dose levels relevant for human exposure can be used as valuable input in PBPK modelling in order to identify whether groups of individuals can be at higher risks due to the presence of allelic variants with altered activity or a different age-related enzyme expression level"*.

<u>UDP-glucuronyl-transferases (UGT)</u>

Among the different recombinant human isoforms, UGT2B15 showed the highest activity over the range of BPA concentrations (1-20 µM) tested. Clearly, a role for UGT2B15 is identified and to a lesser extent also to 2B7 and 1A8 (EFSA CEF Panel, 2010).

Polymorphisms have been identified in the UGT2B15 gene. The polymorphism can result in a modification of the activity for different substrates, with the UGT2B15*1 allelic variant (wild type)

having 2 to 5 fold higher rates of glucuronidation when compared to UGT2B15*2. Polymorphisms of UGT 1A9 and 2B7 have been also identified, whose functional consequence is unclear. The coefficient of variation for UGT2B15, 1A9 and 2B7 in a large human liver bank is 72, 55 and 45% (the lowest among different UGT isoforms), respectively, and therefore, the impact of the polymorphic allelic variants is expected to be limited. Due to the redundancy in UGTs for conjugation and the overlapping substrate specificity, it is expected that a single polymorphism would not significantly affect the total BPA glucuronidation capacity of individuals. At present no specific information about ontogeny in humans is available for UGT2B15. Some information is available on other UGT isoforms (e.g. UGT1A1, which reaches adult activity at 3-6 months of age, or UGT2B7, which is only 5% that of adults at term, but increases to 30% by 3 months of age, and to adult levels by 1 year of age). This pattern has been used in human PBPK models to account for possible limited UGT activity for BPA conjugation during early life. Notably, most information on UGT ontogeny refers to the liver, which is usually endowed with the highest glucuronidation activity. However, in the fetus UGT immunoreactivity in liver and kidney tissue is considerably lower when compared with the red blood cells. For this reason it has been hypothesised that circulating UGTs may substantially contribute to detoxification of xenobiotics in the fetus (EFSA CEF Panel, 2010).

Sulfotransferases

Regarding the role of SULT isoform(s) in the conjugation and deactivation of BPA, human recombinant SULT1A1 has been identified as the major isoform mediating BPA sulfation in the human liver, although recombinant SULT2A1 and 1E1 showed also some activity. The human SULT1A1 gene has common single nucleotide polymorphisms resulting in three allelic variants for which the differences in specific activity can be up to 10-fold, although they are not necessarily translated into the same degree of interindividual variability in in vivo sulfation capacity. The differences due to polymorphism are expected to be covered by the interindividual standard uncertainty factor. For SULT enzymes no age-dependency has been described and consequently, in humans the sulfation activity is comparable at birth and in the adult (EFSA CEF Panel, 2010).

3.1.4.2. Evaluation of recent studies on polymorphisms

Two recent studies were identified in which the consequences of UGT polymorphisms for BPA glucuronidation were investigated.

Hanioka et al. (2011) studied the effect of polymorphic forms of human UGT2B15 expressed in insect cells in vitro. The study demonstrated that among the 7 allelic variants of the gene investigated, the gene product from UGT2B15.1, 2B15.3, 2B15.4, 2B15.6 and 2B15.7 had intrinsic clearances of 140 to 178 μL × min^{-1}/mg insect cell membrane protein. Two other forms (2B15.2 and 2B15.5) had a considerably less intrinsic clearance (17.2 and 6.6 μL x min^{-1}/mg protein, respectively). Since the Km values for all isoforms were approximately similar (2.3 to 5.12 μM), the differences in intrinsic clearance were mainly related to a large decrease in V_{max}, probably as a result of the DNA sequence change in the 2B15.2 and 2B15.5 genes (D58Y; 253G>T) as compared to the wild-type (2B15.1).

Trdan Lušin et al. (2012) studied the glucuronidation of BPA in adult human microsomal preparations. For this purpose, they developed a sensitive analytical method using labeled BPA in HPLC-MS/MS, which enabled simultaneous determination of unconjugated and conjugated BPA. BPA glucuronidation was studied in microsomes prepared from liver, kidneys, intestines and lungs. No BPA glucuronidation could be determined in human lung microsomes. In liver, kidneys and intestines, the microsomal intrinsic clearances were 950, 40 and 24 μL × min^{-1}/mg microsomal protein, corresponding to full tissue intrinsic clearances of 857, 8 and 2 ml × min^{-1}/kg bw, after scaling-up of the microsomal data to full organ weight. These authors also investigated the influence of a polymorphism of human UGT1A1 on the metabolism of BPA. Although this is not the most active form of UGT to contribute to the glucuronidation of BPA (which is UGT2B15), it still has significant capacity. For genotyped microsomes containing only wild-type UGT1A1*1, an intrinsic

clearance of 1 240 µL × min^{-1}/mg microsomal protein was found and for UGT1A1*1/*28 (heterozygous) an intrinsic clearance of 1 190 µL × min^{-1}/mg microsomal protein. However, for the homozygous UGT1A1*28/*28, the intrinsic clearance was only 320 µL × min^{-1}/mg microsomal protein. There were no differences in K_m values for the two allelic variants studied. Thus for the three different genotypes intrinsic tissue clearances of 1 113, 1 075 and 284 ml × min^{-1}/kg bw were calculated. The authors reasoned that this polymorphism of UGT1A1 may have toxicological consequences, since the glucuronidation capacity of the liver may be strongly reduced in UGT1A1*28 homozygous individuals.

In a recent paper Partosch et al. (2013) used published data on V_{max} and K_m from 15 different hepatic cell donors (Kuester and Sipes, 2007) to simulate the individual blood concentration of unconjugated BPA by PBPK modelling. In this human-based PBPK model the estimated highest and lowest peak blood concentrations (C_{max}) were 4.7-fold different and the Area Under the Curve (AUC) varied with a factor of 4.6. In this model, the glucuronidation and the sulfate pathways are negatively correlated: in subjects with low glucuronidation capacity the fraction of dose which is metabolised to the sulfate conjugate is higher than in subjects with high glucuronidation capacity. The results show that the differences are covered by the intraspecies kinetic default assessment factor

The CEF Panel concluded overall that due to the redundancy of UGTs, a single polymorphism is unlikely to significantly affect the total BPA glucuronidation capacity of an individual. The default intraspecies uncertainty factors used to derive a health-based guidance value are considered sufficient to account for possible differences in rates of metabolism of BPA.

3.1.5. Inter-species extrapolation of BPA dosimetrics using an HED Approach

A critical aspect of any risk assessment is the extrapolation of findings from animal toxicology studies with BPA to understand the potential for effects in humans. This extrapolation includes uncertainties surrounding inter-species and intra-species differences in toxicokinetics and toxicodynamics, which are often incorporated by using default uncertainty factors to convert reference points (e.g. BMDL, NOAEL) into health-based guidance values (e.g. TDI). Derivation of a human-equivalent dose (HED) is an accepted method for linking a critical effect from the dose-response relationship in animals to predict a level without harmful effects in humans (US EPA, 2011).

In derivation of the HED, the exposure related to the critical effect (i.e. a BMDL or an NOAEL) found in an animal study is multiplied by a factor that takes account of quantitative differences in toxicokinetics between the animal species used in the study and humans. This factor then replaces the toxicokinetic component in the interspecies assessment factor. The factor by which this toxicokinetic component is replaced can be obtained from allometric scaling, or from comparison of toxicokinetic data as explained below. For the interspecies extrapolation, then of the default factor of 10 only a factor 2.5 remains to take account of differences in toxicodynamics (see Table 5.5 in WHO/IPCS, 2009).

Since target tissue concentrations determine toxicological effects, measurements that define internal dosimetrics (e.g. serum AUC, C_{max}, time above a critical concentration) are most often used as the basis to characterize inter-species differences. In the absence of mechanism of action information that relates a specific dosimetric parameter with the toxicodynamic effect(s), the dose metric most often used for parent compound effects is the AUC in serum since it is readily measured, incorporates both time and concentration elements of exposure, and is predictably related to tissue AUCs. When chemical-specific information is not available, empirically derived allometric relationships between kinetic and metabolic parameters in different species, typically involving body weight to the ¾ power, provide a basis for inter-species extrapolation of internal dosimetrics. Indeed, the US EPA and ECHA

use the ratio of $bw^{1/4}$ (= $[bw_{Animal}/bw_{Human}]^{1/4}$)[11] as a default inter-species toxicokinetic dosimetric adjustment factor (DAF) for cancer and non-cancer endpoints when chemical-specific data are not available or for extrapolation from animals to humans in general (US EPA, 2011; ECHA 2012). PBPK modelling is another accepted method for reducing uncertainty associated with extrapolations between species and dose in regulatory risk assessment.

The dose-adjusted AUC (i.e. AUC/D with D representing the dose) is a common means for inter-species extrapolation of dosimetrics at exposure levels where pharmacokinetic processes are not saturated. Under these conditions, the Human Equivalent Dose Factor (HEDF) is defined by a common relationship between the external dose given to an animal and the resultant AUC and the external dose given to a human and its AUC. The HED represents the multiples of the BPA dose (D) in an animal species by a specified route and lifestage that a human would require to obtain an equivalent AUC from oral administration (D × HEDF = HED). For example, if the same dose (on a body weight basis) administered to either an animal or a human produces a 5-fold higher AUC in the human, 1/5th the animal dose given to a human would produce the same internal dosimetric. Experimentally, AUCs are often determined using the same dose so that the HEDF simplifies to (the ratio of) animal AUC/human AUC, which is 0.2 in the previous example.

For BPA chemical-specific data are available, so that the ratio AUC_{Animal}/AUC_{Human} can be derived. The studies of Doerge et al. (2010a, b ; 2011a) provide BPA measurements obtained using identical experimental protocols for adult and newborn CD-1 mice, Sprague-Dawley rats, and Rhesus monkeys. The AUC data for oral and injected BPA are shown in table 3 for a common external dose of 100 µg/kg bw per day along with AUCs for human adults that were simulated for the same oral dose using the human PBPK model of Yang et al. (2013) which evolved from the monkey-based model of Fisher et al. (2011). For adult mice with oral administration, the published AUC value of 0.1 nM×h (Doerge et al., 2011b) was critically reviewed by the CEF Panel in respect to the uncertainty arising from the high proportion of non-detects among the data, and a recalculated AUC value of 0.244 nM×h was derived (see Section 3.1.6.1). table 3 also lists the respective human-equivalent, allometric scaling-derived DAFs for adults to convert point of departure doses from animal toxicity tests to human-equivalent exposures. table 3 indicates that in an adult mouse, an oral dose of 1 mg/kg bw is equivalent to a human dose of 0.068 mg/kg bw (1 mg/kg bw x HEDF of 0.068 = HED of 0.068 mg/kg bw), i.e. because of the large differences in body weight, a smaller dose is required in humans to achieve the same AUC. table 4 shows the comparable data for human infants, showing that a neonatal rat injection dose of 1 mg/kg bw per day is equivalent to an oral dose of 310 mg/kg bw per day to a human infant (i.e. because of the immaturity of Phase II metabolism in neonatal rodents but not primates and the bypassing of metabolism in the GI tract after injection).

These AUC ratios are chemical-specific adjustment factors that replace the typical default uncertainty factor for inter-species extrapolation of toxicokinetics. As explained above, then only a factor of 2.5 would remain to cover the differences in toxicodynamics . For example, the TDI of 50 µg/kg bw per day established by EFSA for BPA in 2006 was based on a NOAEL of 5 mg/kg bw per day in Sprague-Dawley rats from the Tyl et al. (2002) study. The TDI was obtained by dividing the NOAEL by a 100-fold default combined uncertainty factor, which is comprised of factors of 10 for each toxicokinetics and toxicodynamics. Using the HED approach with a human-equivalent dose factor (HEDF) of 0.72 for orally dosed adult rats (table 3), the HED-derived TDI would be (5000×0.72)/(2.5×10) = 144 µg/kg bw per day. The CEF Panel noted that the use of chemical-specific adjustment factors represents a refinement in risk assessment, but also noted the uncertainties related to the derivation of the HEDF, particularly in the mouse, as discussed further in Section 3.1.6.

For comparison with the non-chemical specific default approach table 3 also shows the DAFs calculated for adult animals based on the EPA default procedure, which is based solely on the animal-

[11] The ¼ exponent results from the application of the $bw^{-1/4}$ scaling to doses in units of mg/kg bw per day (rather than mg per day).

to-human body weight ratio raised to the ¼ power. Comparison with the experimentally derived HEDFs provides some insight about BPA metabolism and disposition affecting dosimetrics beyond the predictable body weight effects: 1) in mouse, the HEDF of 0.068 (= 0.244/3.6) is lower than the DAF of 0.14, which suggests that mouse has greater metabolic capacity, serving to reduce the AUC; 2) in rat, the HEDF of 0.72 exceeds the DAF of 0.24, which could reflect the effect of enterohepatic recirculation in the rat that serves to extend exposure to BPA; 3) in monkey, the HEDF of 0.42 is similar to the DAF of 0.55, which suggests that body weight differences predominate.

Table 3: Determination of HEDF for BPA in human adults. HEDF (= AUC_{Animal}/AUC_{Human}) values were calculated from experimentally determined serum AUCs of unconjugated BPA from adult and neonatal animals for a common gavage or injection dose of 100 µg/kg bw and from AUCs for human adults and infants that were simulated for the same oral dose using a human PBPK model. The HED represent the multiples of BPA dose (D) in an animal species by a specified route and lifestage that a human would require to obtain an equivalent AUC from oral administration (D × HEDF = HED). For comparison, the comparable dose adjustment factors (DAF) are shown which were derived by using the US EPA default of animal-to-human body weight ratios raised to the ¼ power.

Species-Route	**AUC-Adult (nmol × h × l^{-1})**	**HEDF-Adult**	**DAF –Adult $bw^{1/4}$ Scaling**
Mouse-oral	0.244*	0.068 (= 0.244/3.6)	0.14 $= (0.025/70)^{1/4}$
Mouse – IV injection	54	15 (= 54 /3.6)	
Rat-oral	2.6	0.72 (= 2.6/3.6)	0.24 $= (0.25/70)^{1/4}$
Rat – IV injection	95	26 (= 95 /3.6)	
Monkey-oral	1.5	0.42 (= 1.5/3.6)	0.55 $= (6.6/70)^{1/4}$
Monkey – IV injection	180	50 (=180/3.6)	
Human-oral PBPK-simulation; Yang et al. (2013)	3.6 (reference value)	–	–

* Recalculated AUC value, see Section 3.1.6.1

Table 4: Determination of HEDF for BPA in human infants.

Species-Route	**AUC-Neonate (nmol × h × l^{-1})**	**HEDF-Neonate**
Mouse-oral	26	8.7 (= 26/3)
Mouse – SC injection	26	8.7 (= 26/3)
Rat-oral	56	19 (= 56/3)
Rat – SC injection	930	310 (= 930/3)
Monkey-oral	5.7	1.9 (= 5.7/3)
Monkey – IV injection	190	63 (=190/3)
Human-oral PBPK-simulation; Yang et al. (2013)	3.0 (reference value)	–

3.1.6. Evaluation of uncertainties affecting the determination of HEDF for BPA

3.1.6.1. Uncertainty and physiological plausibility of the HEDF for adult mice with oral exposure

The importance of the HEDF for adult mice with oral exposure for the risk assessment of BPA made it necessary to perform a dedicated uncertainty analysis of this parameter and of the underlying AUC for adult mice with oral exposure. The following paragraphs summarise the analytical uncertainty in the toxicokinetic raw data and describe lower-, middle-, and upper-bound approaches for the determination of AUC. The middle-bound AUC estimate was chosen to derive a HEDF for risk assessment. Finally, the physiological plausibility of the middle-bound AUC estimate is evaluated.

The toxicokinetics in adult mice with oral administration was examined by Doerge et al. (2011b). Following the administration by gavage, blood samples were taken at seven time points from 0.25 h to 24 h. Twelve (new) animals of both sex were used per time point (i.e. 84 animals in total plus 12 controls). The authors reported that levels of unconjugated BPA that were above the detection limit were observed only at the earliest three time points, and only in one or two samples out of the twelve determinations at each time. Analysis of the raw data (kindly provided by Daniel R. Doerge, NCTR, U.S. FDA, 2013) revealed the following additional information: there was one missing measurement at 0.25 h. Moreover, detectable levels of unconjugated BPA that were above the LOD (0.2 nM) were observed in 3 of 11 (t = 0.25 h), 2 of 12 (t = 0.5 h), and 1 of 12 (t = 1 h) animals. Finally, five of the six detectable observations were in the range of 0.36–0.84 nM, whereas the sixth observation had an extreme value of 5.85 nM, which occurred at t = 0.5 h and which was regarded by the authors as an outlier to be removed from analysis (Daniel R. Doerge, NCTR, U.S. FDA, 2013, personal communication). The impact of this outlier removal is assessed below.

To reflect the uncertainty that is associated with the estimation of the AUC due to the high proportion of non-detects among the data the CEF Panel decided to derive lower-bound (LB), middle-bound (MB), and upper-bound (UB) estimates of the AUC. The following description outlines the procedures by which these estimates were derived.

The **lower-bound (LB)** approach is based on setting all non-detectable observations of the earliest three time points (0.25 h, 0.5 h, 1 h) to zero. The resulting plasma concentration profile, which is shown in Figure 3A, yielded an area under the curve from time zero to 1 h ($AUC_{0–1h}$) of 0.081 nM×h. For the area under the curve from 1 h to infinity ($AUC_{1h–\infty}$), a value of 0.026 nM×h was calculated from the last concentration value at 1 h and by assuming an exponential decrease with a terminal half-life $t_{1/2}$ of 0.6 h which was reported for adult mice with oral administration (Doerge et al., 2011b). Summing up the two values yielded an AUC (from time zero to infinity) of 0.108 nM×h. Based on this value, a HEDF of 0.030 (= 0.108/3.6) for adult mice with oral administration was derived by taking the AUC of 3.6 nM×h for adult humans with oral administration into account (Yang et al., 2013). Including the outlier at t = 0.5 h (see above) into the calculation would increase the $AUC_{0–1h}$ from 0.081 to 0.262 nM×h, which would then result in an AUC (from time zero to infinity) of 0.288 nM×h and a HEDF of 0.080 which are within the range of uncertainty spanned by the LB and UB estimates (see below).

The **middle-bound (MB)** approach uses the measurements of the first time point at 0.25 h and sets all non-detects to 0.5×LOD (= 0.1 nM) (see Section 4.2.7 in Part I – Exposure assessment). This imputation yields a concentration value of 0.253 nM for t = 0.25 h. The next two data points at 0.5 h and 1 h are extrapolated from the first data point at 0.25 h by assuming an exponential decrease with a half-life of 0.2 h. This half-life was taken from PND 21 mice with gavage (Doerge et al., 2011b) (Figure 4C and see below). This extrapolation yields a concentration value of 0.019 nM for t = 1 h. The terminal elimination phase at >1 h was assumed to follow an exponential decrease with a half-life of 5 h which was based on the evidence from all available toxicokinetic studies on mice with oral administration (Figure 4). Specifically, it was reasoned that the time course for unconjugated BPA should run in parallel to the time course of total (or conjugated) BPA after a certain time point. Figure 4 shows several studies with different age groups and oral administration procedures, for which the time course could be followed up to 24 h post administration, and for which most terminal half-lives for unconjugated and total are in the range of 4–8 h. A value of 5 h was selected. This approach yielded values of 0.108 nM×h for $AUC_{0–1h}$ and 0.136 nM×h for $AUC_{1h–\infty}$. The sum of both values yields an AUC (from time zero to infinity) of 0.244 nM×h. This AUC gave a HEDF of 0.068 (= 0.244/3.6).

The **upper-bound (UB)** approach is based on toxicokinetic data for PND 21 mice administered by gavage (Doerge et al., 2011b) (Figure 4C). As in the experiment with adult mice, blood samples were taken at seven time points from 0.25 h to 24 h with 12 (new) animals per time point. The authors reported that levels of unconjugated BPA that were above the LOD (0.2 nM) were observed only at

the earliest three time points. Analysis of the raw data (provided by Daniel R. Doerge, NCTR, U.S. FDA, 2014) revealed that there were two missing measurements at 0.25 h and three missing measurements at 0.5 h. Moreover, detectable levels of unconjugated BPA that were above the LOD were observed in 10 of 10 animals (t = 0.25 h), 7 of 9 animals (t = 0.5 h), and 1 of 12 (t = 1 h) animals. The CEF Panel took account of these non-detects in the following way: The mean plasma BPA concentration of 1.279 nM at t = 0.25 h is not affected (no non-detects); for the two non-detects at t = 0.5 h, the concentration was set to half the LOD, resulting in a mean plasma BPA concentration of 0.553 nM at this time point. The concentration estimate for t = 1 h as given by Doerge et al (2011b) was discarded, because it is based on only a single quantifiable data point. Instead, the concentration at t = 1 h was estimated to be 0.098 nM by extrapolation from the mean values at t = 0.25 h and t = 0.5 h, assuming a half-life of 0.2 h for the first hour post dosing. This serum concentration time profile was used as a starting point to derive an UB estimate of the AUC in adult mice. For the terminal elimination phase at >1 h, an exponential decrease with a half-life of 5 h was assumed (see MB approach). This approach yielded values of 0.552 nM×h for $AUC_{0–1h}$ and 0.705 nM×h for $AUC_{1h–\infty}$. The sum of both values yields an AUC (from time zero to infinity) of 1.257 nM×h. This AUC gave a HEDF of 0.349 (= 1.257/3.6).

To summarize, lower-bound (LB), middle-bound (MB), and upper-bound (UB) estimates of 0.108, 0.244, and 1.257 nM×h were obtained for the AUC. These estimates translate into HEDF values of 0.030, 0.068, and 0.349. Of the three AUC estimates, the MB estimate of 0.244 nM×h was chosen to derive a HEDF of 0.068 for adult mice with oral administration.

The physiological plausibility of the chosen AUC estimate for adult mice with oral administration was evaluated by taking the AUC values for adult rats, monkeys, and humans and the allometric scaling to toxicokinetic parameters with body weight into consideration. The results of toxicokinetic studies with IV injection in adult mice (Doerge et al., 2012), rats (Doerge et al., 2010a), and monkeys (Doerge et al., 2010b) have shown a close allometric relationship between serum clearance (Cl = BW×dose/AUC) and body weight (BW) with an exponent of 0.78, close to the default value of 0.75, and a coefficient of 3.6 L/h (Figure 8A). The latter corresponds to the predicted clearance for an animal of 1-kg body weight. For toxicokinetic studies with oral administration, the quotient of AUC and dose equals the serum clearance divided by the systemically available fraction (= Cl / f). Plotting the Cl/f values for mice, rats and monkeys against the respective body weights showed a less close allometric relationship (Figure 8B). Fitting this relationship with a restriction of the allometric exponent to 0.78 (i.e. the exponent for the IV data) yielded a regression line with a coefficient of 354 L/h corresponding to an systemically available fraction f (= oral bioavailability) of 1.0% (= 3.6/354×100%). The fact that the data point for rats is below the regression line suggests additional factors contribute to a higher AUC (e.g., enterohepatic recirculation of aglycone BPA; Figure 3B). In contrast, the data point for mice is well above the regression line which indicates a lower AUC for mice (see MB estimate in Figure 8B). There is, however, a relatively large uncertainty in the data point for mice that is reflected by the LB–UB range in Figure 8B. For adult humans, a Cl/f value was calculated from the AUC of 3.6 nM×h (predicted from PBPK modelling; Yang et al., 2013), the oral dose of 100 µg/kg bw per day, and the body weight of 75 kg. This Cl/f value for adult humans compared well with the prediction made by regression analysis of the animal TK data (Figure 8B).

To conclude, in spite of the analytical difficulties in measuring the serum concentration time course of unconjugated BPA in adult mice with gavage administration of 100 µg/kg bw per day, which contributed to a great extent to the uncertainty in AUC, there are also physiological reasons related to the extensive first-pass metabolism in adult mice. This at least partly explains the (relatively strong) discrepancy between observed toxicokinetic values and expected values predicted from default allometric considerations.

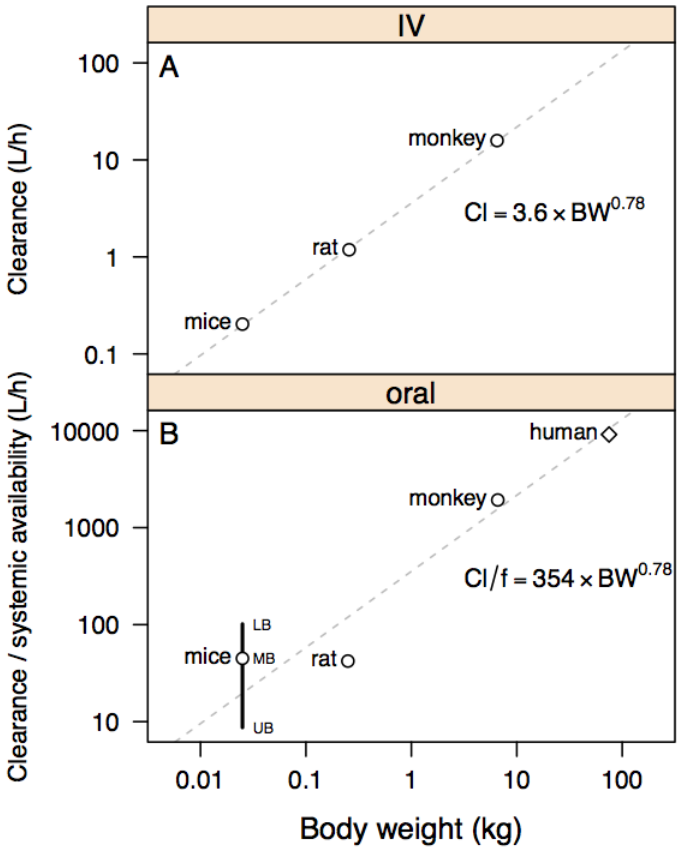


Figure 8: Allometric scaling of serum clearance values for adult mice, rats, monkeys, and humans from oral and IV administration (see also Doerge et al., 2012). For IV data, linear regression analysis was performed on log-log coordinates. For oral data, the regression analysis was performed with a restriction of the allometric exponent to 0.78 (i.e. the exponent for the IV data) and excluding the human data point, which was not experimentally based but but based on PBPK modelling (Yang et al., 2013). For further details, see main text.

3.1.6.2. Main sources of uncertainty in the determination of HEDF in general

The Human Equivalent Dose Factor (HEDF) is used to account for the toxicokinetic portion of the interspecies differences. Multiplying the HEDF by a reference point of a toxicity study yields a human equivalent oral dose that can be used for risk assessment. For the present opinion, HEDF values were calculated from the area under the curve (AUC) of the serum unconjugated BPA concentration in animals and humans (HEDF = AUC_{Animal}/AUC_{Human}) under the standard condition of a common external dose of 100 µg/kg bw per day.

AUC_{Animal} values were obtained from toxicokinetic experiments with oral administration, IV injection or SC injection in adult and newborn CD-1 mice, Sprague-Dawley rats, and rhesus monkeys (Doerge et al. 2010a,b, 2011a,b, 2012). The AUC_{Human} values for human adults and infants with oral dosing were predicted by PBPK modelling (Yang et al., 2013) using a monkey-based PBPK model (Fisher et al., 2011).

The present evaluation of uncertainties affecting the HEDF is focused on animal and human studies with oral administration because these were the most critical and relevant studies for risk assessment. Compared to studies with IV or SC bolus injection, oral administration studies are influenced by potentially more sources of biological variability due to the different administration procedures (e.g., gastrointestinal bolus gavage, oral bolus dosing, exposure *via* diet). For the present opinion, the HEDFs for animal studies with oral dosing were derived from bolus-gavage toxicokinetic studies in

animals (Doerge et al. 2010a, b, 2011a, b, 2012), and from a human PBPK model (Yang et al., 2013), which originated from a monkey-based PBPK model with bolus-gavage dosing (Fisher et al., 2011). The human PBPK model was evaluated against the results of a toxicokinetic study in humans with gelatin-capsule administration (Völkel, et al., 2002).

For HEDF determination, the CEF Panel is of the opinion that toxicokinetic studies in animals and humans should be comparable in respect to the administration procedures, and should permit fast gastrointestinal absorption. Procedures such as gastrointestinal bolus gavage with aqueous solutions or gelatin-capsule administration have the advantage of avoiding important sources of variability arising from the use of non-aqueous vehicles such as corn oil and from absorption-delaying digestion processes following oral bolus dosing or dietary exposure. The delay in the latter results from the inclusion of processes with relatively long time constants (i.e. mechanical and enzymatic food digestion, transport of digested food). From the systems analysis point of view, pulsed inputs (i.e. gastrointestinal bolus gavage, gelatin-capsule administration) are preferred for toxicokinetic studies to reveal the true systems parameter such as the time constants for gastrointestinal absorption, distribution, metabolism, and excretion (ADME). Other administration procedures (e.g., use of a corn-oil vehicle, dietary exposure) are more likely to yield apparent time constants not reflecting elementary (first order) ADME processes. Moreover, they are more prone to sources of variability as mentioned before.

The CEF Panel noted that the HEDF determination for animal studies with oral dosing is based on administration procedures which are somewhat artificial from the consumer exposure point of view. However, these "artificial" procedures apply to the animal and human toxicokinetic studies as well, so that the HEDF in itself is consistent. The question of applicability to the human situation arises when the HEDF is multiplied with the reference point of a toxicity study to yield a human equivalent dose. The question then is whether the type of administration in the toxicity study (e.g. *via* diet) is comparable to the typical exposure situation in humans. The two-generation reproductive toxicity study in CD-1 mice by Tyl et al. (2008), for example, exposed the animals *via* dosed feed. Because of the additional physiological (i.e. digestive) processes involved, the time course of the serum concentration of unconjugated BPA can be expected to deviate from those observed in toxicokinetic studies with gastrointestinal bolus gavage or gelatin capsule administration. Indeed, Sieli et al. (2011) reported a change in the shape of the serum concentration-time profile for unconjugated BPA and also a delayed time to C_{max} when the oral-bolus dosing was changed to dietary exposure. Remarkably, the AUCs were comparable between both types of administration. Since the typical exposure to BPA in humans is *via* dietary exposure, there is no reason to question the application of the HEDF to the PoD of a toxicity study with dietary exposure.

Overall, the main sources of uncertainty in the determination of HEDF are (i) the variabilities in the experimental animals and in the dosing and sampling procedures, and (ii) the uncertainty about the serum concentration-time course of unconjugated BPA in humans as predicted by PBPK modelling. These sources of uncertainty influence the AUC_{Aminal} and AUC_{Human}, which are ratioed to yield the HEDF. An evaluation of the magnitude of uncertainty of the individual sources of uncertainty is given in Appendix D. The overall uncertainty is judged to be within 0.5–2 times the HEDF estimate. A specific case is the toxicokinetic study in adult mice with oral administration, for which a dedicated uncertainty assessment was performed in Section 3.1.6.1.

Multiplying the HEDF with the reference point of a toxicity study with oral administration re-raises the issue of uncertainty in the extrapolation to the human situation. The question of uncertainty is whether the type of oral administration in the toxicity study is comparable to the typical exposure situation in humans. The exposure of animals *via* dosed feed has been shown to lead to a serum concentration-time profile for unconjugated BPA which was different from that observed under oral-bolus dosing (Sieli et al., 2011); the AUC, however, was not affected. Since the typical exposure to BPA in humans is *via* dietary exposure rather than via bolus dosing, there is no reason to assume a

large uncertainty when extrapolating from a toxicity study with dietary exposure to the human situation.

3.1.7. Dermal absorption and penetration of BPA and PBPK modelling of aggregated oral and dermal exposure

As described in Section 3.1.5, the CEF Panel made use of the results of PBPK modelling (Fisher et al., 2011; Yang et al., 2013) to apply the HED concept for converting doses and reference points from animal toxicity studies into human equivalent oral doses, which can directly be compared with oral exposure estimates.

However, because the dermal route of exposure (mainly due to thermal paper and to some extent due to cosmetics) was also an important source of exposure to BPA, it was necessary to carry out an assessment of aggregated oral and dermal exposure to BPA, again using PBPK modelling. It should be noted that this aggregated assessment did not include the contribution to BPA exposure due to inhalation of BPA-containing dust, as this source was considered to contribute only a very small fraction of total BPA exposure (< 1%) and no suitable PBPK model was readily available to provide an estimate of the internal dose metric arising from inhalation.

In order to carry out an assessment of aggregated oral and dermal exposure to BPA, it was necessary to have information on the dermal absorption and penetration of BPA, in order to quantify how an external dermal dose translates into an internal dose metric (e.g., AUC) for unconjugated BPA, the toxicologically active compound. Information of this internal dose metric enables the conversion of the external dermal dose into an equivalent oral dose (i.e. an oral dose that would result in the same AUC as the dermal dose that it represents) that can be summed up with the oral exposure estimates (e.g. from diet) to provide an aggregated exposure estimate that can directly be compared to a health-based guidance value.

So far, no toxicokinetic study in humans involving dermal exposure has been carried out, that provides information about (i) the extent of dermal absorption of BPA and (ii) the internal dose metrics for unconjugated BPA. However, several *in vitro* studies on cutaneous penetration using pig skin and human skin samples and an *in vivo* study in rats with dermal BPA absorption are available. The information available from these *in vitro* studies can, in principle, be used in PBPK modelling to simulate the fate of BPA taken up dermally. The PBPK model of Fisher et al. (2011) does not contain an implementation for a dermal input. However, Mielke et al. (2011) developed a PBPK model for the oral and dermal exposure, which enables predictions of serum concentration-time profiles and estimations of internal dose metrics for unconjugated BPA following oral and dermal exposure. The CEF Panel therefore decided to use the PBPK model of Mielke et al. (2011) to simulate the aggregated oral and dermal exposure. The implications of using a second PBPK model, in addition to the PBPK model of Fisher et al. (2011), which was used for the derivation of the HED, are discussed below.

3.1.7.1. In vitro and in vivo studies on dermal absorption

Appendix D provides an overview of the experimental studies in vitro and in vivo that have been used to derive an estimate of the fraction of an external dermal dose which reaches the systemic circulation, while the following section provides the conclusions of the CEF Panel on the extent of dermal absorption and penetration, based the data from these studies.

3.1.7.2. Conclusion on the extent of dermal penetration and absorption

The available evidence from in vitro dermal absorption studies with human skin explants from breast, abdomen, and upper leg, and also from an in vivo dermal absorption study in rats, suggests a 24-h dermal absorption of 2.3–8.6%. The upper limit of 8.6% was reported by Demierre et al. (2012) as the fraction of the applied dose that passed through human skin explants within 24 h. Demierre et al. (2012) additionally reported a skin deposition of ~35% of the applied dose after 24 h, the main fraction being located in the most external layers of the stratum corneum. The CEF Panel decided to

use a skin absorption of 10% for exposure scenarios with dermal contact to thermal paper. In the EU-RAR (2008), a dermal absorption of 10% was assumed, based on default considerations with respect to lipophilicity and molecular mass. The CEF Panel further decided not to consider the amount deposited in the stratum corneum as becoming available for systemic uptake for reasons emerging from the PBPK modelling of dermal exposure.

For the PBPK modelling of exposure scenarios with dermal contact to thermal paper, a BPA depot (receiving 100% of the external dermal dose) was assumed in the moisture film on the skin surface. It was further assumed (very conservatively) that the BPA depot remains on the skin surface during the whole day and that 10% of the initial depot content is absorbed within 24 h. BPA remaining on the skin surface after 24 h (i.e. 90% of the inital depot content) is assumed to be completely removed by hand washing, and the skin surface depot is then reloaded with 100% of the new dermal dose. In other words, the BPA depot is assumed to be periodically replenished to 100% after 24 h by a new dermal contact to thermal paper. An important consequence of assuming the BPA depot to be depleted to only a small extent within 24 h is that the dermal absorption process is in a steady state with a virtually stable and permanent concentration gradient in the stratum corneum, along which BPA is diffusing through the skin to reach the systemic circulation. These simplifying and conservative assumptions, which were made to keep the PBPK model as simple as possible, show that the fraction deposited in the stratum corneum will remain there as a concentration gradient as long as BPA is available on the skin surface.

The CEF Panel decided not to consider skin metabolism in dermal exposure scenarios as the available information does not enable derivation of a reliable estimate of the extent of skin metabolism. This results in a conservative estimate of the fraction of an external dermal dose of unconjugated BPA reaching the systemic circulation. The CEF Panel noted that the assumption of 10% absorption for the hand contact to thermal paper is also a further conservative assumption, since (compared to human skin explants from breast, abdomen or the dorsal part of the upper leg) the absorption across the skin of the palms can be expected to be lower because of the thicker stratum corneum.

The CEF Panel recognised the potential overestimation of the internal dose metric following dermal exposure, resulting from combining conservative assumptions about the following:

a. the fraction of BPA permeating through the skin, the kinetics following dermal exposure, and the absolute amount of total human exposure to BPA that occurs via the dermal route, particularly when it is realised that the dietary intake assessments alone already exceed the urinary biomonitoring estimates;

b. the assumption of 10% absorption through the fingers and hand in vivo, which is thicker than the skin sections used in the ex vivo studies; and

c. not considering metabolism in the skin prior to systemic distribution.

The CEF Panel noted that ongoing human dermal BPA PK and cashier studies at the NIEHS Clinical Research Unit will help to resolve much of the uncertainty associated with dermal exposure. The above considerations suggest that estimates of internal exposure from the dermal route are both highly uncertain and likely to significantly influence the combined estimate of total systemic exposure to unconjugated BPA. The model used in the current opinion takes into account these uncertainties mentioned above in such a way that it leads to overly conservative internal exposure estimates and thus also to overly conservative oral dose equivalents.

3.1.7.3. PBPK modelling of aggregated oral and dermal exposure

A PBPK model for the aggregated oral and dermal exposure (Mielke et al., 2011) was used to enable estimation of the internal dose metrics for unconjugated BPA for a combined exposure to BPA via diet and house dust (oral route) and via thermal paper and cosmetics (dermal route). The model

structure is shown in Figure 9A. When re-implementing the PBPK model using the statistical computing environment R (R Core Team, 2014) in combination with the deSolve package (Soetaert et al., 2010) the model predictions were checked and agreed with those published by Mielke et al. (2011). For the present opinion, PBPK model predictions were performed for adult males and children (1.5 – 4.5 years), using the model parameters given by Mielke et al. (2011) and Mielke and Gundert-Remy (2009). These two population groups matched the population groups of adult males (18 – 45 years) and children (3 – 10 years) which were used in Part I – Exposure assessment. PBPK model parameters are given in Table 56 in Appendix D).

Compared to the published model version (Mielke et al., 2011), the PBPK model was slightly modified in respect to the simulation of dermal exposure. For the uptake of BPA from cosmetics, a constant uptake rate was assumed, leading to 50% absorption of the external dermal dose within 24 h. For the uptake of BPA from thermal paper, it was assumed that thermal paper is touched once a day and that BPA migrates into a depot in the moisture film on the skin surface within a short duration of 5 min. The amount migrating into this skin-surface depot is 100% of the external dermal exposure (values taken from Tables 22 and 23 in Part I – Exposure assessment). During each day (= 24 h), 10% of the initial depot content on the skin surface is assumed to diffuse across the skin barrier into the skin compartment according to a first-order process with a time constant $k = -\ln(0.9)/(24\ \text{h}) = 0.00439\ \text{h}^{-1}$ which corresponds to a dermal absorption half-life of 158 h. The value for the time constant necessarily results from the assumption of 10% absorption within 24 h. BPA remaining on the skin surface after 24 h (i.e. 90% of the inital depot content) is assumed to be completely removed by hand washing, and the skin surface depot is then reloaded with 100% of the new dermal dose. In other words, the BPA depot is assumed to be periodically replenished to 100% after 24 h by a new dermal contact to thermal paper.

Table 5 shows the oral and dermal doses (D_O and D_D), which were used for the scenarios with average and high exposures of children and adult males to BPA in diet and house dust (oral route) and in thermal paper and cosmetics (dermal route). The exposure to and uptake of BPA from the various sources was implemented differently in the PBPK model. It comprised a continuous and constant dermal uptake of BPA in cosmetics, a single dermal contact to thermal paper per day, and a dietary exposure involving 3 meals per day. For reasons of keeping the PBPK model as simple as possible, the oral uptake via dust was assumed to be synchronized with dietary uptake. Since internal exposure is in the linear range of the toxicokinetics of BPA, this assumption has no influence on the magnitude of the calculated AUCs. The dermal uptake via dust was regarded as negligible (see Section 4.5.3 in Part I – Exposure assessment). PBPK modelling was used to simulate the serum concentration of unconjugated BPA (Figure 9B). The simulation was run for 10 days to reach a steady state. The predicted serum concentration-time profile for the last day was used to determine the area under the curve (AUC). Table 5 contains the predicted serum AUC values of unconjugated BPA for the oral (AUC_O) and dermal (AUC_D) exposures. Since only a reference dose for external oral exposure is available (i.e. a TDI or a t-TDI), for the risk assessment it is necessary to express both oral and dermal exposures on the same basis, thus as external oral equivalents. When exposures via both routes are converted to external oral equivalents, these can also be summed, resulting in an estimate for aggregated exposure. For oral exposure such a conversion is not necessary, since the oral exposures are intrinsically external equivalent oral exposures. To convert the external dermal exposure (D_D) to equivalent oral exposure (D'_D), and thus to allow for the calculation of an aggregated (oral plus dermal) exposure estimate, the following equation was used:

$$D'_D = EF_{D\rightarrow O} \cdot D_D \quad \text{with} \quad EF_{D\rightarrow O} = \frac{AUC_D / D_D}{AUC_O / D_O}.$$

$EF_{D\rightarrow O}$ is the dermal-to-oral route equivalence factor which is calculated from the ratio of the dose-adjusted AUC values. Within each population group and for each dermal source, the obtained $EF_{D\rightarrow O}$ values are virtually the same for the average and high exposure scenarios, reflecting linear kinetics. Moreover, within each population group, the $EF_{D\rightarrow O}$ values are five-fold higher for cosmetics

compared to thermal paper, reflecting the assumption of a five-fold higher dermal absorption for cosmetics compared to thermal paper. Finally, the $EF_{D\rightarrow O}$ values are smaller for children than for adult males (e.g., 0.77 versus 1.20 for thermal paper), possibly resulting from a higher body-weight-specific metabolic rate (clearance) for the children. table 6 shows the dermal doses expressed as equivalent oral doses for the average and high exposure scenarios for children and adult males. The dermal-to-oral route conversion was also performed for adolescents using the $EF_{D\rightarrow O}$ values for adult males.

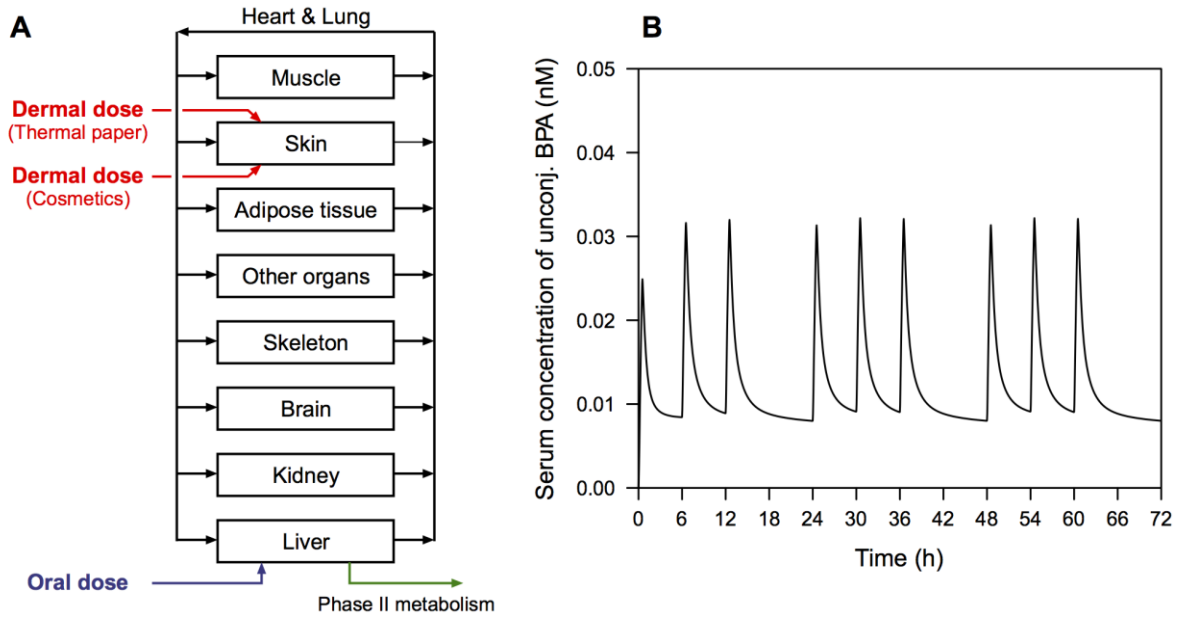


Figure 9: PBPK modelling of aggregated oral and dermal exposure in humans. PBPK modelling of aggregated oral and dermal exposure in humans. (A) Structure of the PBPK model for unconjugated BPA (Mielke et al., 2011). (B) Model prediction of the serum concentration of unconjugated BPA for the high exposure of adult males involving a oral dose of 0.336 µg/kg bw per day (diet plus dust taken up via 3 meals per day), an external dermal dose of 0.542 µg/kg bw per day (finger contact to thermal paper once a day), and an external dermal dose of 0.004 µg/kg bw per day (exposure to trace amounts of BPA in cosmetics). The extent of absorption in this model is 100% for the oral route, but with a first pass effect build in in the model so that the systemic availability is reduced. Extent of absorption is 10% for the dermal route with contact to thermal paper, and 50% for the dermal route associated with cosmetics. For the dermal contact with thermal paper, it is assumed that the depot on the skin surface is refilled to 100% of the external dermal exposure, based on the assumption of 1× hand washing followed by new contact to thermal paper. For cosmetics, a continuous (stable) uptake is assumed.

Table 5: PBPK model-based predictions of the area under the curve (AUC) for unconjugated BPA in serum. Prediction were made for average and high exposure scenarios for two selected population groups. Dermal doses (D_D) represent the individual exposures to BPA in thermal paper (T) and cosmetics (C) whereas the oral doses (D_O) refer to the summed exposures from diet and house dust (table 22 and 23 in Part I – Exposure assessment). Given are the predicted AUC values for the oral (AUC_O) and dermal (AUC_D) exposures as well as the values for the dermal-to-oral route equivalence factor (EF_{DO}).

Exposure scenario	Population group in PBPK modelling	Dermal source	D_O	D_D	AUC_O	AUC_D	$EF_{D\rightarrow O}$
			µg (kg bw)$^{-1}$ d^{-1}		nmol × h × l^{-1}		
Average	Adult male	T	0.127	0.059	0.037	0.021	1.20
		C		0.002		0.004	6.02
	Children 1.5 – 4.5 years	T	0.293	0.069	0.070	0.013	0.77

		C		0.002		0.002	3.83
High	Adult male	T	0.336	0.542	0.098	0.190	1.20
		C		0.004		0.007	6.03
	Children 1.5 – 4.5 years	T	0.818	0.550	0.195	0.101	0.77
		C		0.004		0.004	3.85

Table 6: PBPK model-based conversion of dermal doses from different sources into equivalent oral doses for average and high exposure scenarios for three selected population groups. Dermal doses (D_D) represent the individual exposures to BPA in thermal paper (T) and cosmetics (C). Given are the dermal-to-oral route extrapolation factors ($EF_{D\rightarrow O}$) which were used to translate the dermal doses into equivalent oral doses (D'_D). For adolescents, the exposure estimates of the adolescent population (10 – 18 years) were used in combination with the $EF_{D\rightarrow O}$ values for adult males.

Exposure scenario	Population group in		Dermal source	$EF_{D\rightarrow O}$	D_D	D'_D
	Exposure assessment	PBPK modelling			µg (kg bw)$^{-1}$ d^{-1}	
Average	Adult males 18 – 45 years	Adult male	T	1.20	0.059	0.071
			C	6.02	0.002	0.012
	Adolescents 10 – 18 years	Adult male	T	1.20	0.094	0.113
			C	6.02	0.003	0.015
	Children 3 – 10 years	Children 1.5 – 4.5 years	T	0.77	0.069	0.053
			C	3.83	0.002	0.008
High	Adult males 18 – 45 years	Adult male	T	1.20	0.542	0.650
			C	6.03	0.004	0.024
	Adolescents 10 – 18 years	Adult male	T	1.20	0.863	1.036
			C	6.03	0.005	0.029
	Children 3 – 10 years	Children 1.5 – 4.5 years	T	0.77	0.550	0.424
			C	3.85	0.004	0.016

In the tables 5 and 6, the average exposure estimate of 0.059 µg/kg bw per day for adult males is based on a transfer of 1.375 µg BPA from thermal paper to the finger tips (surface area per finger tip: 2 cm^2, surface dose: 0.69 µg/cm^2), the contact by 3 fingers of one hand only, a single handling event per day, and a body weight of 70 kg (1.375 µg × 3 d^{-1} / 70 kg = 0.059 µg/kg bw per day). The high exposure estimate of 0.542 µg/kg bw per day for adult males was obtained by modifying some of the above assumptions: contact with two hands (i.e. 6 finger tips in total) and 4.6 handling events per day. It is further assumed that each new handling event adds 1.375 µg BPA to the already existing BPA depot in the moisture film on the skin surface, resulting in a total daily surface dose of 4.6×0.69 µg/cm^2 = 3.17 µg/cm^2. Again, only 10% of this surface dose is assummed to be absorbed within 24 h.

Dermal exposure scenarios for children were based on the transfer of 1.375 µg BPA per finger tip, the contact of 3 or 6 fingers, handling events of 0.5 or 2.0 per day, and a body weight of 30 kg.

The CEF Panel noted that the use of a second PBPK model (Mielke et al., 2011) for converting an external dermal exposure into an equivalent oral exposure introduced a (consequential) inconsistency, because the PBPK model of Fisher et al. (2011) was selected as the model of choice for deriving the HED (see Section 3.1.3.4). The CEF Panel quantified the impact of the functional model differences, which turned out to be driven to a major extent by the intestinal first-pass metabolism (present in the Fisher model, but absent in the Mielke/Gundert-Remy model) and to a very minor extent by a slight difference in intrinsic clearance of the liver. The overall impact is that because of this model uncertainty, the dermal-to-oral route equivalence factors ($EF_{D\rightarrow O}$) could be 7.6-fold higher than the equivalence factor used here (see Appendix D).

3.1.8. Conclusions on toxicokinetics

The kinetic data available indicate species- and life stage-dependent differences. Such variability has to be considered when data of different species are compared. Conjugation to BPA-glucuronide, which is a biologically inactive form, is the major metabolic pathway of BPA in humans and animals. A study in humans on high BPA diets (Teeguarden et al., 2011) showed that unconjugated BPA in serum was below the LOD of 0.3 ng/ml (= 1.3 nM), confirming under the condition of the study that internal exposure to unconjugated BPA is low. The percentage of unconjugated BPA in blood is only a few percent of total BPA (sum of conjugated and unconjugated BPA). Based on the analysis of oral (gavage) versus intravenous toxicokinetic data, the oral systemic bioavailability of unconjugated BPA in rats is 2.8 %, in mice 0.45 % and in monkeys 0.9 % (Doerge et al., 2010a,b, 2011a, b, 2012). The concentrations measured in the animal studies and also in the human study render the relevance of serum or blood concentrations in humans, which were measured and reported by some authors in the literature (see Section 4.6.3 in Part I – Exposure assessment) as rather unplausible. Experimental data on the systemic availability of unconjugated BPA in humans has until now not been published. From studies on physiologically based pharmacokinetic (PBPK) modelling it can be concluded, that at relevant oral exposures (e.g. < 1 µg/kg bw per day) the maximum serum concentrations (C_{max}) of unconjugated BPA are in the 3.2–160 pg/ml (7–37 pM) range, depending on the model used (Mielke and Gundert-Remy, 2009; Edginton and Ritter, 2009; Fisher et al., 2011; Yang et al., 2013). BPA does not accumulate in the body even though the concentration of unconjugated BPA is somewhat higher in fat compared to serum.

Some new animal data in particular in mice, rats and monkey give more insight into the kinetics of BPA, in particular into the age-dependent maturation of conjugation reactions. Also, diaplacentar transfer of BPA has been measured in rat and monkey. Data in rats indicate that in early pregnancy transfer to the fetus might be greater compared to later pregnancy after IV injection exposure of BPA (fetus/dam concentration ratios: 2.8 at GD 12, 1.2 at GD 16, 0.4 at GD 20). Unconjugated BPA and BPA-conjugates are measured in the amniotic fluid of rats and rhesus monkeys at low concentrations. BPA is found in milk of rat dams exposed to BPA at a level of 100 ug/kg bw per day in the unconjugated and conjugated forms. The amount delivered to the pups is so small that the concentrations in pup serum are below 0.2 nM (45.6 pg/ml), and therefore pup exposure via lactation is extremely low (1/300 of the maternal dose). These data are in marked contrast to the average concentrations reported for human breast milk (unconjugated BPA~ 0.3 ng/ml; total BPA 1.1 ng/ml)despite the fact that the average human exposure is 1/1000 of the rat exposure exposure (see Chapter 4.6.4. in Part I – Exposure assessment).

Polymorphisms have been described for the enzymes relevant for the conjugation of BPA. Since BPA conjugation can be carried out by several enzymes, a single polymorphism in one gene, resulting in a reduction or loss of enzymatic activity of functional enzymes may result in a change in the plasma levels of unconjugated BPA. Since BPA is glucuronidated by two UTGs and is conjugated not only to glucuronides but also to sulphates, it can be assumed that the increase in blood concentration is modest. This assumption has been confirmed also in the PBPK modelling study by Partosch et al. (2013) showing a 4-fold difference in AUC and C_{max} between the human PBPK models with the highest and the lowest metabolic activity. This difference in sensitivity of BPA in the human population is covered by the assessment factors used in the risk assessment of BPA.

A solid base of toxicokinetic studies in various laboratory animal species (Doerge et al., 2010a,b,c; 2011a,b; 2012) provide internal dose metrics for neonatal-to-adult stages and for different routes of exposure (oral and intravenous/subcutaneous). Moreover, PBPK models have been developed to predict the internal exposures in laboratory animals and humans in a route-specific manner. Overall, this body of information permits extrapolation to humans and the application of the HED concept for providing HEDF which account for the toxicokinetic portion of the interspecies differences. Multiplying the HEDF by a reference point of a critical toxicity study yields a human-equivalent oral dose that is used for risk assessment. The assessment of the physiological plausibility of the derived HEDF values for adult animals with oral dosing revealed a good agreement of the HEDF for monkeys

with the allometric scaling-derived DAF. In rats, the HEDF was 3-times higher than the DAF which can be explained by the effect of enterohepatic recirculation, serving to extent the exposure to BPA. For mice, the HEDF was 2-times lower than the DAF, which suggests a greater metabolic capacity, serving to reduce the AUC. The Panel noted that due to limitations in the analytical detectability of unconjugated BPA in mouse serum, the HEDF for mice may be conservative by a factor of 5.

The available evidence from *in vitro* skin absorption experiments with human, pig and rat skin and from *in vivo* studies on dermal absorption in rats suggests a 24-h dermal absorption for human skin of 2.3–8.6%. For exposure scenarios with dermal contact to thermal paper, the CEF Panel decided to use a skin penetration of 10%. The CEF Panel decided not to consider the amount deposited in the stratum corneum as becoming available for systemic uptake for reasons emerging from the PBPK modelling of dermal exposure. The CEF Panel further decided not to consider skin metabolism in dermal exposure scenarios as the available information does not permit to arrive at a reliable estimate of the extent of skin metabolism. Not to consider skin metabolism is a conservative decision. The CEF Panel noted that the assumption of 10% dermal absorption for the hand contact to thermal paper is also a further conservative decision, since the absorption across the skin of the palms can be expected to be lower than in other body parts because of the thicker stratum corneum. For scenarios with aggregated oral and dermal exposures, PBPK modelling was used to estimate the internal dose metrics for unconjugated BPA and to convert external dermal doses into an equivalent oral doses.

3.2. General toxicity

3.2.1. Animal studies

EU-RAR (2003 and 2008)

In the original 2003 EU Risk Assessment report, the evaluation of the systemic effects of BPA after repeated exposure via the oral route was based on the review of experimental studies in rats, mice and dogs. Changes in body weight gain, liver and kidney were identified as the main systemic effects of BPA after oral exposure in both rodent species. In a 90-day dietary study in dogs, a no effect level of approximately 80 mg/kg bw per day was identified, with increases in relative liver weight being the only other finding observed at approximately 270 mg/kg bew/day.

In the updated report of 2008, a NOAEL of 50 mg/kg bw per day for liver effects from the 2-generation study in mice by Tyl et al. (2008), rather than the LOAEL of 120 mg/kg/day from the Tyl et al. (2002) study identified in 2003 for these effects, was taken forward to the risk characterisation.

EFSA (2006 and 2010)

The EFSA 2006 opinion focused on reproductive and endocrine system-related effects of BPA rather than systemic toxicity *per se*, since these endpoints had been recognized as critical endpoints. As reported in the opinion's summary: *"The available studies cover the majority of endpoints considered relevant for assessment of reproductive effects and other toxicities….The lowest NOAEL of 5 mg/kg bw per day derived in the recent two-generation reproductive toxicity study in mice is based on liver effects. Toxic effects of repeated administration of BPA on the liver in mice have also been observed in previous studies with a LOAEL of 120 mg/kg bw per day, suggesting that liver toxicity is at least as sensitive an endpoint for BPA as reproductive and developmental effects. The NOAEL for liver toxicity in mice is identical to the derived NOAEL for reproductive toxicity of bisphenol A in rats used in the EU RAR, which is based on effects on adult and offspring body weight gain"* (EFSA, 2006).

In 2010, EFSA further confirmed the validity for setting the TDI of the overall NOAEL of BPA of 5 mg/kg bw per day for systemic effects identified in the multi-generation studies in rats and mice by Tyl et al. (2002, 2008).

FAO/WHO (2011)

With regard to repeated exposure studies with BPA, the WHO report concluded: *"Tyl et al. (2002, 2008) conducted two large multigenerational studies in rats and mice using dietary administration of*

BPA over a wide range of doses (1 or 3 µg/kg bw up to 500 or 600 mg/kg bw), allowing for dose–response assessment. These studies demonstrated effects on the liver, kidney and body weight at doses of 50 mg/kg bw and higher. A more recent study by Stump et al. (2010), which also used an expanded dose range and the same animal model as that used by Tyl et al. (2002), demonstrated similar findings (on common end-points examined), with a lowest NOAEL of 5 mg/kg bw. The liver also appeared to be a target organ in a non-rodent model (dog), with a NOAEL of 74 mg/kg bw following oral exposure."

ANSES (2011 and 2013)
The 2013 risk assessment by ANSES specifically dealt with the effects identified as "proven" in animal studies and "suspected" in humans in the 2011 ANSES report, none of which was related to general toxicity effects.

3.2.2. Studies on general toxicity after oral exposure to BPA considered most significant by previous reports published before 2010

Tyl et al. (2002) exposed CD Sprague-Dawley rats (n = 20 females per group) to dietary BPA in a three generation study at 0, 0.015, 0.3, 4.5, 75, 750, 7 500 ppm (giving doses of approximately 0, 0.02, 0.3, 5, 50 and 500 mg/kg bw per day). The exposure started 10 weeks before mating and continued during mating, gestation and lactation until weaning. At weaning (PND21), 30 animals /sex/dose were randomly selected as F1 parents, and exposed to BPA as described for the F0 generation. The selection, number and treatment of the F2 parents were performed as described for the F1 parents. Adult systemic toxicity at 750 and 7 500 ppm in all generations included: reduced body weights and body weight gains, reduced absolute and increased relative weanling and adult organ weight (liver, kidney, adrenals, spleen, pituitary and brain), and mild renal (tubular degeneration) and hepatic pathology (in females only). Reproductive organ histology and function were unaffected, except for reduced ovarian weights, a significantly reduced number of implantation sites and decreased number of pups/litter on PND 0 at 7500 ppm. In the F1, F2 and F3 offspring vaginal patency and preputial separation were delayed, associated with reduced body weight. Adult systemic NOAEL were 5 mg/kg bw per day and reproductive and postnatal NOAEL were 50 mg/kg bw per day.

Tyl et al. (2008) examined dietary BPA in a CD-1 mice (n=28 per group) two-generation study at 0, 0.018, 0.18, 1.8, 30, 300, or 3 500 ppm in feed (giving 0, 0.003, 0.03, 0.3, 5, 50, or 600 mg BPA/kg bw per day). 17β-oestradiol (0.5 ppm) was used as positive control. There were no BPA related effects on adult mating, fertility or gestational indices, ovarian primordial follicle counts, oestrous cyclicity, precoital interval, offspring sex ratios or postnatal survival, sperm parameters or reproductive organ weights or histology. Systemic effects in adults were increased kidney and liver weight, centrilobular hepatocyte hypertrophy, and nephropathy and statistical significant reduction in epididymal sperm concentration (15% reduction) in both F0 and F1 males at 3500 ppm (600 mg/kg bw per day). Centrilobular hepatocyte hypertrophy was also apparent in F0 males at 50 mg BPA/kg bw per day), and kidney weight was statistically significantly increased at this dose level in both F0 and F1 males, while in F1 males there was also a statistically significant increase in kidney weight in animals receiving 0.3 or 5 mg/kg bw per day. Female mice were less sensitive to these effects. Increased kidney and liver weight and centrilobular hepatocyte hypertrophy was observed in F0 females at 3 500 ppm (600 mg/kg bw per day), but nephropathy was not evident on histopathological examination, and centrilobular hypertrophy was the only treatment related change reported in the F1 females. At 3 500 ppm (600 mg/kg bw per day) BPA also reduced F1/F2 weanling body weight, reduced weanling spleen and testes weight (with seminiferous tubule hypoplasia). At lower doses (0.018 to 30 ppm) there were no treatment related effects in adults or F1/F2 offspring. There are no obvious weaknesses in this study.

3.2.3. New studies on general toxicity after exposure to BPA published after 2010

The US National Center for Toxicological Research (NCTR) has recently completed a subchronic toxicity study involving pre- and postnatal administration of BPA to Sprague Dawley rats (US FDA/NCTR, 2013 and Delclos et al., 2014). The rats were exposed to BPA by gavage at doses of 0, 2.5, 8, 25, 80, 260, 840, 2 700, 100 000 and 300 000 µg/kg bw per day from GD 6 to the start of labour and then directly to the pups from PND 1 to PND 90. Ethinyl oestradiol (EE_2) was used as a reference substance and given by gavage at doses of 0.5 and 5.0 µg/kg bw per day. Litters were adjusted to 10 pups (5 males and 5 females) at PND1. The litter was the unit of analysis and the target litter number was 20 per dose group (n = 18-23). Data collection include body weights, weekly food consumption, litter parameters, anogenital distances at PND 1 and PND 90, measures of sexual development (vaginal opening and time to first estrus, nipple retention, testicular descent, and preputial separation, vaginal cytology, clinical chemistry, organ weights and histology.

Dose-related changes in organ weight was observed with statistically significant effect at the highest dose level only in both females and males, with increased liver weight and reduced weights for the following organs: heart, ovary, brain (males only), kidney (males only) and spleen (100 000 µg/kg bw per day in females and 300 000 µg/kg bw per day in males). Changes in clinical chemistry (e.g. cholesterol, leptin, triglycerides) were observed in both sexes and at the two top dose levels.

Due to the limited number of studies available addressing the endpoint general toxicity, this endpoint has not been analysed by the WoE approach. The CEF Panel nevertheless considered that the general toxicity effects of BPA were "likely".

3.2.4. Conclusion on hazard identification for general toxicity of BPA

In summary, BPA effects on the kidney and liver weight were reported both in rats and mice in the multi-generation studies by Tyl et al. in 2002 and 2008. In male mice the increased kidney weight was associated with nephropathy at the highest BPA dose, while the kidney weight changes were less marked in female mice and were not associated with nephropathy. Mild renal tubular degeneration was also observed in female rats at the highest dose. In contrast, Tyl et al. (2002) and the new subchronic rat study including prenatal exposure by US FDA/NCTR, showed reductions in kidney weight. The CEF Panel noted that the mechanisms of the effects in the rodent kidney are not yet understood including whether these are due to the unconjugated or conjugated form of BPA. As it would not be possible to distinguish between effects of conjugated and unconjugated BPA, the CEF Panel assumed that the effects in the kidney were caused by unconjugated BPA as a conservative approach. Liver weight was increased in rats (relative weight) and mice (both absolute and relative weight), the latter species also showing hepatocyte hypertrophy (Tyl et al., 2002; US FDA/NCTR, 2013 and Delclos et al., 2014). These observations support that changes in the kidney and liver are critical endpoints in BPA toxicity, and based on the EFSA evaluations of 2006 and 2010 the CEF Panel considered that these effects were "likely" without performing a WoE. These endpoints were therefore taken forward for hazard characterisation.

3.2.5. Hazard characterisation (dose-response relationship) for general toxicity

Based on the above mentioned robust studies on general toxicity, the reported effects on kidney and liver have been taken forward for hazard characterisation. It should be noted that the US FDA/NCTR (2013) study is of shorter duration than the studies by Tyl and colleagues and effects indicative of general toxicity were only seen at doses higher than those in the Tyl studies, and therefore the latter studies have been selected as the basis for hazard characterisation for general toxicity.

In compliance with the Opinion of the EFSA Scientific Committee on the use of the Benchmark dose (BMD) approach in Risk Assessment (EFSA, 2009), the results obtained on general toxicity in the reproductive toxicity study with BPA in mice have been subjected to statistical dose-response modelling. Given that a NOAEL of 5 mg/kg bw per day was established from both the rat (Tyl et al., 2002) and mouse (Tyl et al., 2008) studies, but that the HEDs from the mice are much lower that for

rats at the same dose levels of BPA, the focus of the BMD analysis was on the study in mice (Tyl et al., 2008). The Tyl et al. (2002) rat study had been used to derive a reference point in the previous EFSA opinion from 2010, by using the NOAEL of 5 mg/kg bw per day. As stated in the EFSA opinion on BMD the NOAEL and BMDL are equally applicable as a reference point: "*It has been suggested that larger or additional uncertainty factors might be appropriate when a BMDL is used as the reference point. The argument used is that the BMDL does not reflect a "no-effect" dose, in contrast to the NOAEL. This argument is based on the false assumption that a NOAEL is associated with the complete absence of any effect. As discussed (in Section 3.5), the default values of the BMR are such that the BMDL on average coincides with the NOAEL. Further, it was shown in section 3.3 that the potential magnitude of the effect at the NOAEL can be even greater than the specified effect size (BMR) associated with the BMD/BMDL. Taking these considerations into account, an additional uncertainty factor, beyond those normally applied is not necessary. The health-based guidance value derived from the BMDL can be expected to be as protective as the one derived from the NOAEL, i.e, on average over a large number of risk assessments. In conclusion, the default values for uncertainty factors currently applied to the NOAEL are equally applicable to the BMDL"* (EFSA, 2009).

The toxicological effects used for defining a reference dose were increased liver weight, increased kidney weight and centrilobular hepatocyte hypertrophy in the adult F0 and F1 generation in male mice (see table 77 in Appendix E). Since the public consultation the individual data from Tyl et al. (2008) have become available, and new BMD calculations on the changes in organ weights have been performed based on individual data.

For all the new dose-response modelling of the mice individual organ weight data (Tyl et al., 2008), the statistical package PROAST (version 40.7) has been used, obtained directly from RIVM, through personal contact. For the previous dose-response modelling of the histopathological data (liver hypertrophy and mammary duct hyperplasia), PROAST version 38.6 was used. This version is no longer available at the website (www.proast.nl), but a more recent version (38.9) can be downloaded. Using the PROAST package, the 95 % lower confidence limit (one-sided) of the Benchmark dose (BMDL) was calculated. For each evaluation, depending on the type of data evaluated, the statistical models for continuous data (organ weights) or for quantal data (histopathology) were used.

A benchmark response (BMR) of 10% was chosen both for the kidney and liver effects, based on the view of the CEF Panel that changes in the kidney and liver weight, and hepatocyte hypertrophy of less than 10% should not be regarded as adverse. The CEF Panel also took into account that the changes in the liver (hepatocyte hypertrophy) were likely to be adaptive in nature, and the pathological changes in the kidney were marginal, only observed at the highest dose level and lacked a clear dose-response.

All the results obtained are reported in detail in Appendix E. table 7 below shows the $BMDL_{10}$ values obtained for liver and kidney effects in the F0 and F1 generations of mice. A lack of dose-response relationship was observed for nephropathy in both sexes. Therefore no model was obtained with acceptable fit and no BMDL could be calculated for this effects. The CEF Panel noted that there is uncertainty regarding the robustness of the BMD modelling for mammary gland hyperplasia, as discussed in more detail in Section 3.9.7.
The analysis revealed large differences in the BMD estimates obtained with the various models and the wide BMD confidence intervals, obtained with some of the models (Appendix E). The CEF Panel concluded that the dose-response relationship in the US FDA/NCTR study (2013) and Delclos et al. (2014) is not suitable to derive with any confidence a reference point for this endpoint.

Table 7: Dose-response relationships for general toxicity of BPA in mice (Tyl et al., 2008)

Study	Species (generation)	Route of administration	Toxic effect	External dose level (µg/kg bw per day)	
				$BMDU_{10}$	$BMDL_{10}$
Tyl et al.,	Mice (F0) males,	Oral feed	Increased absolute liver	456 800	213 100

2008	with sex and F0/F1 as covariate		weight		
Tyl et al., 2008	Mice (F1) males, with sex and F0/F1 as covariate	Oral feed	Increased relative liver weight	328 700	229 000
Tyl et al., 2008	Mice (F0) males, with sex and F0/F1 as covariate	Oral feed	Centrilobular hepatocyte hypertrophy	67 200	3 460
Tyl et al., 2008	Mice (F0) males, with sex and F0/F1 as covariate	Oral feed	Increased absolute mean kidney weight	91 690	3 420
Tyl et al., 2008	Mice (F0) males, with sex and F0/F1 as covariate	Oral feed	Increased relative mean kidney weight	108 900	8 960

Although the lowest $BMDL_{10}$ from the modelling was observed for hepatocyte hypertrophy, the effect of BPA on hepatocyte hypertrophy was regarded by the CEF Panel as adaptive and as a less critical effect than the effect in the kidney. In addition, the analysis of the liver weight changes indicated a much higher level for the BMD for this endpoint, while a relationship between liver weight changes and liver hypertrophy is known to exist. That supports that the observed liver hypertrophy has low relevance. For the absolute liver and kidney weight changes in F0 females very wide confidence intervals around the BMDs were obtained, because of lack of dose-response information in the data. Considering that organ weights are dependent on body weight, adequate dose-response information could be identified for relative liver and kidney for all generations and and sexes, when changes in the body weight were taken into account. In addition the confidence intervals for the BMD calculations were smaller for the relative organ weights, indicating better dose-response relationships and less variability in the data for relative organ weights. The CEF Panel has therefore selected the endpoint of relative mean kidney weight in the mouse as a reference point, for which a $BMDL_{10}$ of 8 960 µg/kg bw per day was derived.

3.2.6. Conclusion on hazard characterisation for general toxicity

BPA was found to affect kidney and liver weight in parental animals and in all the generations of rats and mice examined in multi-generation studies. These effects were considered by EFSA (2006, 2010) as relevant systemic effects for the identification of a NOAEL. In mice the increased kidney weight was associated with nephropathy at the highest BPA dose. Liver weight was increased in rats (relative weight) and mice (both absolute and relative weight). The latter species also showed hepatocellular hypertrophy. The CEF Panel considered the endpoint "general toxicity" for hazard characterisation, using a reference point from the two-generation study in mice, which provided a $BMDL_{10}$ for mean relative kidney weight of 8 960 µg/kg bw per day in male mice of the F0 generation.

3.3. Reproductive and developmental effects

3.3.1. Human studies

3.3.1.1. Summary of previous opinions

EU-RAR (2003, 2008)
No human data were reviewed in the 2003 report. In the 2008 report it was stated that no conclusions could be drawn from a human study investigating the possible association between recurrent miscarriage and BPA exposure.

EFSA (2006, 2010)
No human data were reported in the opinion of 2006.

The 2010 EFSA opinion included evaluation of a number of studies investigating the association between BPA exposure and reproductive/developmental disorders in human subjects that had been

published since 2007 (Itoh et al., 2007; Padmanabhan et al., 2008; Wolff et al., 2008; Braun et al., 2009; Cobellis et al., 2009; Yang et al., 2009; Li et al., 2010a, b; Meeker et al., 2010; Mendiola et al., 2010; Mok-Lin et al., 2010).

The CEF Panel considered that the available studies were limited by their mostly cross sectional design and other methodological issues, and therefore that no relevant conclusions for risk assessment could be drawn from them.

NTP-CERHR (2008)

No human data were available on developmental effects of BPA and only a few studies focused on reproductive endpoints. The NTP expressed the view that the evidence from the limited number of studies in humans exposed to BPA was not sufficient to reach conclusions regarding possible developmental or reproductive hazard.

FAO/WHO (2011)

In respect to male reproduction endpoints, the FAO/WHO report reviewed three epidemiological studies (Mendiola et al., 2010, Meeker et al., 2010, Li et al., 2010a) studying the association of urinary BPA levels with semen quality. Increased urinary BPA concentrations were associated with reduced semen quality in all three studies, although statistical significance was reached in one study only. Additional limitations of these studies included their cross-sectional designs and the incomplete assessment of occupational co-exposure in one of the three studies. In the case of female reproduction, an inverse association between urinary BPA concentration and oocyte yield from women undergoing in vitro fertilization treatment in fertility clinics was reported in a small study only (Mok-Lin et al., 2010), thus preventing any conclusions to be drawn in the absence of data replication. Limited and inconsistent evidence for an association of BPA with altered age of pubertal onset in girls was identified in two epidemiological studies. Therefore, no conclusions could be drawn by the Expert Meeting with repect to an association of BPA with perinatal outcomes.

ANSES (2011, 2013)

In the 2011 ANSES report, experts evaluated human studies and stated that effects of BPA on the male reproductive system were *controversial*, that effects of BPA on oocyte maturation were *suspected* based on good quality studies (Mok-Lin et al., 2010, Fujimoto et al., 2010), and that studies of other parameters (endometrium, ovaries and pregnancy outcome) were *too limited to draw a conclusion* (ANSES, 2011). Since the adoption of the report on the health effects of BPA in 2011, ANSES has reviewed over 20 recently published epidemiological studies, and indicated in their 2013 risk assessment of BPA that the results of these studies did not change their previous conclusions (ANSES, 2013).

3.3.1.2. Evaluation of recent human studies on BPA exposure and reproductive and developmental effects

This section provides an overview of the human studies on reproductive and developmental effects published after July 2010. Some of these studies are also evaluated in relation to hormonal and metabolic effects. A detailed description and evaluation of each study is provided separately in Appendix B.

Since the previous EFSA review (2010), 21 studies have been evaluated.

The studies have been grouped into postulated BPA effects on 1) adult reproduction and health (Fujimoto et al., 2011; Bloom et al, 2011a, b; Ehrlich et al., 2012a, b; Krotz et al., 2012; Buttke et al., 2012; Li et al., 2010a, b; Zhou et al., 2013; Galloway et al., 2010; Kandaraki et al., 2011; Tarantino et al., 2013), and 2) gestational/birth outcomes (Cantonwine et al., 2010; Fénichel et al., 2012; Miao et al., 2011a, b; Choi et al., 2012; Chevrier et al., 2012; Chou et al., 2011; Philippat et al., 2012; Snijder et al., 2013; Lee et al., 2013a).

1) BPA effects on adult reproduction and health

Of seven studies examining BPA exposure in relation to adult reproductive outcomes, five examined indicators such as embryo quality, fertilisation and implantation failure in couples undergoing in vitro fertilization (IVF). The causes of infertility are very different in those who present for IVF compared with both fertile couples and couples seeking investigations and treatment for infertility. Notwithstanding the benefits of those undergoing IVF from a research practicability point of view, caution must therefore be exercised when extrapolating from an IVF study group to the general infertility population, let alone to the fertile population (Hull et al., 1985; Maheshwari et al., 2008; HFEA, 2011).

In a cross-sectional study with 31 women, Fujimoto et al. (2011) found no association between serum unconjugated BPA and oocyte fertilization (number of oocytes) in the full study but an inverse association was seen in the nine Asian women included in the study.

In their first cross-sectional study with 27 couples, Bloom et al. (2011a) found no association between female serum unconjugated BPA and embryo cell number, but reported weak associations for male serum BPA and indicators of embryo quality (lower embryo cell number; p=0.07, lower embryo fragmentation score: p=0.009). In their second cross-sectional study with 44 women (some women overlapping with the first study), Bloom et al. (2011b) found that higher serum BPA was significantly associated with reduced peak oestradiol levels measured as an index of follicular response), but was not associated with oocyte fertilization.

Ehrlich et al. (2012a) collected 1-2 spot urine samples in 137 women during a total of 180 IVF cycles and reported a borderline significant association between increasing urinary total BPA quartiles and implantation failure (p-trend =0.06). In a subsequent study, partially utilising the same study population as Ehrlich et al. (2012a) , Ehrlich et al. (2012b) found that higher urinary total BPA was associated with decreasing number of oocytes, decreasing number of normally fertilized oocytes and decreasing oestradiol levels (all $p<0.01$). The women in both studies (Ehrlich et al., 2012a and 2012b) were part of a larger prospective cohort study designed to investigate the impact of environmental chemicals on fertility and pregnancy outcomes among couples seeking fertility treatment. The authors describe the studies as "prospective", but the time between assessment of exposure and outcome was only a few days.

Krotz et al. (2012a) examined whether phthalates and BPA could be detected in follicular fluid following IVF treatment in five women, BPA was undetectable (phthalates were detected).

The CEF Panel noted that the generalisability of results from IVF studies is uncertain, as women undergoing IVF are likely to also be exposed to BPA from medical plastics during an IVF cycle. The above studies were limited by timing of the exposure, sample size and study design. Four of the studies measured BPA in serum, which may not be a valid measure due to the pervasive contamination from plastic.

A cross-sectional study in occupationally exposed workers in China showed that higher urinary BPA was associated with lower sperm concentration, count, vitality and motility, suggesting negative impact on human fertility (Li et al., 2011). Of 888 men invited, only 58% participated in the study, without reasons for non-participation being known (fertility problem, age, etc.), which may constitute a selection bias. The measurements of sperm quality involved 218 individuals. The results are comparable to a study evaluated in the EFSA CEF PANEL, 2010 opinion (Meeker et al., 2010).

In another cross-sectional study from China, Zhou et al. (2013) evaluated the association between serum bisphenol A and sex hormone levels in 137 male factory workers who were exposed to BPA at the workplace for more than 6 months, and 153 age-matched workers from a tap water factory without occupational exposure to BPA. Increasing serum BPA concentration was associated with decreased androstenedione levels, decreased free testosterone levels, decreased free androgen index, and

increased sex hormone-binding globulin levels. The results are comparable to two studies evaluated in the EFSA CEF PANEL, 2010 opinion (Mendiola et al., 2010; Meeker et al., 2010).

In a cross-sectional study of 715 adult men in the InCHIANTI study in Italy, Galloway et al., 2010 found that higher levels of urinary BPA was associated with increased serum total testosterone concentration (p=0.004), but was not associated with circulating free testosterone (p=0.075) or β-oestradiol. In women, no associations were found between urinary BPA and total testosterone, free testosterone or β-oestradiol, but a positive association was found for sex hormone-binding globulin (SHBG) in premenopausal women (p=0.004).

Two case-control studies examined associations between serum total BPA and existence of polycystic ovary syndrome (PCOS) in women. Kandaraki et al., 2011 included 71 PCOS and 100 normal women and found that serum BPA was higher in the PCOS group compared with controls (1.05 vs 0.72 ng/ml, p<0.001).Tarantino et al. (2012) included 40 PCOS and 20 normal women and found that higher serum BPA was associated with hepatic steatosis and markers of low-grade inflammation in the women with PCOS. The studies have several statistical concerns. Furthermore, the CEF Panel considered that serum BPA can be an unreliable measure due to the pervasive contamination from plastic.

Buttke et al. (2012) studied associations between exposures to multiple endocrine-disrupting chemicals, including BPA, and age of menarche in adplescent girls in NHANES 2003-2008. Urinary total BPA concentration was not associated with age of menarche.

2)BPA effects on gestational/birth outcomes

Eleven studies examined BPA exposure in relation to gestational or birth outcomes.

In a nested case-control subset with 30 cases and 30 controls in a study of environmental toxicants in Mexico City, high urinary BPA levels were associated with increased risk of delivery before week 37 (p<0.05) (Cantonwine et al., 2010).

Five studies reported significant associations between prenatal BPA exposure and fetal growth, of which three showed reduced fetal growth with higher BPA exposure (Miao et al., 2011a, Chou et al., 2011 and Snijder et al., 2013), one showed weakly increased fetal growth with higher BPA exposure (Lee et al., 2013) and one showed increased head circumference with increasing BPA (Philippat et al., 2012). In a study from China (Miao et al., 2011a), parental exposure to BPA in the workplace during pregnancy was associated with decreased birth weight in infants (p=0.02 for maternal occupational exposure). The study estimated BPA exposure by combining work place air monitoring and recall of employment history and change in work environment. The characterisation of the exposure is questionable and confounding by diet or concurring exposure factors was not considered.

In a cross-sectional study by Chou et al. (2011) total BPA was measured in maternal and umbilical cord plasma in 97 mother-newborn pairs in a birth cohort in Taiwan. In male neonates only, high maternal BPA was associated with reduced birth weight. The CEF Panel noted that the study was limited by cross sectional design and cord blood BPA measurement.

A prospective study with 219 mother-child pairs within a pregnancy cohort in Rotterdam found no significant association between maternal urinary total BPA (prenatal BPA exposure) and fetal weight or head circumference (Snijder et al., 2013). However, when the analyses were restricted to 80 women for whom three repeat urinary samples were available, the results showed that higher urinary BPA was significantly associated with intrauterine growth restriction. The outcome was assessed by fetal growth rates based on repeat ultrasound biometry and birth size. The estimated difference between altered mean values at birth between the upper and lower category of urinary BPA was –683 g (20.3% of mean) for birth weight and –3.9 cm (11.5% of mean) for head circumference. The study found that increasing the number of urine samples per subject strengthened the exposure–response estimates.

The relatively small sample size limited the power to examine associations with fetal growth in different time windows. The statistical analyses included sensitivity analyses and evaluation of the effect of the number of measurements per subject on the observed associations. The analyses included adjustment for potential confounders, but no dietary factors other than alcohol consumption was considered.

Contrary to the above studies which showed that increased maternal urinary BPA concentrations were associated with reduced fetal growth, a prospective study with 757 mother-children pairs in Korea found that higher maternal urinary BPA concentration in the third trimester was weakly associated with increased birth weight and ponderal index in neonates. The associations differed by gender. No associations were found between urinary BPA measured in the first trimester and birth outcome (Lee et al., 2013a).

Philippat et al., 2012 examined urinary BPA and phthalate exposure and birth outcomes in a case-control study on malformations of the male genitalia nested in two French mother-child cohorts. The study sample comprised 191 infants and cases and controls were treated as one group. Increasing urinary BPA concentrations were associated with increasing head circumference (p-trend 0.01), and also suggested an association with increased birth weight. The study is limited by the choice of study group and the clinical relevance of the association between BPA exposure and head circumference is not clear.

In a study from China (Miao et al., 2011b), maternal occupational BPA exposure was associated with shortened anogenital distance in 153 boys (p-trend: 0.008). As for the study of birth weight in this population (Miao et al,. 2011a) the outcome was compared between groups reflecting maternal exposure, paternal exposure or no work place exposure, based on work place air monitoring and recall of employment history and change in work environment. The characterisation of the exposure is questionable and confounding factors such as diet and/or concurring exposure factors were not considered.

In a study from Fénichel et al. (2012) using a methodologically questionable RIA method to quantify cord blood BPA there was no difference in cord-blood BPA between boys with undescended testis (n=46) and controls (n=106)).

A case-controlled study of exposure to five phthalates and BPA in infants with hypospadias and controls was conducted in Korea (Choi et al., 2012). Phthalates and total BPA were measured both in urine and plasma in 80 children with hypospadias, in 80 control children and in 40 mothers of children with hypospadias. Urinary BPA in children was not associated with hypospadias, whereas plasma total BPA was higher in children with hypospadias than in controls ($P < 0.001$). No relationship was seen between levels of BPA in urine or plasma in the mothers and the occurrence of hypospadias. The study is limited by statistical handling, the measurement of BPA in plasma and very limited description of sampling.

Another case-controlled study from South Korea examined plasma concentrations of several endocrine disrupting chemicals in 39 infants with congenital hypothyroidism and 20 controls. There was no difference in plasma BPA concentration between patients and controls (P=0.2).

In a study by Chevrier et al. (2012) maternal urinary BPA was measured twice during pregnancy and examined in relation to maternal and infant thyroid function. The study sample comprised 476 women in an immigrant Mexican-American population with low socioeconomic status. The results suggested that exposure to BPA during pregnancy was related to reduced total T4 in pregnant women and decreased TSH in male neonates. The average of the two maternal BPA concentrations was associated with reduced TSH in boys ($p<0.01$) but not in girls. This association was stronger when BPA was measured in the third trimester of pregnancy and decreased with interval between BPA and measurement of TSH. Iodine status was taken into account, but potential confounders, such as diet

and/or concurring exposure factors, were not considered. BPAwas not measured in the urine of the neonates.

3.3.1.3. Summary of BPA exposure and reproductive and developmental effects in humans

In their 2010 EFSA opinion, the CEF Panel concluded that the studies then available were not sufficient to draw any conclusion regarding BPA exposure and reproductive and developmental effects in humans. This conclusion was based on studies limited by mostly cross-sectional design, small sample size and other methodological weaknesses. Since then, a number of studies have been reported, but the limitations noted in the previous opinion are still prevalent. Of 22 new studies, only six had a prospective design. Some of the new studies were well powered (i.e. Galloway et al., 2010; Li et al., 2011; Miao et al., 2011a), but had large uncertainty in either exposure or outcome assessment. There are indications from several prospective studies that BPA exposure during pregnancy may have effects on fetal growth (two studies showed reduced fetal growth with increasing maternal BPA exposure, while one study reported increased fetal growth). There are also weak indications that BPA exposure during pregnancy may be associated with maternal and infant thyroid function. It cannot be ruled out, however, that these results are confounded by diet or concurrent exposure factors. The associations do not provide sufficient evidence to infer a causal link between BPA exposure and reproductive effects in humans.

3.3.2. Animal studies

3.3.2.1. Summary of previous reviews of the reproductive and developmental toxicity of BPA

In recent years, the reproductive and developmental toxicity of BPA has been thoroughly evaluated at national, European and international level as reported below.

EU-RAR (2003, 2008)

In the original risk assessment report of 2003, it was stated that the effects of BPA on fertility and reproductive performance had been investigated in three good quality studies: a 2-generation study and a multigenerational study in the rat, and a continuous breeding study in the mouse. These studies had shown similar qualitative and quantitative toxicological profiles of BPA for effects on fertility, i.e. reductions in litter size, both in rats, i.e. at 500 mg/kg bw per day and mice, i.e. at 600 mg/kg bw per day. It was also concluded that there was no convincing evidence that BPA was a developmental toxicant in standard development studies in rats (maternal LOAEL and fetal NOAEL of 160 and 640 mg/kg bw per day, respectively), and mice (maternal and fetfetal NOAELs of 250 and 1,000 mg/kg bw per day, respectively). An overall NOAEL of 50 mg/kg bw per day identified from the rat multigenerational study was then used for risk characterisation purposes, in relation to effects on fertility and provisionally for developmental effects. However, given the uncertainties surrounding the potential for BPA to produce adverse effects on development at low doses, the outcome of further testing was awaited.

The 2-generation study in mice conducted by Tyl et al. (2008) provided a comprehensive investigation of the reproductive effects of BPA at exposure levels of 0, 0.003, 0.03, 0.3, 5, 50 and 600 mg/kg bw per day. Fertility was not affected by any BPA dose tested. In the absence of any adverse effect of BPA in the µg/kg bw per day dose range on the male reproductive tract development, the EU-RAR considered that the study resolved the uncertainties surrounding the potential to produce adverse effects on development at low doses. The study results confirmed the provisional NOAEL of 50 mg/kg bw per day for reproductive and developmental toxicity, based on the effects that were detected at the next dose level of 500-600 mg/kg bw per day, namely slightly longer gestation, reduced pup body weight during lactation, a slight increase in the incidence of undescended testes at weaning, seminiferous tubule hypoplasia in offspring at weaning, and delayed acquisition of preputial separation.

EFSA (2006); EFSA CEF Panel (2010)

In the EFSA 2006 opinion it was stated "*In reviewing the earlier and the recently published studies on BPA, the Panel noted that some studies indicated differences in behaviour or reproductive parameters between control and treated animals at dose levels lower than the previously accepted overall NOAEL of 5 mg/kg bw per day. However, the Panel had considerable reservations both about the biological significance of the reported observations and the robustness of the studies".* The study by Rubin et al., 2001, used as a pivotal study in the risk assessment of ANSES (ANSES, 2013) was evaluated in the EFSA opinion 2006, and the AFC Panel noted some shortcomings of the study: *"The Panel notes that doses were stated to be approximately 0.1 mg and 1.2 mg BPA/kg bw per day, 6 dams/dose, but it is likely that there was underestimation of exposure due to an assumed low water consumption. Offspring exposed to BPA in utero (n= 12 – 28 offspring/group, but only six dams treated) exhibited an increase in body weight. In addition, female offspring exposed perinatally to the higher dose of BPA exhibited altered patterns of ooestrous cyclicity (changes not defined) and decreased levels of plasma LH in adulthood".*

The 2010 EFSA CEF Panel opinion mainly considered peer-reviewed "low dose" in vivo studies published between January 2007 and July 2010. The opinion focused upon the oral route of administration, developmental exposure and the testing of several doses, including at least one below the oral NOAEL of 5 mg/kg bw per day established by Tyl et al., 2008.

The conclusions of the CEF Panel on reproductive toxicity studies in animals exposed to BPA during development applied to both males and females. In particular the CEF Panel concluded: *"The studies on developmental and reproductive toxicity reporting effects at doses lower than 5 mg/kg bw per day including the study by Salian et al. (2009) have severe shortcomings and were considered to be invalid. The Panel considers that the valid studies do not raise concern regarding reproductive and developmental toxicity of BPA at doses lower than 5 mg/kg bw per day."*

NTP-CERHR (2008)

The NTP-CERHR monograph stated that there was clear evidence of adverse developmental effects on survival and growth at "high" doses of BPA, based on reduced survival in fetuses or new-borns (≥ 500 mg/kg bw per day), reduced fetal or birth weight or growth of offspring early in life (≥ 300 mg/kg bw per day), and delayed puberty in female rats (≥ 50 mg/kg bw per day) and male rats and mice (≥ 50 mg/kg bw per day).

With respect to reproductive toxicity, NTP stated that there was some evidence of adverse effects in animal studies, based on possible decreased fertility in mice (≥875 mg/kg bw per day), altered ooestrous cycling in female rats (≥600 mg/kg bw per day), and cellular effects on the testes of male rats (235 mg/kg bw per day). In the case of "low" dose developmental toxicity, the NTP concluded that there was limited evidence of adverse effects based on various neural and behaviour alterations (≥10 µg/kg bw per day), lesions in the prostate (10 µg/kg bw per day) and mammary glands (2.5–1000 µg/kg bw per day), altered prostate gland and urinary tract development (10 µg/kg bw per day), and early onset of puberty (24 and 200 µg/kg bw per day).

FAO/WHO (2011)

Based on a review of the studies published since 2008, the Expert Meeting concluded that there is considerable uncertainty as to whether BPA has any effect in rodents on conventional reproductive or developmental end-points at doses below 1 mg/kg bw per day by the oral or subcutaneous route. The only evidence for adverse reproductive and developmental effects of oral BPA came from studies in rats or mice with no relevant evidence from humans, non-human primates or domestic animals. Species-related differences, e.g. in timing of developmental periods of sexual differentiation and involvement of different hormones, limited a straightforward translation of findings from rodents to humans. Important data gaps in the reproductive and developmental toxicology of BPA in experimental animals included the lack of a thorough assessment of critical developmental reproductive end-points following direct exposure of the neonate to BPA, and of the effects of BPA in alternative animal models, including non-human primates, lagomorphs and other non-rodent species,

that might be more relevant to human development for a few specific issues (e.g. effects on the prostate).

ANSES (2011 and 2013)

In the 2011 report, concerning the effects of BPA on the male reproductive system in animal studies, ANSES concluded that there were proven alterations of sperm production after 5 weeks exposure during adulthood (Chitra et al., 2003 and Herath et al., 2004), suspected reductions in plasma testosterone concentrations and altered sexual behaviour due to pubertal exposure. In their 2013 report, ANSES evaluated several new animal studies on the effects of BPA on the male reproductive system (D'Cruz et al., 2012a; Doshi et al., 2011; Kobayashi et al., 2012; Lopez-Casas et al., 2012; Nanjappa et al., 2012) and expressed the view that overall these data did not call into question the conclusions of the 2011 report.

In 2011, the ANSES report on the health effects of BPA concluded that in vivo animal studies indicated proven effects of BPA on the female reproductive system, consisting of increased occurrence of ovarian cysts (Newbold et al., 2007, 2009; Signorile et al., 2010), endometrial hyperplasia (Mendoza-Rodríguez et al., 2011; Markey et al., 2005), earlier onset of puberty (Honma et al., 2002; Howdeshell et al., 1999; Nikaido et al., 2004; Adewale et al., 2009 and Fernandez et al., 2009) and changes in the hypothalamic-pituitary-gonadal (HPG) axis (Savabieasfahani et al., 2006; Evans et al., 2004; Collet et al., 2010) after pre- and/or early postnatal BPA exposure.

In 2013, ANSES concluded that the female reproductive toxicity studies in animals published since the 2011 ANSES report supported the conclusion that developmental exposure (*in utero* in the mouse and the monkey and early postnatal in the ewe) to low BPA doses could disrupt the meiotic processes and cause early folliculogenesis possibly leading to a reduction in the follicular reserve, and unknown functional reproductive consequences in the adult. Furthermore, in ANSES' view certain recent studies have reinforced that BPA can disrupt the HPG axis and cause histological changes and acceleration of the puberty process during early neonatal exposure.

3.3.2.2. Studies on reproductive and developmental effects following oral exposure to BPA considered by previous reports published before 2010

The WoE approach that has been taken in the current opinion has necessitated the re-evaluation of a number of studies on reproductive and developmental effects of BPA that has already been evaluated in the previous risk assessments summarised above, namely the study by Rubin et al. (2001), used as a pivotal study for reproductive and developmental toxicity in the ANSES report (ANSES, 2013), the studies of Tyl et al. (Tyl et al., 2002; 2008) that were used by EFSA as a basis for the derivation of a TDI (EFSA, 2006, 2008; EFSA CEF Panel, 2010) and the study of Salian et al. (2009). These studies have been briefly summarised here (more detail is provided in Appendix B) and are also included in the WoE section as starting points.

Rubin et al. (2001) measured the effect of BPA on the offspring of Sprague-Dawley female rats exposed to BPA in drinking water at concentrations of 1 mg/l and 10 mg/l (approximately 0.1 and 1.2 mg BPA/kg bw per day) from GD6 throughout lactation. Patterns of ooestrous cyclicity were determined in the female offspring by daily examination of vaginal cytology at 4 and 6 months of age. A statistically significant increased body weight of the offspring from day 4-11 was observed in both sexes. From day 22 and onwards, only females showed an increased body weight, the effect being greater in the 0.1 mg/kg bw per day group than in the 1.2 mg/kg bw per day group. A statistically significant and dose-dependent reduction in the percentage of animals with regular cycles and in the mean number of regular 4 or 5-day ooestrous cycles per animal was found at the highest BPA exposure level. There were some shortcomings in the study performance. The number of mated dams (n=6) was low, and it was not reported whether the litter was used as statistical unit.

In the Tyl et al. (2002) multigenerational dietary study of CD Sprague-Dawley rats, described in detail in section 3.2.2, reduced ovarian weights, a significantly reduced number of implantation sites and

decreased number of pups/litter on PND 0 were reported in the F0 females at the top dose of 7500 ppm BPA in the diet (estimated to be equivalent to 500 mg/kg bw per day) compared with controls. In the F1, F2 and F3 offspring, at this dose level only, vaginal patency and preputial separation were delayed, and associated with reduced body weight. No effects on reproductive organ histology and function were reported at lower dose levels of BPA, and the NOAEL for reproductive effects was therefore 50 mg/kg bw per day.

In the Tyl et al. (2008) two-generation dietary study of CD-1 mice, described in detail in section 3.2.2, no BPA-related effects were observed on adult mating, fertility or gestational indices, ovarian primordial follicle counts, ooestrous cyclicity, precoital interval, offspring sex ratios or postnatal survival, sperm parameters or reproductive organ weights or histology. The reproductive/ developmental NOAEL was 300 ppm (50 mg/kg bw per day), based on the effect in the testes of F1/F2 offspring.

Salian et al. (2009) performed a 3 generation-study to assess the effects of very low oral doses of BPA (1.2 or 2.4 µg/kg bw per day administered by gavage) in Holtzman rats. Fertility was assessed in adult F1-3 males by mating them with unexposed females. The authors reported a significant increase in post implantation loss in the F3 offspring and a decrease in litter size in F1-3 offspring at both BPA concentrations was observed, but a dose-response relationship was only evident for the decrease in litter size. Sperm count and motility were significantly reduced in the F1, F2 and F3 male offspring, with a dose related reduction in sperm count. A reduction in testicular expression profiles of steroid receptors was also reported.

Tinwell et al. (2002) studied the effects of BPA (0.02, 0.1, 50 mg/kg bw per day) administered by oral gavage from GD6-21 on two strains of rat (Sprague-Dawley and Alderley Park). Litter size, sex ratio, pup birth weights, timing of puberty, AGD were unaffected in both sexes. In females at PND98 only the highest dose affected timing of puberty (delayed) and then only in Alderley Park animals. In males at PND90 the only significant effects were at the highest BPA dose, the Alderly Park strain and were reduced daily sperm production.

In a combined, extensive, study with good numbers of litters and offspring, Howdeshell et al. (2008) and Ryan et al. (2010a) reported the sexes separately from the same where the same Long Evans hooded rats dams, split into two study arms (the second with a more extensive dose range of EE positive control groups), received BPA by oral gavage (2, 20, 200 µg/kg bw per day) from GD7 to PND18. In males (Howdeshell et al., 2008), only one statistically significant effect was observed: and only in the first arm not the second: an increased incidence of testicular degeneration (7/20 males at 200 µg BPA/kg bw per day vs 1/31 in controls), although without any concomitant effect on testis size or sperm numbers. In females (Ryan et al., 2010a), BPA had no significant effect on any parameter, including a forced breeding study with the F1 offspring.

3.3.2.3. Animal studies on reproduction and developmental effects after non-oral exposure published before 2010 previously evaluated by EFSA in 2006 or 2010

These studies have been briefly summarised here (more detail is provided in Appendix B) and are also included in the WoE section as starting points.

Studies in mice

Two studies by Nikaido et al. (2004 and 2005) reported effects of BPA on CD-1 mice offspring after maternal exposure at GD15 and in female CD-1 mice after prepubertal exposure at 15 days of age. BPA was given subcutaneously in doses of 0.5 (maternal exposure only) and 10 mg/kg bw per day for four consecutive days in both experiments. Vaginal opening, ooestrous cyclicity and mammary gland development were studied. According to the authors, maternal exposure to BPA did not accelerate puberty onset or modify the ooestrous cycle. Effects of perinatal exposure to BPA (25 and 250 ng/kg bw per day, but reported as 25 and 250 µg/kg bw per day) from sc administration was investigated following administration of BPA by Alzet mini-pumps to CD-1 mice from day 9 of pregnancy until

postnatal day 4 (Markey et al., 2005) as reported in EFSA 2006, Appendix B). Decreased vaginal weight, small increases in the incorporation of bromodeoxyuridine into DNA of endometrial gland epithelial cells, and increased expression of oestrogen receptor-alpha (ERalpha/ESR1) and progesterone receptor (PGR) in the luminal epithelium of the endometrium and subepithelial stroma were reported in BPA-exposed animals.

Low-dose effects of BPA on sexual maturation and reproduction of offspring were also investigated in female ICR/Jcl mice after sc injection of BPA (2 and 20 µg/kg bw per day), diethylstilboestrol (DES, 0.02, 0.2 and 2 µg/kg bw per day) or oil vehicle once a day to pregnant dams from GD11-17 (Honma et al., 2002). Age at vaginal opening was significantly earlier (reduced from 27.3 in controls to 26.2 days) in females exposed to 20 µg/kg bw per day BPA in utero and all DES exposed animals. The first vaginal oestrus was earlier (from app. 27.8 to 27.0 days) in female offspring exposed to 20 µg/kg bw per day BPA and, at all DES dose levels, adult AGD was significantly increased. None of the BPA doses affected mating, gestation, pups/litter or pup sex ratio in F1 females. Similarly, in Rubin et al. (2006) CD-1 mice were treated on GD8 via osmotic minipumps delivering 50% dimethylsufoxide as vehicle control or BPA at doses of 25, 250 ng BPA/kg bw per day until PND16. Litters were culled to 4 male and 4 female pups/dam and before PND22-24 tyrosine hydroxylase positive neurons in the rostral periventricular preoptic area (AVPV) important for controlling ooestrous cyclicity were counted. BPA had no significant effect on pups/litter or sex ratio. The number of sections through the AVPV was significantly higher in control female than control male brains but not in the BPA exposed groups although the number of sections was significantly reduced in BPA-treated females at the highest dose. Similarly, the numbers of tyrosine hydroxylase positive neurons were significantly different between male and female offspring in the control and lowest BPA dose groups and significantly reduced at the highest BPA dose in females. Taken together the authors concluded that this suggests a loss of sexual dimorphism following BPA exposure.

Effects of exposure of BPA on sperm quality in mice were reported in several non-oral studies. Aikawa et al. (2004) reported a decreased percentage of motile sperm and an increased incidence of malformed sperm in mice at 10 weeks of age given sc injections of BPA (approximately 0.3 and 30 mg/kg bw per day) for the first five days after birth. No change in testicular histology was reported.

Toyama and Yuasa (2004) reported effects in the steps 2-3 spermatids and the acrosomal granule and nucleus were deformed, after sc exposure of BPA to new born (n=3-4) mice (0.6 to 66 mg/kg bw per day) and rats (0.2 to 120 mg/kg bw per day). However, fully mature animals did not show any of these testicular effects and the animals were fertile.

Studies in rats

In Kato et al. (2006) Sprague-Dawley male rats were administered 2, 11, 56, 277, 97,000 µg BPA/kg bw per day from PND0 (day of birth) -PND9 by subcutaneous injection. Oestradiol (0.9 mg/kg bw per day) was used as a positive control and induced effects on most of the parameters assessed. The pups were regularly investigated up to adulthood, including necroscopy, on PND 10, 35, 150). The only observed effect of reproductive development, morphology and endocrinology was a reduction in the proportion of abnormal sperm at two of the BPA doses (one ≤3.6 mg/kg bw per day BPA HED). The latter cannot be considered adverse and none of the BPA treated animals displayed any impairment in fertility.

The following studies were not included as starting points in the WoE tables as starting points because they did not include at least one BPA dose ≤3.6 mg/kg bw per day HED: Aikawa et al. (2004) and Nikaido et al. (2004).

3.3.2.4. Animal studies on reproductive and developmental effects of BPA published after 2010 or excluded from previous EFSA evaluations

This section provides an overview of the experimental animal studies on reproductive and developmental effects published after 1st August 2010 that met the inclusion criteria set by the CEF Panel (see Section 2 and Appendix A). For this endpoint, however, an additional exclusion criterion was set for "high dose BPA" studies, as outlined in the following section, *Oral Human Equivalent Doses*. A detailed description and evaluation of each study is provided separately in Appendix B.

The assessment has considered (a) exposure to BPA during development (via maternal and/or lactational routes), where the offspring are the subject of the investigation, and (b) exposure to adult (post weaning) animals, followed by assessment of reproductive health and function. Thirty-two studies were included in the assessment following the application of inclusion and exclusion criteria (see Section 2 and Appendix A). Note that some studies contributed to multiple sections below (adult vs developmental exposure, male vs female).

Oral Human Equivalent Doses

The CEF Panel noted that the study of Tyl et al. (2002) offered a well-established oral NOAEL of 5 mg BPA/kg bw per day in the rat, with a higher NOAEL of 50 mg BPA/kg bw per day for reproductive effects. For its assessment of this endpoint, therefore, the CEF Panel focussed on studies reporting effects on reproductive parameters at dose levels below the NOAEL of 5 mg BPA/kg bw per day in the rat. Because of the range of species used in these studies and the various routes of administration employed, the CEF Panel decided to calculate an oral HED (see Section 3.1.5) for each dose level used in a particular study, using the HED conversion factors shown in table 3. This enabled comparison between and integration of information from different studies. Any study employing BPA doses with HEDs $\geq$3.6 mg BPA/kg bw per day has not been included in the assessment below, unless it also included a dose level or dose levels below a HED of 3.6 mg BPA/kg bw per day. The data for sheep were not available to calculate the HED and therefore a dose given to a sheep has been considered equivalent to the same oral dose given to a human, which led to the inclusion of 1 study and exclusion of 1 study. This assumption is supported by allometric scaling considerations.

As a consequence of this analysis the following 11 studies were excluded from further evaluation because the doses used all exceeded the HED of 3.6 mg BPA/kg bw per day: Adewale et al., 2009; Aikawa et al., 2004; Crawford et al., 2012; Doshi et al., 2013; El-Beshbishy et al., 2012; Karavan et al., 2012; Norazit et al., 2012; Quignot et al., 2012a; Salian-Mehta et al., 2013; Salloum et al., 2013; Tainaka et al., 2012. Additionally the following three studies in which BPA was tested as part of a mixture of chemicals, but not on its own, were excluded (Christiansen et al., 2012; Xi et al., 2011; Manikkam et al., 2013).

In the following summary of the studies reviewed, dose levels are only given as doses administered to the animals by the chosen route. The calculated HED for each dose level in a particular study is given in the description of each study in Appendix B. As already indicated the studies below all included a dose level or dose levels below a HED $\leq$3.6 mg BPA/kg bw per day, and a number of studies also included BPA doses with HEDs $\geq$3.6 mg BPA/kg bw per day (e.g. the 2013 study of US FDA/NCTR/Delclos et al., 2014). Where this was the case, the findings at the higher dose levels have also been summarised, in order to provide an overview of high dose effects of BPA on reproductive function.

a) Effects of BPA on reproductive function following exposure during development, includingexposure via the mother or direct exposure during post-natal development

Under this heading, the CEF Panel has assessed 25 studies including at least one BPA HED $\leq$3.6 mg/kg bw per day. The following overview of the studies is divided into studies investigating the effects of BPA on the male and female reproductive systems respectively, and is further subdivided

into studies considered to show (a) no effects attributable to BPA, (b) some limited effects of BPA, (c) possibly relevant effects of BPA.

Male reproductive system
Ten studies focussing on testis development and/or function (e.g. sperm count and sperm motility) and masculinisation (e.g. nipple-retention, ano-genital distance, androgens) have been evaluated. Only one of these studies involved an investigation of the functional fertility of the exposed offspring (Zhang et al., 2013).

Four studies showed no significant adverse effects attributable to treatment with BPA on the male reproductive system, as follows.

In the large and well performed study of Ferguson et al. (2011) Sprague Dawley rats were administered 2.5 or 25.0 µg/kg bw per day BPA or 5.0 or 10.0 µg/kg bw per day ethinyl oestradiol by gavage on GD 6–21 (dams) and PND 1-21 (offspring gavaged individually). No treatment-related effects were seen on birth weights of the pups (although pre-weaning body weights decreased), nor on anogenital distances (AGD), AGD index, developmental landmarks, or serum hormones. There were also no effects on hormonal measures at weaning.

Larocca et al. (2011) administered 2.5 or 25 µg BPA/kg bw per day to pregnant C57/B16 mice by oral gavage from GD 12-PND 21. A positive control (DES, 2 µg/kg bw per day) was included. No BPA-related effects were seen on pregnancy outcome and on reproductive development of male offspring, including testis gene expression and morphology and measures of masculinisation (circulating testosterone and AGD).

Lopez-Casas et al. (2012) exposed CD-1 mice to BPA (0.16; 16 or 64 mg/kg bw per day or 17-beta-oestradiol (E2: 0.006; 0.012 or 0.048 mg/kg bw per day) via oral administration in the drinking water of the dams. The effects of mono-(2-ethylhexyl)-phthalate, zearalenone and lindane were also investigated. There were three exposure groups: (A) during the two weeks before mating; (B) exposure continued until birth or (C) exposure was continued until four weeks after birth. Body weight, testis weight, testicular morphology, apoptosis and testis gene expression were investigated. The only effect reported for BPA was an increase in germ cell apoptosis at 64 mg/kg bw per day in exposure group (C).

Horstman et al. (2012) administered BPA (0.02, 0.5, and 400 mg/kg bw per day) in dimethyl sulfoxide by subcutaneous injection to pregnant Sprague Dawley rats from GD 8-20. EE was used as a positive control. Fetuses were harvested at GD 16, 18 and 20, at which times no effects of BPA or EE on testis morphology were observed.

Three studies reported some limited effects of BPA on the male reproductive system, as follows.

In Kobayashi et al. (2012), Sprague Dawley rats were administered 3 doses of BPA (0.33, 3.3, 33 mg/kg in diet, equivalent to 0.02, 0.17 or 1.65 mg/kg bw per day from GD 6 to PND 21. F1 offspring were examined at 5 weeks and 3 months postnatally and body and organ weights, AGD, reproductive hormones and sperm counts were quantified. The only BPA-related effect in males was a statistically significant decrease in epididymal weights in the 3-month old male animals receiving 1.65 mg BPA/kg bw per day.

Nanjappa et al. (2012) administered BPA to pregnant/lactating Long Evans rat dams (2.5 and 25 µg/kg bw per day by oral gavage up to PND21 and investigated adult male offspring studied at three time-points. Stimulation of Leydig cell division was observed in the pre-pubertal period and Leydig cell numbers were increased on day 90, but without any overall effect on testosterone levels. Increased expression of some developmental/reproductive proteins was also reported (e.g. HSD17B3, AMH).

In the US FDA/NCTR sub-chronic toxicity study (2013)/Delclos et al. (2014), the experimental design of which is reported in Appendix B, at PND 90 the AGD index of Sprague Dawley rat males in the 300 000 µg BPA/kg bw per day group was approximately 6.5% greater than that of the vehicle control group. Interpretation of this finding was however made difficult by a similar change in the male naïve control compared with the vehicle control. Testicular descent was significantly delayed by 5% in the 260 µg BPA/kg bw per day dose groups. The incidence of seminiferous tubule giant cells increased from 0/20 in vehicle controls to 5/23 at the 2.5 µg/kg bw per day dose only. No other effect of BPA was reported on male reproductive organ weights, AGD, reproductive endocrinology or sperm production.

A further three studies reported possibly relevant effects of BPA on the male reproductive system, as follows.

In Zhang et al. (2013), postnatal male CD-1 mice were given 0, 20 or 40 µg BPA/kg bw per day by subcutaneous injection on PND 3-21, 3-35, 3-49. The authors reported a range of treatment-related effects on spermatogenesis, including a significant increase in germ cells in the testis at 3 weeks in mice treated with 40 but not 20 µg/kg bw BPA/day, followed by a significant decrease at both 5 and 7 weeks in mice receiving 20 or 40 µg/kg bw per day BPA. These changes were accompanied by a decrease in the population of germ cells entering meiosis. BPA-related increases in diameter of seminiferous tubules were reported in mice at 3 weeks, followed by decreases at 5 and 7 weeks. Morphological abnormalities were seen in the sperm of the BPA-treated animals, together with decreased motility. Changes in gene expression were also reported. Finally, exposure of male mice to 40 but not 20 µg/kg bw per day BPA followed by mating with untreated females resulted in a reduction in offspring body weight and size at PND 14, 21 and 35, together with a reported increased rate of dystocia and poor body condition. The CEF Panel concluded that the results of this study support an effect of dose levels ≤3.6 mg BPA/kg bw per day HED on postnatal testis development in the mouse.

In Christiansen et al. (2014) Wistar rat dams were administered BPA at dose levels of 0.025, 0.25, 5, 50 mg/kg bw per day in corn oil from GD7 to PD22. Very few statistically significant effects of BPA were observed. Male pup AGD was significantly decreased (7% max) at all except the lowest BPA dose and nipple retention increased at the highest dose (4-fold, but dose-dependent). Of the organs weighed, the only significant effect was an increase in retroperitoneal fat pad weight in male pups at the highest BPA dose. While the decrease in male AGD and increased nipple retention (although only statistically significant >3.6 mg BPA/kg bw per day HED) probably is indicative of some impairment of masculinisation it is not known from this study whether there is any decrease in subsequent fertility.

In the study of deCatanzaro et al. (2013), adult female CF0-1 mice maintained on either high or low phytoestrogen diets received, in 1 g of peanut butter, either vehicle (peanut oil) or 0.175, 1.75, 17.5 µg BPA/g peanut butter/day, or, with the high phytoestrogen diet only, 17.5, 175, 1 750 µg BPA/g peanut butter/day from GD 9 to PND 1. Pups were weaned on PND 27 and males were maintained on the same phytoestrogen diet as their mother until PND 60 or 90. Male offspring AGD, reproductive organ weights, capacity to inseminate and urinary hormone levels were measured. In the second study with high phytoestogen diet only, none of the BPA doses affected these body/reproductive organ indices and urinary testosterone, oestradiol and creatinine were also unaffected. At the 17.5 µg BPA/day dose there were reductions in intromission number (also at the 175 µg BPA/day dose) and ejaculations by around 50%. The CEF Panel considered that the erratic appearance of mostly minor effects of BPA only at the high phytoestrogen dose made the study difficult to interpret in terms of human risk.

Overall summary of studies investigating the effects of BPA on the male reproductive system and involving developmental exposure

Of the 10 studies evaluated, as described above, four studies were considered to show no adverse effects on the male reproductive system attributable to treatment with BPA, three studies reported

some limited effects, while three showed one or more consistent effects. Few studies involved prolonged post-natal exposure and a number of methodological concerns were raised. Overall the CEF Panel considered that there was some evidence, with considerable inter-study variation (in quality and outcomes), of an effect on the male reproductive system following developmental exposure at dose levels below a HED of 3.6 mg/kg bw per day. The most convincing effects were those reported by Christiansen et al. (2014).

Female reproductive system
The CEF Panel evaluated 17 studies investigating the effects of BPA in females as a result of developmental exposure.

Three rodent studies showed no effects attributable to treatment with BPA on the female reproductive system at dose levels below a HED of 3.6 mg/kg bw per day, as follows.

In the study of Ferguson et al. (2011) described above (and also in Appendix B), there were no BPA-related effects on birth weight of the pups, although pre-weaning body weights decreased. There were no effects of treatment on anogenital distances and AGD index in females, or on developmental landmarks or measures of serum hormones. There were also no effects on hormonal measures at weaning.

Xiao et al. (2011) administered daily subcutaneous injections of BPA in sesame oil to provide doses of 0, 0.025, 0.5, 10, 40, and 100 mg/kg bw per day from gestation days 0.5-3.5 to C57BL/6 mice and examined the effects of BPA on implantation. Although there were significant effects on implantation at dose levels of 40 mg BPA/kg bw per day and above, together with increased gestation periods, reduced litter size, reduced postnatal survival rate and continued expression of progesterone receptors (PGR) in the luminal epithelium of the uteri, no significant effects were observed in mice receiving ≤3.6 mg BPA/kg bw day HED.

To examine the effects of BPA on implantation (Berger et al., 2007), mice were exposed via either sc injection or two dietary administration routes (not gavage). Subcutaneous injections of BPA (0.016, 0.047, 0.14, 0.45, 1.3, 3.9, 35.2, 105.5 mg/kg bw per day) in peanut oil were administered daily to adult female primiparous CF-1 mice on gestation days 1-4. Oral administration via either (1) dietary supplement (calculated daily intake: 36, 278, 550, 453 mg BPA/kg bw per day) or (2) food contamination (calculated daily intake: 2,223, 2,293 mg BPA/kg bw per day) was also performed on GD1-4. Implantation rate, litter size, number of implantation sites and uterine histology (at the highest BPA dose only) were assessed. Although adverse effects on parturition rates and litter size were observed at the two highest sc doses, BPA at HED ≤3.6 mg/kg bw per day had no significant effect on any of the measures assessed. None of the sc or oral doses had any significant effects at HED ≤3.6 mg/kg bw per day.

Seven rodent studies reported some limited effects of BPA on the female reproductive system at dose levels below a HED of 3.6 mg/kg bw per day, as follows.

In Kobayashi et al. (2012), Sprague Dawley rats were administered 3 doses of BPA (0.33, 3.3, 33 mg/kg in diet, equivalent to 0.02, 0.17 or 1.65 mg/kg bw per day) from GD 6 to PND 21. F1 offspring were examined at 5 weeks and 3 months postnatally and body and organ weights, AGD and reproductive hormones were quantified. A reduction in female AGD and ovary weight was reported at the two higher doses at 5 weeks, but the CEF Panel considered that this finding had no clear significance without further data on the reproductive performance, also noting that the effect had normalised by 3 months (adulthood).

Signorile et al. (2010) & Signorile et al. (2012). The 2010 study dosed pregnant female BAlb-C mice with 100 or 1000 µg/kg bw per day of BPA by subcutaneous injection throughout pregnancy and up to day 7 postnatally. There was no positive control in the 2010 study. While all groups contained

animals with ovarian cysts, the incidence was significantly higher in the two BPA-exposed groups (9-10/20) compared with vehicle controls (2/20) although corpora lutea numbers were unaffected. The incidence of endometrial hyperplasia was significantly increased (from 2/20 to 10/20) at the higher BPA dose. The authors report that "endometriosis-like" abnormalities were significantly increased in incidence by BPA (from 1/20 to 6-7/20). In the 2012 study further morphological analysis of the same ovaries as Signorile et al. (2012) ovaries was carried out when the animals were 3 months of age. Follicle classes were counted and related to an endometriosis-like phenotype reported in the same animals in Signorile et al. (2010). The authors reported a higher incidence of endometriosis-like characteristics in animals with fewer primordial and developing follicles and more atretic follicles, which were significantly adversely affected by both BPA doses.

Mendoza-Rodrigues et al. (2011) dosed adult female Wistar rats with BPA (approximately 1.2 mg/kg bw per day) via their drinking water from GD6-PND21 in a study without positive controls. The female offspring were examined at age 3 months for oestrousooestrous cyclicity and ovarian and uterine morphology. Although the ovaries were morphologically normal, the BPA exposed animals showed a significantly higher incidence of abnormal cycles. Uterine epithelial and stromal thickness increased in the BPA-treated group and more females showed reduced rates of uterine apoptosis.

Two studies from the same group first studied the effects of immediate post-natal BPA exposure (Newbold et al., 2007) and gestational BPA exposure (Newbold et al., 2009). In Newbold et a. (2007), doses of 10, 100, 1,000 µg BPA/kg bw per day were administered by subcutaneous injection to female CD-1 pups on PND1-5. There was no positive control. Reproductive tracts were examined at 18 months post-natal and two statistically significant effects were observed, both at 100 µg BPA/kg bw per day: (i) increased incidence of ovarian cysts (from 7/18 in control to 14/20) and (ii) increased incidence of cystic endometrial hyperplasia (from 1/18 in control to 9/20). There were no other convincing abnormalities. In Newbold et al. (2009), pregnant CD-1 mice were administered BPA at 0.1, 1, 10, 100, 1,000 µg/kg bw per day by subcutaneous injection, from GD9-16. There was no positive control and necroscopies of the F1 females were performed at 16-18 months post-natal. Cystic ovaries were seen in all groups and only approached significance (p=0.05) in the 0.068 µg BPA/kg bw per day group while uterine abnormalities were not significantly altered by BPA exposure. The total number of animals exhibiting reproductive tract lesions, if mesonephric remnants were included, was significantly higher at the two lowest doses of BPA.

In the US FDA/NTCR subchronic toxicity study (2013)/ Delclos et al. (2014) the design of which is reported in Appendix B, BPA did not affect the time of vaginal opening in Sprague Dawley rat females or the body weight at which the landmark was achieved. Nor was any effect observed on time to first oestrus. The dose level of 300,000 µg BPA/kg bw per day significantly increased the proportion of animals showing abnormal cycles in a manner similar to that of EE_2 and ovarian weights (absolute and adjusted for brain weight) were decreased at this dose level, accompanied morphologically by depletion of corpora lutea and antral follicles. The CEF Panel noted that there were vehicle effects compared with naïve controls, including some very minor alterations in female offspring cyclicity. However, there were no statistically significant reproductive effects in female at BPA doses ≤3.6 mg/kg bw per day HED on PND90, including an absence of any significant effects on AGD, ovarian and uterine morphology, ovarian cyclicity or reproductive endocrinology.

The only non-rodent studies investigating prenatal BPA exposure were those by Hunt et al. (2012) and Veiga-Lopez et al. (2013). Hunt et al. (2012) used pregnant Rhesus macaques to investigate reproductive parameters in the female offspring. Two routes of administration were used: (1) oral in diet, 400 µg BPA/kg bw per day (single daily dose) or (2) subcutaneous implant tested to yield 2.2-3.3 ng unconjugated BPA/ml plasma in non-pregnant females (continuous exposure). Two exposure windows were investigated for each route: (1) early GD50-100, the onset of meiosis and (2) late GD100-term, the period of follicle formation. Only the results for the oral route were considered for evaluation because of the inadequate number of animals dosed via the subcutaneous route (only 2 monkeys in the control group). BPA at 400 µg BPA/kg bw per day was associated with a modest but

statistically significant increase in the proportion of multi-oocyte secondary or antral follicles but had no significant effect on incidence of meiotic defects reportedly seen in the implant group). The significance of the increased incidence of multi-oocyte follicles for subsequent fertility in monkeys or humans remains to be conclusively demonstrated, although it is likely negative.

In Veiga-Lopez et al. (2013) adult Suffolk ewes received 0.5 mg BPA/kg bw per day subcutaneously from GD30 to GD90 (total gestation period 147 days) and controls received corn oil alone. BPA levels in arterial umbilical blood samples were monitored at GD90. The authors reported that levels of unconjugated BPA increased from 0.4 ng/ml in controls to 2.6 ng/ml in BPA-exposed fetuses. CYP19A1 and SRD5A1 were reduced at GD65 but not GD90 in BPA exposed ovaries but had no effect on the pattern of transcript changes between GD65 and GD90. BPA exposure down-regulated 45 miRNA at GD65 but only 11 miRNA at GD90. The CEF Panel considered that the consequences of these changes are not obvious.

A further four rodent and one primate studies reported possibly relevant effects of BPA on the female reproductive system at dose levels below a HED of 3.6 mg/kg bw per day, as follows.

A further rodent study by Cabaton et al. (2011) performed an important forced/continuous breeding experiment. CD-1 female mice were exposed to BPA via subcutaneous osmotic minipumps (0.025, 0.25, 2.5 µg/kg bw per day) from GD6-PND16. DES (10ng/kg bw per day) was used as a positive control. Female F1 offspring were force-bred from 2 months post-natal for 32 weeks. The lowest and highest BPA doses were associated with significantly reduced cumulative pup numbers. At the highest BPA dose numbers of litters and the proportion of females with ≥6 litters were significantly reduced.

Cynomolgus monkeys were given BPA (10 µg/kg bw per day) (n=18) or vehicle (n=19) subcutaneously by implanted pump prenatally to mothers during pregnancy. No effects of BPA were observed on delivery, stillbirth, premature birth, gestational length or body weight of offspring (Nakagami et al. 2009).

Nah et al. (2012) administered a single subcutaneous injection, at dose levels of 0.1, 1, 10, 100 mg BPA/kg bw to postnatal female ICR mice on PND 8. Body weight gain, onset of puberty and ooestrous cycling were investigated at PND 25, 30, 70. Ovary weights and age of puberty (vaginal opening) were reduced significantly at all BPA doses, but other significant effects (ooestrous cycling, uterus weight) were only seen at the two higher doses of BPA. However, these differences were not seen on PND70.

In Christiansen et al. (2014) Wistar rat dams were administered BPA at dose levels of 0.025, 0.25, 5, 50 mg/kg bw per day in corn oil from GD7 to PD22. Very few statistically significant effects of BPA were observed in females although female pup AGD was significantly decreased (9% max) at all doses (a similar effect was seen in male pups, see above). The CEF Panel considered that the decrease in AGD in females at ≤3.6 mg/kg bw per day HED is indicative of an effect of BPA on genital development at all doses administered in this study, but the reproductive significance of decreased AGD in the females is uncertain.

The study by Zhang et al. (2012a) was designed to assess the effects of BPA on germ cell cyst breakdown and primordial follicle formation in CD1 mice. Pregnant mice were given 0, 20, 40 and 80 µg BPA/kg bw per day by subcutaneous injection from 12.5 days to 18.5 days postcoitum. The ovaries of the female offspring were variously analysed at 13.5, 15.5, 17.5 and 19.5 (=PND1) days postcoitum and at PND 3, 5, 7 for meiosis progression, bisulphite sequencing, immunohistochemistry and histology for meiosis progression markers. Dose-dependent effects of BPA were observed, with retention of oocytes in nests (cysts) and reduced primordial follicle numbers. However, numbers of oocytes were higher in the PND3 ovaries, possibly linked with delayed meiosis progression and

decreased levels of increasingly methylated Stra8. Progression to meiosis prophase I of oocytes was delayed in the 80 µg/kg/day treated group.

Overall summary of studies investigating the effects of BPA on the female reproductive system and involving developmental exposure

Of the 17 studies evaluated, as described above, four studies were considered to show no effects attributable to treatment with BPA on the female reproductive system, seven studies reported some limited effects, while six showed one or more consistent effects, with the caveat of very variable study quality and outcomes. Few studies involved prolonged post-natal exposure and a number of methodological concerns were raised. Overall the CEF Panel considered that there was some evidence of an effect on the female reproductive system following developmental exposure at dose levels below a HED of 3.6 mg/kg bw per day, with the most convincing effects reported by Christiansen et al. (2014).

Effects of BPA on bone development following developmental exposure

Pelch et al. (2012) examined the effect of developmental exposure to low doses of diethylstilboestrol (DES), BPA or ethinyl oestradiol (EE_2) on bone geometry and torsional strength in the offspring at 10 and 13 weeks of age (females) or 23 weeks (males). C57BL/6 mice were given 0.1 µg/kg bw per day diethylstilboestrol, 10 µg/kg bw per day BPA, 0.01, 0.1, or 1.0 µg/kg bw per day ethinyl oestradiol or vehicle from gestation day 11 to post-natal day 12 via a mini-osmotic pump. Exposure to DES, BPA or low dose EE_2 increased adult femur length by small increments (approximately 2.5%). Exposure to the highest dose of EE_2 did not alter femur length, which the authors considered provided evidence of a non-monotonic dose-response. Exposure to EE_2 and DES, but not BPA, decreased femur tensile strength, while no changes were seen in bone collagen content.

b) Effects of BPA on reproductive function following exposure during adult life

Male reproductive system

The CEF Panel has evaluated seven studies published since 2006, reporting effects on the testis (6 studies) or prostate (1 study).

Dobrzynska and Radzikowska (2013) administered 5, 10, 20 or 40 mg BPA/kg bw per day in drinking water to male mice for 2 weeks. Decreases in sperm counts, sperm motility, increases in abnormal sperm morphology were reported at all BPA doses except 5 mg/kg/day, which only caused a 3% increase in abnormal sperm. Increased DNA damage in somatic and germ cells was seen at all BPA doses including 5 mg/kg bw per day.

In the study of Qiu et al. (2013), adult male Sprague-Dawley rats (8 weeks old) were administered BPA at dose levels of 0.0005, 0.5, 5 mg/kg bw per day for 8 weeks and examined at the end of the dosing period. BPA did not affect organ or body weights, serum biochemistry or hepatonephric function. While circulating testosterone was unaffected, BPA reduced intratesticular testosterone at 5 mg/kg bw per day. This dose also reduced sperm numbers, seminiferous tubule epithelial height, numbers of round spermatids and the ratio of round spermatids/Sertoli cells, although sperm motility was unaffected. Changes in spermatogenesis-related genes and proteins were also reported. The results suggest only limited effects of BPA below 3.6 mg/kg bw per day HED.

Jin et al. (2013) administered BPA by gavage at a single dose level of 2 µg/kg bw per day to adult male Sprague-Dawley rats for 14 days. Separate groups of rats were administered testosterone propionate (TP) at 0.1 mg/rat/day or a mixture of BPA+TP. BPA was reported to reduce sperm counts and seminiferous tubule numbers of all stage VII germ cells. The BPA-exposed seminiferous tubules had an increased apoptotic index that was unaffected by co-administration of TP. Serum and intra-testicular testosterone were reduced in BPA-exposed animals and the negative effect of BPA on sperm counts was partially reversed by TP, as were numbers of mPSc and 7Sd stage VII germ cells. BPA-exposed rats had lower follicle-stimulating hormone (FSH) and increased luteinizing hormone

(LH), and brain preoptic area GnRH expression was also reduced, while expression of a number of testicular genes was increased.

In the study of Liu et al. (2013), adult (9 weeks old) male Wistar rats were exposed to BPA at dose levels of 2, 20, 200 µg/kg bw per day by gavage for 60 days. E2 (10 µg/kg bw per day) administered sc was used as a positive control. Treatment continued for 60 days. No effect of BPA on testicular parameters was reported at BPA dose levels lower than 200 µg/kg bw per day. BPA (200 µg/kg bw per day) and E2 increased stages VII and IX sperm and decreased stage VII sperm, an effect blocked by ER antagonism using the ER antagonist fulvestrant. Both BPA (200 µg/kg bw per day) and E2 reduced the percentage of leptotene and zygotene spermatocytes and increased the proportion of pachytene spermatocytes, again blocked by ER antagonist administration. Extensive analysis of germ cell meiosis indicated that BPA (200 µg/kg bw per day) and E2 induced disruption of meiosis and increased germ cell apoptosis. Despite methodological and reporting deficiencies, the CEF Panel noted that the study indicates a potential oestrogenic action of BPA in the adult male rat.

Tiwari and Vanage (2013) administered 2 dose levels of BPA (10 µg/kg bw per day and 5 mg/kg bw per day orally to adult Holtzman male rats once per day for 6 days. The males were then repeatedly mated (8 times) with untreated females up to 56 days post-treatment, and treatment-related effects on fertility were investigated. The 5 mg/kg bw per day dose reduced implantation/embryo survival indices in the offspring of treated males during a single (22-28 days) post treatment interval only, and there were no effects on mating or gestation indices. The same dose at the same interval increased post-implantation loss but the effect was not statistically significant on the "dominant lethal mutation". Both BPA doses were associated with reduced sperm production, count and motility although the latter only achieved significance at the higher dose, which also caused DNA damage to the sperms. However, the CEF Panel noted the lack of any effect on fertility, considering the implications of the testicular effects to be unclear.

In a preliminary and inadequately quantified study, El Ghazzawy et al. (2011) administered a single low dose level of 20 µg BPA/kg bw per day, ±pomegranate juice and appropriate controls, by oral gavage to adult male albino rats for 8 weeks. The authors reported a quantified reduction in caudal epididymis sperm numbers (1.8-fold lower in the BPA dose group) and qualitative morphological observations were made about caput epididymis and sperm structure and ultrastructure.

In a well powered, well performed study with valid endpoints, Castro et al. (2013) exposed adult male Wistar rats to 0, 25, 50, 300, 600 µg BPA/kg bw per day for 4 days by sc injection. Testosterone increased, oestradiol decreased and the testosterone/oestradiol ratio was skewed by exposure to BPA at all doses tested.

Overall summary of studies investigating the effects of BPA on the male reproductive system and involving exposure of adult animals

Of the seven studies published after 2010, six investigated effects of BPA on testicular function although only one of these examined whether the changes found impacted on the fertility of the animals (and reported no effects). Effects on sperm parameters, hormone levels, testicular gene and protein expression were reported in oral studies employing doses in the range of 5 mg/kg bw per day (HED of 3.6 mg/kg bw per day), but several authors reported effects at much lower dose levels, in one study as low as 2 µg BPA/kg bw per day. Overall the CEF Panel considered that there was some limited evidence of an effect on the male reproductive system following developmental exposure at dose levels below a HED of 3.6 mg/kg bw per day.

The CEF Panel noted however that no effect of BPA was reported on male reproductive organ weights and sperm production at any dose level in the robust subchronic toxicity study in rats conducted by US FDA/NCTR (US FDA/NCTR Report 2013/Delclos et al., 2014), which involved exposure during both prenatal and adult life, although testicular descent was significantly delayed by approximately 1 and 2 days, respectively, in the 260 and 300,000 µg BPA/kg bw per day dose groups.

Similarly, in the multigenerational studies in rats and mice conducted by Tyl et al. (2002, 2008), effects were only reported at a dose level of 600 mg/kg bw per day in mice, with a statistically significant reduction in epididymal sperm concentration (15% reduction) in males. BPA also reduced F1/F2 weanling testis weight (with seminiferous tubule hypoplasia). The NOAEL for reproductive effects in both studies was 50 mg/kg bw per day.

Female reproductive system
Two studies published after 2006 have been evaluated by the CEF Panel, one investigating BPA effects on delivery and placental signalling and one on ovarian effects.

In Tan et al. (2013) 6-8 week old pregnant female ICR mice were administered 0, 2, 20, 200 mg BPA/kg bw per day in ethanol/corn oil by gavage from GD 13 – GD 16. Blood samples and tissues were harvested at E17. Mice exposed to 20 and 200 mg/kg bw per day had significantly elevated E2, testosterone and CRH although only the highest dose was associated with increased placental crh transcript and cyp19a1 was not affected. Placental CREB protein was increased in all BPA groups, as was the PKC zeta/gamma ratio, while PKC delta was only affected at the highest dose. The study suggests that BPA exposure in pregnant mice may disturb the endocrine and PKC signalling pathways in the placenta although limited effects were seen at ≤3.6 mg/kg bw per day HED.

In a well performed and well powered study Lee et al. (2013b) administered BPA by oral gavage at 0.001 or 0.1 mg/kg bw per day for 90 days to adult (8 weeks old) female Sprague-Dawley rats. A positive control, 0.001 mg oestradiol benzoate (EB)/kg bw per day was included. Circulating E2 and T was reduced by both dose levels of BPA and also by EB and the duration of the oestrus phase increased. Follicular and corpora luteal atresia was increased by BPA although EB only affected luteal atresia as determined by caspase-3 analysis. Theca cell cyp19 was decreased by BPA and StAR decreased by BPA and EB. Circulating and pituitary LH levels, but not FSH levels, were increased by BPA at both dose levels, without a marked dose-response.

Summary of studies investigating the effects of BPA on the female reproductive system and involving exposure of adult animals
The two studies evaluated, as described above, showed limited effects of BPA on placental and ovarian function at dose levels considerably below a HED of 3.6 mg/kg bw per day.

3.3.2.5. Summary of reproductive and developmental effects of BPA in animal studies

Overall, the better -powered, better-conducted studies, especially those including several dose levels ≤3.6 mg BPA/kg/day HED, reported only limited evidence of effects of in utero exposure to BPA on reproductive development. However, on balance, the evidence remains contradictory and highly variable between studies. Overall the CEF Panel considered that there was some evidence of an effect of BPA on both the male and female reproductive systems following developmental exposure.

There is also some evidence for effects of exposure of adult animals to BPA on aspects of reproductive health/performance, in particular for a possible effect on testicular function in males at dose levels below 5 mg/kg bw per day, although again these effects were modest and follow-up to establish reduced fertility in adulthood is quite limited. In several cases the assessed studies investigated molecular biology endpoints but not functional/morphological endpoints. It appears that molecular endpoints often exhibit greater sensitivity to BPA. While these outcomes are considered relevant, an adverse phenotypic outcome is of greater significance for risk assessment and it is important that studies to be included in risk assessment should report both sets of endpoints.

The CEF Panel noted that ovarian cysts and endometrial hyperplasia have been reported in female animals exposed to BPA in a number of studies published before 2010, and that other assessments such as that of ANSES (2011, 2013) have considered the two phenomenon important. Certainly these two adverse effects are highly relevant to human reproductive competence and health. However, the CEF Panel considered that more recent studies considered for this opinion did not report any

significant effects of BPA at an HED ≤3.6 mg/kg bw per day on the incidence of ovarian cysts or endometrial hyperplasia.

Finally, the CEF Panel noted the very wide variability in the incidence and/or magnitude of adverse effects, if any, between the studies assessed. There are a number of contributing reasons for this, including: (i) use of different species and strains, (ii) use of different ages categories, (iii) onset and termination of exposure regimens varies hugely between studies, (iv) widely varying durations of exposures to BPA, (v) considerable variation in study quality and statistical power, (vi) extensive variations in vehicle and/or solvents used, (vii) diverse selection of end-points assessed, (viii) low number of forced/continuous breeding or two-generation studies, (ix) wide range of cages, bedding diets and exposure routes employed, (x) few studies have confirmed in-animal BPA doses levels – i.e. how much BPA is reaching target organs? All these factors will contribute to the variability in the findings reported in the studies included in this assessment.

3.3.3. In vitro studies

Several recent studies (Ye et al., 2011; Guo et al., 2012; Quignot et al., 2012a) on the expression and activities of steroidogenic enzymes suggest that BPA has an inhibitory effect only at micromolar concentrations. Slight differences in the results from studies using microsomes and cells from different species (human, mouse, rat) suggest a cell- and species-specific BPA effects on these enzymes. Results from a study using organotypic culture of human fetal testis, N´Tumba-Byn et al. (2013), suggests that human fetal testes may have a greater sensitivity to the inhibitory effects of BPA (10^{-8} M) on testosterone production. However, considering that the number of human fetuses in the study was low and that the effects of BPA on enzyme activities were only observed at higher concentrations the findings of N´Tumba-Byn et al. (2013) still need to be confirmed. Other studies were performed on the BPA-induced mechanisms of proliferation in a human ovarian epithelial cancer cell line (Ptak et al., 2012) and in mouse spermatogonial cells (Sheng et al., 2011). These cells were sensitive to concentrations of BPA at the pico- and nanomolar range, which are still high concentration considering internal dose levels, and non-genomic signalling pathways were involved. The significance of these in vitro findings has to be further investigated and the impact on the in vivo reprotoxic effects of BPA is not yet clear.

3.3.4. WoE of developmental and reproductive effects of BPA in humans, animals and in vitro

For interpretation of these tables always refer to Appendix A.

Table 8: Overall table of WoE evaluation of reproductive and developmental effects of BPA in humans and animals.

Human studies	
Overall conclusion on Likelihood of reproductive effects of BPA in humans: An association between BPA and embryo quality and implantation success during IVF, semen quality, sex hormones or age of menarche in humans is considered unlikely	**Unlikely**
Overall conclusion on Likelihood of gestational /birth outcomes of BPA in humans: There are indications from prospective studies that BPA exposure during pregnancy may be associated with effects on fetal growth, and weak indications that BPA exposure during pregnancy may be associated with maternal and infant thyroid function. However, it cannot be ruled out that the results are confounded by diet or concurrent exposure factors. For fetal growth, two studies showed reduced fetal growth, while one study reported increased fetal growth with increasing maternal BPA exposure. The associations do not provide sufficient evidence to infer a causal link between BPA exposure and reproductive effects in humans. Potential effects are considered to be as likely as not.	**As likely as not**
Animal studies	

Overall conclusion on Likelihood for reproductive and developmental effects in animals when exposed during their adult life (post-pubertal) only at doses ≤ HED of 3.6 mg/kg bw per day: As more studies emerge with doses ≤3.6 mg BPA/kg bw per day HED, there are increasing indications of some negative effects of adult exposure to BPA on gonadal or reproductive tract physiology. Very few studies have assessed the effects of long term exposure of adults to such doses of BPA (human scenario) on the reproductive gold standard – fertility and reproductive ageing. Since only single studies on ovary and prostate gland fit the methodological criteria used in this opinion, no conclusion can be drawn on BPA-related effects on these organs. Taken together the studies assessed here suggest that BPA at an HED of ≤3.6 mg/kg bw per day may have adverse effects on testis function, especially various measures of spermatogenesis. There is less evidence to support a conclusion that BPA will significantly impair testis morphology or reproductive endocrinology, especially in the longer term. **Note: Alteration of reproductive capacity are likely at high doses (above an HED of 3.6 mg/kg bw per day)**	**As likely as not**
Overall conclusion on Likelihood for reproductive and developmental effects in animals when exposed during development (prenatally and pre-pubertally) ≤ HED of 3.6 mg/kg bw per day: Taken overall, there are some data suggesting negative effects of doses of BPA ≤ an HED of 3.6 mg/kg bw per day on reproductive development in animals. However, the lack of agreement between studies results in a high degree of uncertainty. The most consistent findings are generally of small magnitude (e.g. reduced male AGD) and often not accompanied by associated changes (e.g. disturbed ovarian cyclicity would be expected to be associated with ovarian abnormalities). Given difficulties in determining whether molecular changes are causal or due to adaptation or morphological changes, the weight given to studies presenting molecular findings without accompanying morphological data is low. The single non-human primate ovary study included was hampered by inadequate numbers of animals per group. **Note: Alteration of reproductive development are likely at high doses (above an HED of 3.6 mg/kg bw per day)**	**As likely as not**

3.3.5. Conclusions on reproductive and developmental effects

In relation to reproductive and developmental effects in humans, the CEF Panel concluded that there are indications from prospective studies that BPA exposure during pregnancy may be associated with disturbed fetal growth, and weak indications that BPA exposure during pregnancy may be associated with maternal and infant decreased thyroid function, but it cannot be ruled out that the results are confounded by diet or concurrent exposure factors. The associations found in the human studies are not sufficient to infer a causal link between BPA exposure and reproductive effects in humans. Potential effects are considered to be as likely as not.

Overall, the better powered, better conducted studies in animals found few consistent effects of in-utero exposure to BPA on reproductive development at dose levels at or below 3.6 mg BPA/kg/day HED. On balance, the evidence remains contradictory and highly variable between studies. The CEF Panel noted that there is some evidence for effects of BPA exposure on several parameters indicative for changes in the reproductive system in adult male animals at dose levels below 3.6 mg/kg bw per day, although these effects were modest. It is not possible to conclude that these changes are reflective of changes in reproductive performance, since the studies rarely included a forced/continuous breeding phase in adulthood to establish reduced fertility. However, in several multigenerational studies no effects were observed at dose levels as low as 3 μg/kg bw per day up to at least 50 mg/kg bw per day.

Using a WoE approach, the CEF Panel assigned a likelihood level of "as likely as not" to reproductive and developmental effects of BPA at low doses (below the HED of 3.6 mg/kg bw per day). Since the likelihood level for this endpoint is less than "likely" (see Appendix A), this endpoint was not taken

forward for assessing the toxicological reference point, but was taken into account in the evaluation of uncertainty for hazard characterisation and risk characterisation (Section 4.3).

3.4. Neurological, neurodevelopmental and neuroendocrine effects

3.4.1. Human studies

3.4.1.1. Summary of previous opinions

EU-RAR (2003 and 2008)
No human data were available at the time of these reports.

EFSA (2006); EFSA CEF Panel (2010)
No human studies were reviewed in the opinion of 2006.

The 2010 EFSA opinion included evaluation of a study by Braun et al. (2009) which found that the concentration of BPA in maternal urine from early pregnancy was associated with adverse externalising behaviour in 2-year old girls. This was the first epidemiological study suggesting an association between BPA exposure and neurodevelopmental effects. Several methodological limitations were noted and the CEF Panel could not draw any relevant conclusion for risk assessment from the study.

NTP-CERHR (2008)
No human studies on the effects of human developmental exposure to bisphenol A were then available.

FAO/WHO (2011)
The Expert meeting reviewed the prospective cohort study by Braun et al. (2009) and concluded that the results suggest that prenatal BPA exposures (especially in early pregnancy) are associated with the later development of externalizing behaviours, e.g. aggression and hyperactivity, particularly in girls. The Expert meeting also indicated replication of this study using large prospective birth cohorts with serial measures of urinary BPA during pregnancy as a high-priority research need.

ANSES (2011, 2013)
In 2011, the ANSES experts concluded that the human data then available were insufficient to draw a conclusion on the effects of BPA on human behaviour.

The ANSES report evaluated the Braun et al. (2009) study and noted the methodological flaws, which limit the value of the study. A study by Miodovnik et al., 2011 was also evaluated in the ANSES report. Miodovnik et al.sought to correlate the urinary levels of BPA and of phthalates analysed during pregnancy with the sociability of multiethnic city children aged 7 to 9, in 137 children. No significant association was found between urinary levels of BPA and social problems assessed by the Social Responsiveness Scale (SRS).

3.4.1.2. Evaluation of recent human studies on BPA exposure and neurological/behavioural, neurodevelopmental and neuroendocrine effects

Six new studies were identified in the literature and evaluated (Miodovnik et al., 2011; Yolton et al., 2011; Braun et al., 2011; Perera et al., 2012; Harley et al., 2013a; Hong et al., 2013). A detailed description and evaluation of each study is provided separately in Appendix B.

In a group of 404 mother-child pairs, Miodovnik et al. (2011) examined prenatal BPA exposure (maternal urinary BPA concentrations) in relation to child social behaviour (Social Responsiveness Scale) at age seven- to nine years in a group of inner-city children in New York. The results showed suggestive associations for prenatal BPA with autistic spectrum type behaviours, but were not statistically significant.

Yolton et al. (2011) studied 350 mother/infant pairs and examined maternal urinary BPA concentration and infant neurobehaviour measured at five weeks of age using the NICU Network Neurobehavioural Scale (NNNS). The NNNS is a tool with proven sensitivity to both overt and subtle differences in infant neurobehaviour. There was no significant association with prenatal BPA exposure and infant neurobehaviour.

In 2009 Braun et al. reported that higher prenatal BPA exposure (maternal urinary BPA concentrations) was associated with more externalising problem behaviour in girls at age 2 years, but not in boys (reviewed by EFSA CEF Panel, 2010). A follow up study by Braun et al. (2011) in the same children at age 3 years showed that higher prenatal BPA exposure (as judged by maternal urinary BPA) was significantly associated with more anxiety, depression, and hyperactivity behaviours in girls, but not in boys. The outcome measures were assessed by parent reports using the Behavior Assessment System for Children 2 (BASC-2) and the Behavior Rating Inventory of Executed Function-Preschool (BRIEF-P). In this study, no associations were found for children's urinary BPA concentrations and later behaviour. The study by Yolton et al. (2011) and the two studies by Braun et al. (2009, 2011) used the same study population (a prospective birth cohort in the Cincinnati, Ohio, metropolitan area designed for the study of low-level environmental toxicant exposures).

Perera et al. (2012) investigated the association between prenatal BPA exposure (maternal urinary BPA) and child behaviour in children between three and five years of age in 198 mother- child pairs in a low-income minority population in New York. In contrast to the studies by Braun et al. (2009 and 2011), which found associations between prenatal BPA exposure and child behaviour mainly in girls, Perera et al. (2012) found that higher prenatal exposure to BPA was significantly associated with increased emotional reactivity and aggressive behaviour in boys.On the contrary, in girls prenatal BPA exposure was associated with reduced scores for anxiety/depression and aggressive behaviour. The associations were adjusted for postnatal BPA exposure (child urinary BPA) and the authors suggest that the prenatal period is a critical time window for potential adverse effects of BPA on children's neurodevelopment. Childhood urinary BPA concentration was negatively associated with one out of seven syndrome scores (Emotionally Reactive) in the full sample, but no associations were seen for any outcome in boys and girls separately.

Harley et al. (2013a) studied prenatal and early childhood BPA concentrations in urine and behaviour in children at age 7 years using data from 292 mothers and children participating in the longitudinal birth chohort CHAMACOS, a low income Mexican-American agricultural population in California. Children's behaviour was assessed by maternal and teacher reports (BASC-2 and CADS, scales validated in English and Spanish) at age 7 and by direct assessment at age 9 by using the Connors' Continuous Performance Test (CPT). Higher urinary BPA concentrations in mothers during pregnancy were associated with increased internalising problems, including anxiety and depression, in boys (BASC-2), while no associations were found in girls. The findings were consistent using both mothers' and teachers' report. No associations were seen in boys or girls on the CADS at 7 years or in boys or girls with any behaviour at age 9 (CPT).

Higher urinary BPA concentrations in children at age 5 years were associated with increased internalising problems and increased (ADHD) behaviour in both boys and girls and increased externalising behaviors, including conduct problems, in girls at age 7 (BASC-2 and CADS). The associations with childhood BPA were mainly seen with teacher reports. No associations were seen with BPA concentrations at 5 years and any behaviour at age 9 (CPT).

In a cross-sectional study in Korea with 1008 children aged 8-11 years, Hong et al. (2013) examined urinary BPA and behaviour and learning. Behaviour and learning were assessed by the Korean version of the Child Behavioural Checklist (CBCL) and the Learning Disability Evaluation Scale (LDES). Higher urinary BPA concentrations in the children were associated with higher CBCL total

problem score and with lower LDES listening and learning scores. There was no interaction effect between urinary BPA concentration and gender.

A recent review (Rochester, 2013) identified 91 epidemiological studies showing associations between environmental BPA exposure and various adverse effects in humans, including neurodevelopmental effects. However, the authors concluded that these studies were primarily supportive and that a causal relationship was still difficult to obtain (Rochester, 2013).Summary of neurological, neurodevelopmental and neuroendocrine effects in humans after prenatal and postnatal/childhood BPA exposure

Although most of the new studies were prospective, the uncertainty related to the relationship between exposure to BPA and the outcomes limits the conclusions that can be drawn from studies showing associations. In particular, the CEF Panel noted that in the Miodovnik et al. (2011), Braun et al. (2011) and Perera et al. (2012) studies, neuropsychological functioning and social function were assessed by parents' reports of child behaviour. Parental reports, even when using validated questionnaires/scales, may present more variability than standardised neurodevelopmental scales administered by child psychiatrists. The Perera et al. (2012) and Braun et al. (2009, 2011) studies, although inconsistent, indicate that prenatal BPA exposure may affect child behaviours in a sex-dependent manner. In contrast, the Miodovnik et al. (2011) and Yolton et al. (2011) studies did not indicate that prenatal BPA exposure was associated with infant or childhood behaviour. In a study with 292 mothers and children in the CHAMACOS, Harley et al. (2013a) linked prenatal BPA exposure to behavioural problems in boys at age 7. Significant associations were seen for both mothers' and teachers' reports of child behaviour and the study took into account other environmental contaminants, childhood BPA exposure and important covariates reflecting socioeconomic status and home environment. This study also found significant associations between higher childhood BPA exposure (at age 5 years) and problem behaviours at age 7 years in both boys and girls. However, no associations of BPA with any behaviour at age 9 were found when directly assessed (CPT). No reliable associations between childhood BPA exposure and behavioural effects were reported in the other studies (Braun et al., 2011; Perera et al., 2012; Hong et al., 2013). More prospective studies with larger sample size, repeated BPA measurements, inclusion of dietary data and standardized neuropsychological testing are needed.

In summary, there are indications from prospective studies that prenatal BPA exposure (BPA exposure during pregnancy) may be associated with child behaviour in a sex-dependent manner. However, the associations were not consistent across the studies, and it cannot be ruled out that the results are confounded by diet or concurrent exposure factors. The associations do not provide sufficient evidence to infer a causal link between BPA exposure during pregnancy or childhood BPA exposure and neurodevelopmental effects in humans.

3.4.2. Animal studies

3.4.2.1. Summary of previous opinions

The neurobehavioural effects of developmental exposure to BPA have been reviewed on a number of occasions (EU-RAR, 2003, 2008; EFSA, 2006, 2010; NTP-CERHR, 2008; FAO/WHO, 2011; ANSES, 2011, 2013). BPA has been tested in rats and mice in a number of experimental in vivo models, taking into account medium and long-term behavioural, neurochemical, neuroendocrine and gene expression effects, together with shorter term studies in in vitro models. The outcome of these reviews is summarised as follows.

EU-RAR (2003, 2008)

The EU-RAR reviewed 34 animal studies, published between 1999 and 2007, the majority of which used the oral route for BPA exposure. These studies focused on developmental neurotoxicity endpoints, including behavioural studies as well as studies assessing the effects of pre- and /or postnatal BPA on brain development. Many developmental neurotoxicity endpoints were evaluated:

locomotor and exploratory activity; grooming, cognitive, emotional, social, sexual and maternal behaviour; behavioural response to pharmacological challenge; brain morphology, immunohistochemistry, and receptor/gene expression. The overall assessment draws attention to a low level of confidence in the reliability of the studies and a lack of consistency in the results of behavioural testing, such that no firm conclusions can be drawn on any of the parameters considered. However, the environmental regulatory agencies of three countries, Denmark, Sweden and Norway, did not agree with this conclusion, considering that a number of studies on developmental neurotoxicity, namely Adriani et al., 2003, Negishi et al., 2004, Carr et al., 2003, and Ryan and Vandenberg, 2006, were sufficiently reliable for regulatory use.

NTP-CERHR (2008)

The NTP-CERHR (2008) monograph reported that perinatal or pubertal exposure to "low" doses of BPA may cause neural and behavioural alterations in rats and mice, especially related to the development of normal sexual dimorphisms. However, the literature could not be fully interpreted for biological or experimental consistency, or for relevance to human health. In summary, the NTP considered that there is some concern for effects of BPA at low doses on the developing brain and behaviour, but identified the need for additional research to more fully assess its functional and long-term implications and relevance to humans.

EFSA (2006); EFSA CEF Panel (2010)

In 2006, EFSA noted that BPA given orally at low doses during gestation and/or lactation was reported to cause effects on some of the behavioural endpoints assessed. Overall, however, the AFC Panel (2006) considered that there were no consistent treatment-related effects in the behavioural endpoints and apparently contradictory observations were published, citing as an example the fact that neophobia was found as an effect in one study (Adriani et al., 2003) in females and not in males, while in the other study of Negishi et al. (2003) no effect was found in the open field, which should show an effect if neophobia is present in male offspring. The AFC Panel also noted methodological deficiencies in the available studies.

The 2010 EFSA opinion included evaluation of toxicological data published between 2007 and July 2010. For the in vivo animal toxicity studies the focus was on low dose oral studies employing several test doses including at least one less than 5 mg/kg bw per day and involving developmental exposure. The CEF Panel (2010) noted the impact of BPA on development of sexually dimorphic behaviour as addressed in the study by Ryan et al. (2010a), who observed a male-like reduced saccharin preference and inhibition of lordosis behaviour in female rat offspring from oestrogen-treated but not from BPA-treated dams. The CEF Panel (2010) concluded that the effect of BPA on learning and memory behaviour as explored in the study of Stump et al. (2009), which was designed according to OECD Guideline 426 and focused on learning and memory assessed in the Biel Maze, was inconclusive due to large variability in the data.

The 2010 EFSA CEF Panel opinion also considered that significant methodological shortcomings (e.g. small numbers of samples, lack of consideration of the litter effect) applied to a number of studies addressing other neurobehavioural endpoints (e.g. learning and memory behaviour, anxiety-like behaviour and sex-specific behaviour) made the reviewed studies invalid or inadequate for risk assessment purposes. Overall the CEF Panel (2010) concluded that currently available data did not provide convincing evidence of neurobehavioural toxicity of BPA. The CEF Panel (2010) noted that potentially significant biochemical changes, e.g. altered receptor expression in different brain regions, such as changes in N-methyl-D-aspartate (NMDA), oestrogen receptors and alteration in the basal level of aromatase have been reported. However, in the absence of a correlation with a functional adverse effect, the CEF Panel did not consider the available data as convincing evidence of neurobehavioural toxicity of BPA. A minority opinion was expressed by a CEF Panel member, based on uncertainties raised by some recent animal studies suggesting changes in brain receptor programming which may have functional behavioural consequences.

An entire Section of the 2010 EFSA CEF Panel opinion was dedicated to a review of a study by Stump (2009) who performed a developmental neurotoxicity study with BPA, in accordance with OECD guideline 426 and in compliance with GLP. As stated in the EFSA CEF Panel (2010) opinion *"BPA was administered to pregnant rats via the diet at concentration ranging from 0.15 to 2250 mg/kg feed equivalent to 0.01-164 mg/kg bw per day during gestation and to 0.03-410 mg/kg bw per day during lactation. Dams were evaluated for general signs of toxicity, and offspring were evaluated for general toxicity including developmental landmarks and for neurological effects, including behaviour and brain histopathology.*

Based on the body weight effects on dams and offspring and also taking into account the occurrence of seizures and convulsions in the two highest dose groups, which were not observed at the lower dose levels, the study supports the NOAEL which was derived from multigenerational studies in the past (5 mg/kg bw per day), leading to a TDI of 0.05 mg/kg bw per day. The neurodevelopmental toxicity study by Stump (2009) covers motor activity, learning and memory (spatial behaviour), auditory startle response, brain histopathology and morphology. The study does not cover some specific aspects of learning and memory (i.e. avoidance learning, schedule-controlled behaviour, and impulsiveness), anxiety-related behaviour or sexual dimorphic behaviour, but this does not invalidate the study. No statistically significant effects were observed in tests on motor activity or auditory startle or in brain histopathology and morphology. Stump also concluded that there were no changes in learning and memory based on the results of the Biel Maze test. However, the Panel considered that this test on learning and memory was inconclusive due to large variability in the data and of limited value in the risk assessment of BPA."

FAO/WHO (2011)

The Expert Meeting reported changes in brain biochemical signalling, morphometric and cellular end-points within sexually dimorphic anatomical structures and neuroendocrine end-points at dietary exposures of BPA below 5 mg/kg bw per day. Based on the available data, changes in anxiety-like behaviour and convergence of anatomical brain sex differences were identified as end-points suggestive of effects with potential human relevance. In particular, the report called for additional research to examine anxiety-related behavioural end-points (and the underlying mechanisms) following developmental exposure. It recommended to employ multiple validated protocols for testing of anxiety-like behaviour at multiple ages using multiple doses in both sexes; to examine the association of impacts on brain sex differences with functional (behavioural or physiological) end-points, and to conduct dose–response analysis for anatomical brain sex differences in both sexes.

ANSES (2011, 2013)

In 2011, ANSES stated as i) proven the effects of BPA on neurogenesis and brain development due to pre or peri-natal exposure including changes in the neurodifferentiation profile, in glutamatergic and monoaminergic systems, in the expression of oestrogen receptors α and β and in the number of neurons responsive to oxytocin and serotonin, ii) controversial (and thus needing further investigation) the effects on anxiety, explorative behaviour and some sexual dimorphisms within this framework. Finally, the ANSES report considered as suspected the modification of maternal behaviour evidenced in some studies. Altogether, the evidence was considered as sufficient to support the developmental neurotoxicity of BPA resulting from pre- and perinatal exposure and this endpoint was considered as critical and used for risk characterisation in the 2013 risk assessment.
In the ANSES risk assessment of 2013 the oral study by Xu et al. (2010) in mice was taken as the key study for neurodevelopmental toxicity, where the critical effects were the alteration of memory and learning functions paralleled by a decrease in the expression of glutamate NMDA receptors.

3.4.2.2. Evaluation of recent animal studies on BPA exposure and brain and behavioural effects

Several studies in the past decade have indicated neurobehavioural effects of developmental exposure to BPA. In particular, a number of studies have reported the influence of developmental exposure to BPA on anxiety-like behaviours in rodents (e.g. Cox et al., 2010; Tian et al., 2010; Patisaul and Bateman, 2008; Ryan and Vanderbergh, 2006; Gioiosa et al., 2007; Fujimoto et al., 2006). The results

of these studies were largely inconsistent, possibly because of differences in the doses, exposure periods and age at testing used. Both increases and decreases in anxiety levels have been reported, with either significant or not significant sex differences. Most of these studies have been thoroughly reviewed in the previous opinions listed above, which reached various conclusions regarding the neurobehavioural toxicity of BPA. So far limitations in the studies' design, and inconsistency of results made it impossible to establish the reliability of these observations. This section provides an overview of the experimental animal studies on brain and behavioural toxicity published after 1st August 2010 (EFSA CEF Panel, 2010). A detailed description and evaluation of each study published after August 2010 is provided separately in Appendix B.

a. Effects on anxiety-like behaviour

Eighteen studies investigating anxiety-like behaviour in rodents following exposure to BPA have been identified, the majority being developmental studies with oral administration of BPA to rodent dams during gestation and/or lactation. All of the studies included either an open field test procedure or an Elevated Plus Maze (EPM) test for evaluation of anxiety-like behaviour. Six studies used both of these tests whereas a few used additional tests like free exploratory open field, novelty preference, novel home environment, Forced Swimming test, light/dark test/box/transition, or mirrored maze.

A short review of the identified studies is given below. For study details such as number of animals, see Appendix B.

The study by Diaz Weinstein et al. (2013) reported an increased anxiety-like behaviour of adolescent Sprague Dawley rats (7 weeks of age) in the open field (6 min) test and EPM (5 min) tests after twelve days of subcutaneous exposure to one dose level of 40 µg/kg bw BPA. BPA exposure affected both female and male rats to about the same extent. BPA additionally impaired spatial memory in both sexes as evaluated with an object placement procedure, and increased sucrose preference.

Ferguson et al. (2012) exposed Sprague Dawley dams to low doses of BPA (2.5 or 25 µg/kg bw per day) or vehicle by gavage from gestational days 6-21, and then the pups from birth until weaning with the same BPA dose also by gavage. A naïve control group was not treated. Offspring were behaviourally evaluated in a novelty preference test (PND 29), open field test (PND 40-42), motor coordination (PND 43-44), Barnes maze (PND 47-50), acoustic startle response (PND 54, and Morris water maze (PND 75-79). In the open field test, males exposed to both doses of BPA were more active compared to the vehicle control, while no effect was observed in the females. However, the activity of naïve control groups showed approximately the same activity as the BPA treated groups. The authors reported that activity in other assessments (e.g., novelty preference) did not indicate BPA-induced hyperactivity and thus, that this particular effect deserves replication. Littermates of animals used in this study have been investigated for molecular markers for BPA (Cao et al., 2013; He et al. 2012, see below).

Fujimoto et al. (2013) examined anxiety-like behaviour of Wistar rat offspring after postnatal BPA exposure through dams milk using an open field test, an Elevated Plus Maze (EPM) and a Forced swim task at the age of six, seven and nine weeks, respectively. The first seven days after delivery, dams were orally exposed to one dose level of BPA through drinking water (0.1 ppm, equivalent to about 24 µg/kg bw per day). Open field results suggested hyperactivity induced by BPA in males and thereby eliminating the sex differences seen in controls. BPA did not enhance anxiety-like behaviour in the EPM in any sexes, but females were more active than males and the author suggested an anxiolytic effect of BPA. In the FST, BPA induced a depression-like response in both sexes since the latency time to immobility was decrease in both sexes compared to controls, and the time spent immobile was increased in males.

Gioiosa et al. (2013) attempted to identify specific windows of susceptibility to BPA for anxiety-like behaviour by comparing gestational versus lactational exposure in CD-1 mice by use of a cross-fostering procedure. Dams were orally exposed to one dose level of BPA (10 µg/kg bw per day) from

gestational day 11 to lactational day 8. At delivery, offspring were cross-fostered to ensure gestational and lactational exposures only. Juvenile offspring (PND 28-30) were assessed in a novelty test, and adult offspring (PND 70) both in a free exploratory open field and in an EPM to measure emotional response to novelty and anxiety-like behaviour. The control females were less anxious, more active and more prone to explore a novel environment than control males and BPA-treated females. The direction of the behavioural changes was consistent and the effects of pre- or postnatal exposures were similar, although with a greater effect associated with postnatal exposure in females. BPA did not have a primary effect per se on the behavioural end points considered, but consistently eliminated the sex dimorphism in anxiety-like/exploratory response.

Inagaki et al. (2012) made use of an acute exposure paradigm and exposed adult female Sprague Dawley rats, intact or ovariectomised (OVX), to BPA by subcutaneous injections ranging from 0.4 µg/kg to 400 µg/kg bw. Some groups received BPA co-administrated with 17-α E2 or 17-β E2. In this study, two types of hippocampal-dependent memory tasks, object placement (OP) and object recognition (OR), were used. In order to assess influence of anxiety-like behaviour on performance in a memory task, rats were tested in the Elevated plus maze. Neither 20 µg E2/kg bw (enhanced OP memory) nor 40 µg BPA/kg bw (antagonised E2), alone or in combination, influenced anxiety-like behaviour in the Elevated plus maze. Inagaki et al. (2012) also examined learning and memory in the Morris water maze and dendritic spine density in the CA1 area of the hippocampus and layer II/III of the prefrontal cortex (PFC) (see below).

Jasarevic et al. (2013) used outbred deer mice (Peromyscus maniculatus bairdii) whose dams were fed a diet supplemented with either ethinyl oestradiol or one of three doses of BPA (50 mg, 5 mg, or 50 µg/kg feed weight) starting from 2 weeks before mating up to the end of the lactation period. Compared to controls, adult male offspring exposed to ethinyl oestradiol and to the two upper doses of BPA showed increased anxiety-like behaviour in the EMP.

Jones and Watson (2012) used an EPM and a Forced Swimming test to investigate anxiety-like behaviour in adult Long-Evans rats perinatally exposed to BPA. From gestational day 7 until lactational day 14, dams were orally exposed to BPA at 5, 50, 500, or 5000 µg/kg bw per day or corn oil vehicle (controls) by spontaneously drinking from a syringe. The normal sex differences seen in control rats were eliminated in low dose BPA-exposed adults (5 µg/kg bw per day) but not at the higher doses in an EMP (see Section 1.2). No significant differences across doses of BPA or sexes were seen in the Forced Swimming test. The same study also assessed spatial learning capacities in the Morris Water Maze and reported no effects of either BPA doses per se or any interaction of BPA with sex.

Kundakovic et al. (2013) exposed female Balb/c mice orally to BPA (0, 2, 20 or 200 µg/kg bw per day; presumably by gavage) from the day of mating to the end of pregnancy. Offspring were tested for social behaviour (dam/pup interaction, adolescent home cage, and adult social approach and aggression) and anxiety-like behaviour as adults (open field test). BPA exposure induced persistent, largely sex-specific effects on anxiety-like behaviour, leading to disruption of sexually dimorphic behaviours in adult mice. BPA exposure increased anxiety-like behaviour in females and decreased anxiety-like behaviour in males. This study also examined possible epigenetic disturbances of BPA, see below.

Matsuda et al. (2012) assessed the effects of BPA on anxiety-like behaviour (open field test) and brain biochemistry in the offspring of C57BL/6J mice exposed subcutaneously from gestational day 10 until weaning of the offspring to BPA at 0.25 µg/kg bw per day. Exposure to BPA increased anxiety-like behaviour in both juveniles (4 week) and adult (8 week) male offspring, as evaluated by significantly decreased the time spent in the centre area of the open field. In brains of adult male offspring, BPA significantly altered DA turnover. Similar changes were not seen in the female offspring. This study also examined possible neurochemical effects of BPA, see below.

Nakamura et al. (2012) reported hypoactivity but no anxiety-like behaviour in ICR/Jcl mice perinatally exposed to BPA. Dams were injected subcutaneously with vehicle (sesame oil) or 20 µg BPA/kg body weight through gestation and the lactational period. Offspring were tested as juveniles and as adults in open field and EPM, and as adults for learning and memory in Morris water maze.

Patisaul et al., (2012) exposed Wistar rat dams to BPA via drinking water (1 mg/L) from gestation day 6 through lactation and provided soy-based or soy-free diets. Offspring continued on the same diet and BPA exposure regimen via drinking water from weaning until puberty (estimated dose of BPA received: between 100 and 1000 µg/kg bw per day). Positive control rats were given ethinyl oestradiol (EE, 50 µg/L) and fed a soy-free diet. When fed soy-free diet, BPA-exposed juveniles showed slightly increased anxiety-like behaviour in the light/dark box and the EMP, and BPA-exposed adult offspring showed disappearance of the normal sexual dimorphism in exploratory behaviour in the EMP. Administration of a soy-enriched diet appeared to mitigate the BPA effects. This study also examined possible effects of BPA on gene expression in dimorphic brain structures, see below.

Viberg et al. (2011) reported no anxiety-like behaviour (EMP) in adult NMRI male mice neonatally exposed to a single oral dose (gavage) of BPA at 0.32, 3.2 or 4.8 mg/kg bw. However, BPA was found to induce hyperactivity and lack of expected habituation profile in males when tested in a novel home environment at 2 and 5 months of age (see below). The BPA effects were found not to be mediated by alterations in the cholinergic system since all mice responded to nicotine with increased activity. At 4 months of age, testing in the Morris water maze for spatial learning showed no effects of BPA. In another study (Viberg et al., 2012), the same author reported effects of BPA on brain markers, see below.

Wolstenholme et al. (2011a; 2012) found no changes in anxiety-like behaviour in young C57BL/6J mice prenatally exposed to BPA and tested in an EMP (Wolstenholme et al. 2011a, 1.25 mg BPA/kg diet; Wolstenholme et al. 2012, 5 mg BPA/kg diet). Wolstenholme et al. (2013) noted no differences in the open field for F1 mice, but increased activity in F3 generation of the BPA-line mice compared to controls following dietary exposure to BPA of the parenteral generation at 5 mg per kg diet. These studies mainly addressed social behaviour but reported also on possible effects of BPA on gene expression in dimorphic brain structures, see below.

Xu et al. (2012) attempted to contrast in ICR mice the effects of gestational vs lactational exposure to BPA (0.4 or 4 mg/kg bw per day) by the oral route. Both exposure periods (GD 7-20 or PND 1-14) and both doses increased anxiety- and depression-like behaviours in mice of both sexes in one or more of the following tests: the EMP, open field test, dark/light transition task and mirrored maze. The authors suggested that gestational BPA exposure exhibited stronger effect than lactational on anxiety-like behaviour in females. In another study, Xu et al. (2011a) reported abolished (or induced) sex difference of BPA compared to controls (sesame oil) in adolescent ICR mice orally exposed (intragastric, ig) to BPA at 40, 400 µg/kg/d for eight weeks and thereafter tested in an open field and EMP (ca. 90 days old). The mice were additionally tested for learning and memory in the Morris water maze and by use of step-down Passive avoidance task (see below). In a further study, Xu et al. (2013a) exposed adult ICR mice orally to BPA at 0.4, 4, or 40 mg/kg bw per day, or arachis oil, for 12 weeks by gavage, and studied anxiety-like behaviour in the open field test. Males spent significantly more time in the open area after BPA exposure of 40 mg/kg bw/ day, indicating that BPA reduced anxiety-like behaviour in males. No effect of BPA treatment was observed in females. Both Xu et al. (2011a) and Xu et al. (2013a) also reported on learning and memory (see below).

The CEF Panel noted that several of the above studies report increased anxiety-like behaviour in one or both sexes after BPA exposure, whereas others found no differences or a decrease of such behaviour. The results from different studies were thus considered inconsistent. Additionally, the studies were confounded by limitations in study design as indicated in Appendices B and C.

b. Effects on learning and memory

Previous studies have reported that developmental exposure to BPA can interfere with learning and memory capacities in different learning tasks in rodents, including spatial learning, passive avoidance learning and object recognition (for review see EFSA CEF Panel, 2010). These studies have previously not been considered suitable by EFSA for risk assessment due to methodological shortcomings. In addition, Stump et al. (2009; see description of tests applied and results above) did not report any effects of BPA on learning and memory as examined in the Biel Maze test, but the CEF Panel (EFSA CEF PANEL, 2010) found the study inconclusive.

In the ANSES risk assessment of 2013, the oral study by Xu et al. (2010b) in mice was taken as the key study for neurodevelopmental toxicity, where the critical effects were the alteration of memory and learning functions paralleled by a decrease in the expression of glutamate NMDA receptors. Therefore this study has been included by the CEF Panel for the current evaluation. In addition to this, thirteen studies examining effect of BPA on memory and learning have been identified. Seven of the fourteen studies included have investigated effects of BPA following developmental exposure, some studies used subchronic exposure in adolescent/adults and a few used acute exposure regimens. The majority of the studies used tests like the Morris water maze and/or a passive avoidance task (step down), but also object recognition (one new of two objects), object placement (one of two objects in new position), modified Barnes maze and fear memory tasks. Several of the studies investigated neurochemical markers of the brain in parallel with the functional tests.

A short review of the identified studies is given below. Studies using exposure to adolescent/adults are separated from those which used developmental exposure. For study details such as number of animals, see Appendix B.

b.1 Effects on learning and memory – adolescent/adult exposure

Diaz Weinstein et al. (2013; see above) reported impaired spatial memory in both sexes adolescent SD rats tested in an object placement task following nine days subcutaneous injections of BPA at 40 µg/kg bw. In line with this, Eilam-Stock et al. (2012) reported impaired visual and spatial memory following acute subcutaneous BPA injection (40 µg/kg bw) in adult male rats as evaluated by an object recognition and an object placement task. In both tasks, BPA was injected immediately after the initial sample trial and memory retention was tested after two hours. These two tasks were performed four days apart and groups were switched between tasks so that controls in the object recognition task were exposed to BPA in the object placement test and vice versa. Neural brain markers were also examined (see below).

In contrast to the results by Diaz Weinstein et al. (2013) and Eilam-Stock et al. (2012), Inagaki et al. (2012) reported that acute subcutaneous injections at 0, 1, 4, 40, 120, 240 and 400 mg BPA/kg bw did not impair memory retention in adult ovariectomised female SD-rats in the object recognition and the object placement memory tasks, but that BPA blocked the enhancing effects of 17β-oestradiol on learning and memory performances at doses from 4 µg/kg in the object placement and at doses from 40 µg/kg in the object recognition tasks. 17-αE2-induced object placement (OP) memory enhancement was inhibited by all doses BPA. The ovariectomised females were exposed to BPA 30 min before the sample trial and immediately thereafter, and tested every tenth day for about 2 months. An additional group of normally cycling rats was exposed to 40 µg BPA/kg bw, which did not affect the performance in the object placement task at any phase of the ooestrous cycle. Object recognition memory was impaired by BPA only on prooestrous when endogenous E2 levels are highest. Inagaki et al. (2012) also examined brain markers, i.e. dendritic spine density in the CA1 area of the hippocampus and layer II/III of the prefrontal cortex (see below).

Kim et al. (2011) exposed 5 week old male mice to BPA (0, 1, 5, 20 mg/kg bw per day) by oral gavage for 2 weeks, and assessed learning and memory in the Morris water maze test following seven consecutive days of training. The latency time to reach the hidden platform was only significantly increased at the highest BPA dose (20 mg/kg bw) at day 7. The total swimming distance was not significantly affected by the treatment. Neural brain markers were also examined (see below).

Xu et al. (2011a; see above) found no overall effect of oral BPA treatment (0.04, 0.4 mg/kg bw per day) for eight weeks and thereafter tested in the Morris water maze. However, at days 3 and 4 of the 4-days-acquisition part of the Morris water maze test, the BPA low dose males showed a longer escape time than control males and as such abolished sex difference within the low dose BPA group. In the step-down avoidance test, BPA low dose males showed a shorter latency to step down compared to control male and as such induced a sex difference as no effects were seen in females in both of these tests. In Xu et al. (2013a; see above) adult ICR mice were orally exposed to BPA (0.4, 4, or 40 mg/kg bw per day) or arachis oil for 12 weeks. No learning and memory impairment of BPA were reported for females tested in the Morris water maze and the passive avoidance test. For the Morris water maze, no effects were seen on the probe day for any BPA doses. In males, BPA at doses 0.4 and 40 mg/kg/day extended the average escape path length to the hidden platform in Morris water maze task during the training days, while no effect were seen at 4 mg/kg bw per day. BPA at the high dose of 40 mg/kg bw per day shortened also the step-down latency in males 24 h after a footshock. This study examined also neural brain markers (see below).

b.2 Effects on learning and memory – developmental exposure

In Ferguson et al. (2012), low doses of BPA (2.5 or 25 µg/kg bw per day) to Sprague Dawley dams by oral gavage on gestational days 6-21 and then to the offspring from birth to weaning, did not show any significant effects on spatial learning in adolescent/adult offspring neither in the Barnes maze (PND 47-50) nor in the Morris water maze (PND 75-79). In agreement, Jones and Watson (2012; see above) used the Morris water maze and found no spatial learning effects in adult offspring (PND 90-150) of Long-Evans rat dams orally exposed to BPA (5, 50, 500, or 5000 µg/kg bw per day) during gestation and lactation. Nakamura et al. (2012; see above) reported no effect on learning of perinatal exposure to BPA in adult ICR/Jcl mice as evaluated by the Morris water maze. Dams were injected subcutaneously with vehicle (sesame oil) or 20 µg BPA/kg body weight through gestation and the lactational period. In agreement with the three above mentioned studies, Viberg et al. (2011; see above) found no effect on learning and memory (Morris water maze) in 6 month old mice orally exposed as neonatal to single doses of BPA (0, 0.32, 3.2 or 4.8 mg/kg bw). This study used pharmacological challenge with nicotine in order to elucidate mode of action of BPA (see below). Xu et al. (2010b) used the Morris water maze and reported normal memory retention (probe day) in male ICR mice perinatally exposed to BPA (0.05, 0.5, 5 and 50 mg/kg bw) tested as weanlings (PND 21-25) and as adults (PND 56-60), but increased escape lengths (training days) were seen for the upper two doses in weanlings and the upper three doses in adult males. In weanlings, BPA at 5 and 50 mg/kg bw increased error frequency and shortened the step-down latency in males 24 h after a footshock, whereas only BPA 50 mg/kg bw did so in adults. This study examined also neural brain markers (see below).

Jang et al. (2012) exposed pregnant C57BL/6 mice by daily intraperitoneal injection to BPA at 0, 0.1, 1 and 10 mg/kg bw per day from GD6 to GD17. Female BPA offspring were mated with control male offspring to obtain F2 generation, which females were subjected for behavioural testing and investigated for hippocampal neurogenesis. No group differences were seen in the Morris water maze. The passive avoidance test revealed that high-doses BPA (1 mg/kg and 10 mg/kg) decreased the latency time in F2-females without a clear dose-response relationship. For the investigation of hippocampal neurogenesis in F2-females at postnatal day 44, see below.

Jasarevic et al. (2013; see above) reported that male deer mice orally exposed during gestation and lactation to BPA (0.05, 5 or 50 mg/kg feed, equivalent to 0.25, 25 or 250 µg/kg bw per day) had impaired learning performance in the Barnes Maze, while females outperformed males.

Matsuda et al. (2013) reported that perinatal exposure to BPA (dams were exposed subcutaneously (SC) to 250 ng BPA/kg/day during gestation and lactation) enhanced fear memory in juvenile female C57BL/6J mice, but not in adult female or males, as evaluated as number of freezing episodes when returning to a chamber which gave electrical foot-shock the previous day. This study used a pharmacological challenge with sertraline in order to elucidate mode of action of BPA (see below).

The CEF Panel noted that several of the above studies report impaired learning and memory of BPA exposure in one or both sexes, whereas others found no such differences. The results from different studies were thus considered inconsistent. Additionally, the studies were confounded by limitations in study design and performances as indicated in Appendices B and C.

c. Social behaviour

Previous publications have studying social behaviour after BPA exposure (Gioiosa et al., 2007; Palanza et al., 2008; Patisaul and Bateman, 2008; Cox et al., 2010; Tian et al., 2010). Overall, the direction of the effects was not consistent and ranged from pro-social effects to reduction of social motivation. Although most of these studies have been reviewed by EFSA in 2010, the social behaviour endpoint was not addressed separately in the EFSA CEF Panel 2010 opinion.

Four studies examining the effect of BPA on social behaviour have been identified, and three of these are published by the same group. Social behaviours were evaluated by use of e.g. home-cage observations, Social interaction test, Social preference test, and a Social approach and aggression task by use of stimulus animal. In some of these tests, several parameters are recoded – mostly manually. Social behaviour may be influenced by many factors. All four studies applied one or two tests to elucidate whether anxiety-like behaviour in the animals influenced social behaviour or not. A short review of the identified studies is given below. For study details such as number of animals, see Appendix B.

Kundakovic et al. (2013, see above) gave BPA (0, 2, 20 or 200 µg/kg bw per day) to pregnant Balb/c mice from the day of mating to the end of pregnancy. BPA effect on home-cage social behaviour in adolescents offspring of both sexes were increased sniffing and allogrooming at the 2-µg dose (compared with vehicle), and reduced chasing in males at the highest dose (to female level). Dyadic social interactions with a same-sex stimulus mouse at PND70 showed that BPA had moderate effects on aggression and social dominance. This study also examined markers for gene expressions in offspring brains (see below). The CEF Panel has assessed this study with regard to NMDR claimed by the author (see Section 1.2).

In the study by Wolstenholme et al. (2011a; see above), C57BL/6J mice dams were given BPA-supplemented diet during pregnancy (about 1.25 mg BPA/kg diet estimated to be equivalent to approximately 120 µg/kg bw per day). A cross-fostering procedure was used to ensure prenatally exposure only. The prenatally exposed female offspring showed slightly increased social interactions in a free 30-min social interaction test at weaning. The effect of BPA on males was in the same direction but less significant. However, BPA did not affect social preference for a stimulus animal when compared to an inanimate object when tested a few days later. Expression of selected genes involved in neurobehavioural plasticity was also evaluated in this study, see below.

Wolstenholme et al. (2012; see above) exposed C57BL/6J mice (F0 generation only) to BPA (about 5 mg/kg diet, equivalent to approximately 1.0 mg/kg bw per day[12]) through pregnancy. Subsequent generations, obtained by breeding brother-sister pairs, were not exposed to dietary BPA. F1 juveniles of both sexes underwent evaluation for social interaction on PND 20-21 (10 minutes) and social preference on PND 24 (10 minutes), whereas juveniles of the F2 and F4 generation were assessed in the free social interaction test. The results on social behaviour throughout the generations were inconsistent (social interaction decreased in the F0 generation, but increased in the F2 and F4 generations). The study also examined effects on gene expression, see below.

Wolstenholme et al. (2013; see above) exposed C57BL/6J mice (F0 generation only) to BPA (about 5 mg/kg diet) prior to mating and throughout pregnancy. A cross-fostering procedure was used to ensure prenatally exposure only of F1 generation. Subsequent generations, obtained by breeding

[12] Time of adoption of the 2010 EFSA Opinion on BPA dealing with hazard identification and characterisation (EFSA CEF Panel, 2010).

brother-sister pairs, were not exposed to dietary BPA. Juvenile F1 and F3 mice from BPA-exposed-lines displayed higher levels of investigation than controls during the eight first trials in the Social recognition task. In the last trial, F3 male of the BPA exposed line, spent less time with the novel stimulus ovarioectomized mouse compared to other groups. When tested, F3-generation mice displayed similar olfactory discrimination.

The CEF Panel noted that examination the effect of BPA on social behaviour may be challenging as many environmental and genetically factors influence on the animals and its behaviour. The results from these four studies were considered inconsistent, and the studies were confounded by limitations in study design as indicated in Appendices B and C.

a. Effects on sensory-motor functions

Overall, based on previous opinions, there appears to be no convincing evidence of a consistent BPA-related effect on sensory-motor function. The OECD guideline for developmental neurotoxicity advises sensory-motor function to be examined in detail at least once for the adolescent period and once during the young adult period (OECD 426, 2007), and gives the following few examples of tests: extensor thrust response, righting reflex, auditory startle habituation, and evoked potentials. Stump (2010) covered the auditory startle response as sensory-motor endpoint in offspring at PNDs 20 and 60 without any statistically significant effects of BPA.

Two new studies reporting on changes in sensory-motor function following pre and postnatal exposure have been included in the present opinion. Ferguson et al. (2012; see above) assessed the acoustic (auditory) startle response in adult rats (at PND 54) and found that males of the naïve control, both BPA groups and the EE_2 group exhibited significantly less response than vehicle control males. This effect appeared to be due to an aberrant heightened response in the vehicle control since it also was appeared in the naïve control. Wolstenholme et al. (2013; se above) tested F3-generation of BPA-exposed mice for olfactory discrimination and found no effect of BPA (only parent generation exposed).

The CEF Panel noted that the reported effects of BPA on sensory-motor function in adult male rats, not in females, in the Ferguson et al. (2012) were questioned by the author. The auditory startle response is not known as a sexually dimorphic behaviour (Meyer, 1998). The results from the Ferguson (2012) study were considered inconsistent. However, both studies described above were confounded by limitations in study design as indicated in Appendices B and C.

b. Other effects

Note that the studies mentioned below are thoroughly described in Appendix B.

Concerning studies on spontaneously motor activity, Ferguson et al. (2012; see above) found dose-related effect of BPA (2.5 or 25 µg BPA/kg bw per day) on open field activity of male SD-rats (PND 40-42), activity being significantly increased relative to vehicle controls, with EE showing a stronger response. The author commented that since activity in other tests (e.g., novelty preference) did not indicate BPA-induced hyperactivity, this particular effect deserves replication. In the study by Fujimoto et al. (2013; see above), open field results suggested hyperactivity induced by lactational exposure to BPA (maternal exposure 24 µg/kg/bw day) in males Wistar rats (ca. PND 42) and thereby eliminating the sex differences seen in controls. Ishido et al. (2011) assessed the effects of acute neonatal (intracisternal on PND 5) exposure of 20 µg BPA to Wistar rats by an automated motor activity test at age 4-5 weeks, and found significant but marginal nocturnal hyperactivity. Viberg et al. (2011; see above), exposed neonatal (PND 10) male NMRI mice to a single oral dose (gavage) of BPA at 0.32, 3.2 or 4.8 mg/kg bw and assessed spontaneous behaviour in a novel home environment for 1 hour at 2 and 5 months of age. A dose dependent alteration in activity and habituation profile was found, with decreased activity during the first 20 min of the test, and hyperactivity in the last 20-min period. However, very few litters were studied in the paper by Viberg et al. (2011). In contrast to the above mentioned studies, Nakamura et al., (2012; see above) concluded that gestational and

lactational BPA exposure (maternal dose 20 µg BPA/kg bw per day) caused hypoactivity in adult mice, as evaluated by total travelled distance in an open field and EPM.

Jones et al. (2011) reported persistent deficit in the sexual behaviour of adult male Long-Evans rat, but not females, following perinatal exposure to BPA. Dams were orally exposed to corn oil (controls), 5 µg/kg, 50 µg/kg, 500 µg/kg or 5 mg/kg body weight (bw) PBA from gestation day 7 until lactation day 14 (3 dams/group). Impairment of sexual performance was reported by the author to follow a NMDRC since males perinatally exposed to one of the lower doses BPA (50 µg/kg bw per day) seemed more affected than males exposed to the highest dose (5 mg/kg bw per day). This study has been included in Chapter 1.2. The CEF Panel noted that BPA may alter the processes affecting sexual behaviour due to its estrogenic properties, but that the study identified was confounded by limitations in study design as indicated in Appendices B and C.

c. Morphological, molecular and biochemical changes in the brain

The EFSA CEF Panel 2010 opinion recognized as potentially significant the biochemical changes. However, in the absence of a correlation with a functional adverse effect, the relevance of these observations for human health could not be assessed. Fourteen studies addressing functional changes, i.e. behavioural endpoints, in parallel with the effects of BPA on brain (effect on neurotransmitters, neurogenesis, on gene expression, on the morphology of certain brain regions, etc.) have been included (Eilam-Stock et al., 2011; Inagati et al., 2012; Jang et al., 2012; Kim et al., 2011; Kundakovic et al., 2013; Matsuda et al., 2012; Matsuda et al., 2013; Patisaul et al., 2012; Viberg et al.; 2011; Wolstenholme et al, 2011a; Wolstenholme et al, 2012; Xu et al., 2010b; Xu et al., 2012; Xu et al., 2013a). Further, six new studies investigating solely the effects of BPA on animal brains have been found (Cao et al., 2012; Cao et al., 2013; He et al; 2013; Komada et al., 2012; Viberg et al., 2012; Xu et al., 2013b).

Three studies investigation the possible influence of BPA on neurotransmitters in the brains of animals being behaviourally tested (Viberg et al. 2011; Matsuda et al. (2012; 2013)) have been identified. Viberg et al. (2011, see above) reported that both BPA (3.2 or 4.8 mg/kg bw) exposed and control mice responded with increased activity to administration of nicotine, and therefore the observed hyperactivity and lack of expected habituation profile were not mediated by altered functionality of the cholinergic system. Matsuda et al., 2012 found decreased levels of dopamine metabolites in the brain of male C57BL/6J mice perinatal exposed to BPA (0.25 µg/kg bw) and related this to the findings of anxiety-like behaviour of males in the open field (see above). In another study with the same exposure regimen (Matsuda et al., 2013,), changes in the serotoninergic system (5-HT) were linked to enhance fear memory in juvenile female mice perinatal exposure to BPA.

Seven studies (Cao et al., 2012b, 2013; Kundakovic et al., 2013; Patisaul et al., 2012; Xu et al., 2010b; Wolstenholme et al., 2011a; Wolstenholme et al., 2012) have reported changes in the gene expression of the oestrogen receptors after BPA exposure, for experimental details see Appendix B.

The study by Cao et al. (2012b) in neonatal SD-rats shows that subcutaneous injections of BPA (0.05 mg/kg bw, 50 mg/kg bw) from postnatal day (PND) 0 to PND 2 had regional and sex-specific alterations of gene expression of oestrogen receptor alpha (ERα), beta (ERβ) and kisspeptin (Kiss1) in anterior and mediobasal hypothalamus that were both decreased or increased in a region-specific fashion on PNDs 4 and 10. The responses of BPA were very different from those of oestradiol, suggesting that effects of BPA involve mechanisms different from its oestrogenic action. A more recent "mode of action" study by Cao et al. (2013) found that new-borns (PND1) of SD-rat dams receiving BPA orally (2.5 or 25 µg/kg bw per day, GD6-PND21) showed significant changes in oestrogen receptors α and β in hypothalamus and amygdala (in situ hybridisation). The inclusion of a group of unhandled pregnant rats highlighted a significant effect of the gavage procedure that appears to reduce the baseline ESRs expression in the offspring at birth. Both BPA and ethinyl oestradiol (5 and 10 µg/kg bw per day) administrations were shown to have the power of counteracting such a

"gavage effect". Sibling animals used by Cao et al. (2013) were assessed for functional outcome by Ferguson 2012 (see above) with marginal effects on anxiety-like behaviour and learning and memory.

Kundakovic et al. (2013, see above) showed in Balb/c mice exposed to BPA (2, 20, and 200 µg/kg bw during pregnancy) induced sex-specific, dose-dependent, and brain region-specific changes in expression of genes encoding oestrogen receptors (ERs, ERα, Erβ, Erγ). The effects measured in this study appeared to be of different extent and direction depending on BPA dose, suggesting that low doses can be more effective in disturbing social behaviour than the higher ones (non-monotonic responses), see Section 1.2.

Patisaul et al. (2012, see above) reported reduced expression of oestrogen receptor beta (Esr2) and two melanocortin receptors (Mc3r, Mc4r) in amygdala and elimination of sex differences in Kiss1 expression in juvenile Wistar rats following perinatal BPA exposure (estimated dose of BPA received: between 100 and 1000 µg/kg bw per day in drinking water). No such changes were seen in adult offspring. The author linked the genetic changes seen in juveniles to the functional changes of BPA seen in anxiety-like behaviour. However, for the transcriptional effects of BPA, a mode of action different from the estrogenic activity may be considered, because the positive control (ethinyl oestradiol, EE) did not show the same effects.

Xu et al., (2010b) reported that the expressions of hippocampal oestrogen receptor beta (ERbeta) in both PND 21 and PND 56 ICR-mice were markedly down-regulated by perinatal exposure to BPA at 0.5, 5, and 50 mg/kg bw per day and possible involved in avoidance memory.

In Wolstenholme et al. (2012; see above), lower gene expression of several oestrogen receptors, vasopressin and oxytocin, were observed in embryotic brains (F1-generation) of C57BL/6J mice (F0-generation) exposed to BPA (equivalent 1.0 mg BPA/kg bw per day in diet) through pregnancy. In this study, a cross-fostering procedure was used to ensure gestational exposure. Furthermore, offspring were not given BPA and were mated to produce subsequent generations (F2- to F4-generations). Embryotic brains of the F4-generation showed decreased vasopressin mRNA and oxytocin (only males). The author linked the finding to changes in social behaviour.

In another study, Wolstenholme et al. (2011a; see above) showed no change in the expression of oestrogen receptor genes in embryonic brains of C57BL/6J mice exposed to dietary BPA during gestation (about 1.25 mg/kg diet, equivalent to approximately 0.140 mg BPA/kg bw per day), but oxytocin receptor gene was reduced in males. Gene expression analysis also revealed that mRNA for the glutamate transporter, Slc1a1, was enhanced by exposure to BPA in female brains and that expression of two of the three DNA methyltransferase genes, Dnmt1 and Dnmt3a, was modulated by BPA. Results were linked to slight increase of social interaction in weanling females. However, no effects of BPA were found for social preference at the same age.

Overall the changes in gene expression of estrogene receptor after BPA exposure are not consistent and are also inconsistent with the results from functional tests. In addition the effects are mostly inconsistent with the results after treatment with ethinyl oestradiol (EE2), indicating that any proposed changes in functional tests have other MOA than changes in the estrogen receptor.

EFSA CEF PANEL, 2010 reviewed the study by Xu et al., 2010a, which reported inhibition of the expression of N-methyl-D-aspartate receptor (NMDAR) subunits in male SD-rats perinatally exposed to BPA 0.05, 0.5, 5, 50, 200 mg/kg bw per day without parallel functional testing of the rats (see Appendix B). Xu et al., 2010b also reported decreased hippocampal expression of NMDAR subunits NR1, NR2A, NR2B, and of protein expression for ERβ in male offspring of ICR mice orally exposed to 0.05, 0.5, 5 and 50mg/kg bw during gestation and lactation. The changes were linked to impaired learning and memory in the Morris water maze and passive avoidance (step-down) tests at postnatal days 21 and 56.

Three new papers by Xu and co-workers (Xu et al. 2012, 2013a, 2013b) have examined the effect of BPA in the dose range 0.04 to 40 mg/kg bw on behaviour and brain, and have addressed the same hippocampal NMDAR subunits NR1 as Xu et al., 2010b in ICR mice of the same age. Xu et al., (2012; see above) used as cross-fostering design and reported, in contrast to Xu et al. (2010b), no effect of BPA on NMDAR subunit NR1 in hippocampus and amygdala of 56 days old male ICR mice exposed pre- or postnatally to 0.4 and 4 mg BPA/kg/d, but on AMPAR GluR1 in the same brain areas, except at the highest dose in amygdala (both exposure periods) and the highest dose in hippocampus (postnatal exposure). In females, NMDAR subunit NR1 in hippocampus and amygdala were decreased at the lowest postnatal dose and enhanced at the highest prenatal dose, respectively. The AMPAR GluR1 in hippocampus and amygdala was down-regulated in females, except in amygdala at the highest postnatal dose. The changes were linked to increased anxiety- and depression-like behaviours in mice of both sexes (Xu et al., 2012). Xu et al. (2013b) exposed male ICR mice perinatally to 0.04, 0.4 or 4 mg BPA /kg/bw day and found down-regulation of hippocampal NMDR subunit NR1 at PND 21 (upper two doses) and PND 56 (all doses), and hippocampal AMPAR subunit GluR1 at PND 14, 21, and 56 (upper two doses). No functional tests were done in this study. Xu et al. (2013a) exposed adult ICR mice to 0.4, 4 or 40 mg BPA/kg/d for 12 weeks. The lowest and highest BPA doses down-regulated hippocampal NMDR subunit NR1 and AMPAR subunit GluR1 in males, whereas no effects were seen in females. Slight learning and memory impairments were revealed in males and none in females (Xu et al., 2013a).

Overall, the studies by Xu et al. in mice showed an inconsistent change in hippocampal NMDAR subunits between age and sex after exposure to BPA, and no conclusion can be drawn from these findings.

Synaptic density and ultrastructure were investigated in the studies by Xu et al., (2013a, b). In Xu et al. (2013a), the adult male mice orally exposed to BPA (0.4, 4 and 40 mg/kg bw per day) showed decreased synaptic density of pyramidal cells in hippocampus CA1 at 0.4 and 40 mg/kg bw per day, whereas females at the same dose levels showed increased levels compared to males. The middle dose, 4 mg BPA/kg bw per day, showed no such effects. In Xu et al. (2013b), perinatal exposure to BPA at 0.04 and 4 mg/kg bw per day, but not at 0.4 mg/kg bw per day, decreased synaptic density in hippocampal pyramidal cells in 14 and 56 days old male mice (females not included). No effects of BPA on the synaptic width of pyramidal cells in hippocampus CA1 was found, whereas in the Xu et al. (2013a) study, it was increased in adult males at all BPA doses compared to controls. The effects of BPA on synaptic density were inconsistent between doses and sexes.

The possible effects of BPA on proteins implicated in the regulation of neurotransmitter release at synapses have been addressed by Xu et al. (2013a; 2013b) and Viberg et al., (2012). In Xu et al. (2013b) (no functional tests), down-regulation of hippocampal presynaptic synapsin I on PNDs 21 and 56 were seen in males mice perinatally exposed to BPA (0.4, 4 or 40 mg/kg bw per day), but on PND 14 effect was observed only at the high dose. PSD-95, a synaptic marker, in hippocampus was reduced on PND 56. In Xu et al. (2013a), the hippocampal expressions of presynaptic synapsin I and PSD-95 were significantly down-regulated by BPA at all dose levels (0.4, 4 and 40 mg/kg bw per day) in adult male ICR mice, but not in females, which was in parallel with the slight learning and memory impairments revealed in males and not in females. Eilam-Stock et al. (2011, see below) also reported decreased PSD-95 in brains of SD-rats tested in memory trials following acute BPA exposure (40 μg/kg bw) and linked it to memory deficiencies. Viberg et al. (2012) used no functional tests, but investigated proteins important for brain development and reported increased cerebral synaptophysin (synaptic vesicle protein) in both sexes of adult NMRI mice after a single neonatal exposure to 4.8 mg BPA/kg bw, and for male also at 3.2 mg/kg BPA. In adult female, but not in males, BPA caused decreased levels of calcium/calmodulin-dependent kinase II (CaMKII), a protein important for normal brain development, in both cerebral cortex and hippocampus.

Five studies have, in addition to other issues, addressed the possible effects of BPA on neurogenesis (Eilam-Stock et al., 2011; Inagati et al., 2012; Jang et al., 2012; Kim et al., 2011; Komada et al.,

2012). Eilam-Stock et al., (2011, see above) reported that acute exposure to BPA at 40 µg/kg bw significantly decreased dendritic spine density in pyramidal cells in CA1 and medial prefrontal cortex (mPFC) in SD-rats tested in memory trials, but not in untested rats. In the tested rats, BPA also significantly decreased PSD-95, a synaptic marker, in the hippocampus and increased cytosolic pCREB, a transcription factor, in mPFC. Inagati et al. (2012; see above) reported that BPA did not alter E2-dependent increases in dendritic spine density in prefrontal cortex (Golgi impregnation and microscopic evaluation) following an acute exposure regimen of 0.4 µg/kg to 400 µg/kg bw BPA and/or 17-α E2 or 17-β E2 in adult female SD-rats. However, in the hippocampus, BPA blocked E2-dependent increases in basal spines at 4 h and was additive with E2 at 30 min. Serum E2-level was elevated after an acute E2 injection (20µg/kg), both at 30 min and at 4 h, as analysed by RIA kit. The author concluded that BPA can alter E2-dependent functions in adult female and linked the finding to effects on learning and memory (see above). Jang et al., 2012 (see above) reported that intraperitoneal injection of BPA (10 mg/kg GD6-17) to C57BL/6 mice could adversely affect hippocampal neurogenesis in future generations (F2) by modulating the ERK and BDNF–CREB signalling cascades. In F2-females at postnatal day 44, BPA lowered the levels of phospho-ERK, brain-derived neurotrophic factor (BDNF), and phospho-CREB in hippocampi compared to controls. The findings correlated with altered DNA methylation of the CREB regulated transcription co-activator 1 (Crtc1) generated in F2-females and were suggested linked to decreased cross-over latency time in the passive-avoidance test (see above). Two additional studies (Kim et al., 2011, and Komada et al., 2012) reported changed neurogenesis after BPA exposure to adult C57BL/6J male mice and fetal C57BL/6J mice, respectively. Kim et al., (2011; see above) exposed the adult male mice to 1, 5 and 20 mg BPA/kg orally by gavage for two weeks. While the high dose (20 mg/kg) decreased hippocampal neurogenesis and impaired spatial learning in the Morris water maze, the lower dose enhanced hippocampal neurogenesis and had no effect on learning abilities. Komada et al. (2012) gavaged pregnant C57BL/6J mice with 200µg/kg bw BPA for 5 days (gestational day (GD) 8.5 - 13.5). At GD 14, dams were scarified and foetuses subjected to histological evaluation, which showed association between BPA and accelerated hippocampal neurogenesis.

Several biochemical and morphological changes have been reported in the brain after BPA treatment, but the results were inconsistent between treatment, age and sex, and no conclusion can be drawn regarding the relevance of these effects.

3.4.2.3. Summary of neurobehavioural effects of BPA in animals after prenatal and postnatal exposure

In their 2010 EFSA opinion, the CEF Panel concluded that the studies available were not sufficient to draw any conclusion regarding BPA exposure and neurobehavioural effects. The CEF Panel noted at that time that potentially significant biochemical changes, e.g. altered receptor expression in different brain regions, such as changes in NMDA, oestrogen receptors and alteration in the basal level of aromatase have been reported; however, the relevance of these findings was limited by the lack of information on whether functional adverse effects may be associated.

Eighteen new experimental studies on anxiety-like behaviour of BPA using different tests have been identified. Several of the above studies report on increased anxiety-like behaviour after BPA exposure, and some report no effect on such behaviour, but the studies are confounded by limitations in study design, inappropriate statistics and in addition, the results from different studies are inconsistent.

Fourteen new studies have examined effects of BPA on learning and memory, mostly by use of Morris water maze and passive avoidance (step-down) test. Some studies reported significant impairment of either learning and/or memory capacities (both in spatial and non spatial learning tasks), whereas other did not. The effects of BPA on learning and memory abilities of laboratory rodents are not fully consistent, as both positive and negative effects are reported in different papers for both for adult and developmental exposure. Seemingly, BPA administration to adult animals produces more impairment than developmental exposure to BPA. The studies present methodological

shortcomings, such as small sample size, lack of consideration of the litter effect, not properly controlled variability of exposure through diet and inadequate statistics.

Four studies (Wolstenholme et al., 2011a, 2012, 2013; and Kundakovic et al., 2013) evaluated the effects of BPA on social behaviour and reported both positive and negative results. The CEF Panel noted that the studies have methodological shortcomings (litter effect not properly considered, potential variability of exposure not controlled for), although the social behavioural analysis is performed in a scientifically-valid way.

As for the potential effects of BPA on sensory-motor function, two studies (Ferguson et al., 2012; Wolstenholme et al. 2013) reported no likely effect as evaluated by auditory startle response and olfactory discrimination, respectively.

Five studies addressing effects on spontaneous motor behaviour (Ferguson et al. 2012; Fujimoto et al. 2013; Ishido et al., 2011; Nakamura et al., 2012; Viberg et al., 2011) have reported inconsistent results but mostly towards increased activity, especially in male rodents. In contrast, Nakamura et al., (2012) reported hypoactivity following perinatal BPA-exposure. One of these studies (Ishido et al., 2011) presents major methodological shortcomings, including small sample size and the use of a single administration. The findings of Viberg et al. (2011) indicate very limited changes in motor parameters in one test only, while the study by Ferguson et al. (2012) is methodologically sound.

The EFSA CEF PANEL, 2010 opinion recognized as potentially significant the biochemical changes, e.g. altered receptor or protein expression, in different brain regions. A number of new studies address the effects of BPA on brain development (effect on neurogenesis and on gene expression, neuroendocrine effects, effects on the morphology of certain brain regions, etc.). Several biochemical and morphological changes have been reported in the brain after BPA treatment, but the results were inconsistent between treatment, age and sex, and no conclusion can be drawn regarding the relevance of these effects.

3.4.3. WoE of neurological, neurodevelopmental or neuroendocrine effects of BPA in humans, animals and in vitro

Whether BPA induces neurological, neurodevelopmental or neuroendocrine effects in humans and animals was considered using a tabular format for weighting different lines of evidence (WoE evaluation). The overall outcome of this WoE evaluation is presented below, while the WoE evaluation tables for these endpoints are presented in full in Appendix C. For interpretation of these tables always refer to Appendix A.

Table 9: Overall table of WoE evaluation of neurological, neurodevelopmental or neuroendocrine effects in humans and animals.

Human studies	
Overall conclusion on Likelihood of neurodevelopmental effects of BPA in humans: There are indications from prospective studies that prenatal BPA exposure (BPA exposure during pregnancy) may be associated with child behaviour in a sex-dependent manner. However, the associations were not consistent across the studies. It cannot be ruled out that the results are confounded by diet or concurrent exposure factors. The associations do not provide sufficient evidence to infer a causal link between BPA exposure during pregnancy and neurodevelopmental effects in humans. Potential effects are considered to be as likely as not.	**As likely as not**
Overall conclusion on Likelihood of neurological/behavioural effects of BPA in humans: There are indications from one prospective study that childhood BPA exposure may be associated with behavioural problems in both girls and boys. It cannot be ruled out that the results are confounded by diet or concurrent exposure factors. The associations do not provide sufficient evidence to infer a causal link between childhood BPA exposure and neurological effects/behavior in humans. Potential effects are considered to be as likely as not.	**As likely as not**

Animal studies	
Overall conclusion on Likelihood on Anxiety-like behaviour in animals after pre- and/or postnatal exposure to BPA: Several studies report on increased anxiety-like behaviour in laboratory rodents after exposure to BPA. Due to the limitation in study design and statistics, and the inconsistency in the reported results, potential effects are considered to be as likely as not.	**As likely as not**
Overall conclusion on Likelihood on Learning and memory in animals after pre- and/or postnatal exposure to BPA: The effects of BPA on learning and memory abilities of laboratory rodents are no fully consistent, as both positive and negative effects are reported in different papers. The papers have methodological shortcomings, such as underpowered sample size, lack of consideration of the litter effect, or not properly controlled variability of exposure through diet, and inadequate statistics. Potential effects are considered to be as likely as not.	**As likely as not**
Overall conclusion on Likelihood on Social behaviour in animals after pre- and/or postnatal exposure to BPA: The few new studies evaluating the effects of BPA on social behaviour as end point have some methodological shortcomings (e.g. litter effect not properly red, potential variability of exposure not controlled for). Due to the limitation in study design and statistics, and the inconsistency in the reported results, potential effects are considered to be as likely as not	**As likely as not**
Overall conclusion on Likelihood on Sensory-motor function in animals after pre- and/or postnatal exposure to BPA: One of the two studies considered reported some positive effects of BPA on sensory-motor function in adult male rats, not in females. The auditory startle response is not known as a sexually dimorphic behaviour and the study results were considered inconsistent. The potential effects are considered to be as likely as not.	**As likely as not**

3.4.4. Conclusions on neurological, neurodevelopmental and neuroendocrine effects

There are indications from prospective studies in humans that prenatal BPA exposure (BPA exposure during pregnancy) may be associated with altered child behaviour in a sex-dependent manner. However, the associations were not consistent across the studies and it cannot be ruled out that the results are confounded by diet or concurrent exposure factors. The associations reported do not provide sufficient evidence to infer a causal link between BPA exposure during pregnancy or childhood and neurodevelopmental effects in humans.

A number of new studies report changes that may indicate effects of BPA on brain development (effect on neurogenesis and on gene expression, neuroendocrine effects, effects on the morphology of certain brain regions, etc.). Whether such changes are mechanistically related to the neurobehavioral responses reported following exposure is attempted addressed by some studies but with inconsistent results.

Several new animal studies investigated anxiety-like behaviour, learning and memory, social behaviour and sensory-motor function. Some studies report changes in anxiety-like behaviour after BPA exposure. Some, but not all, studies reported significant impairment of either learning and/or memory capacities. A few studies also report effects on social behaviour and sensory-motor function. However, the studies present methodological shortcomings, such as small sample size, lack of consideration of the litter effect, not properly controlled variability of exposure through diet and inadequate statistics. Using a WoE approach, the CEF Panel assigned a likelihood level of "as likely as not" to neurological, neurodevelopmental and neuroendocrine effects of BPA level for this endpoint is less than "likely" (see Appendix A), this endpoint was not taken forward for assessing the toxicological reference point, but was taken into account in the evaluation of uncertainty for hazard characterisation and risk characterisation (Section 4.3).

3.5. Immune effects

3.5.1. Human studies

3.5.1.1. Summary of previous opinions

EU-RAR (2003, 2008)
In an evaluation made in 2003, and updated in 2008 (EU-RAR, 2003, 2008) no information on toxicity for the immune system was presented. Concern was expressed for skin sensitisation in occupational exposure scenarios where there is the potential for skin contact with high concentrations (>30%) of BPA, as there were anecdotal industry reports suggesting that workers handling BPA have in the past experienced skin, eye and respiratory tract irritation. The risk assessment indicated that it could not be determined whether the reported skin reactions were related to skin sensitisation or irritation, but that animal data clearly indicate that BPA is a skin sensitiser, albeit a very weak one, being able to sensitise the skin of mice only at concentrations higher than 30%. The assessment also concluded that it is unlikely that BPA in foodstuffs poses a risk of skin sensitisation

EFSA (2006); EFSA CEF Panel (2010)
In a literature survey by EFSA (2006) and EFSA CEF Panel (2010) no human studies on immune effects were available for evaluation.

NTP-CERHR (2008)
This monograph only presents a summary of the previous evaluation carried out on this endpoint in the EU-RAR of 2003 (see above).

FAO/WHO (2011)
In an opinion by FAO/WHO no human data on effects on the immune system were provided. The opinion expressed concern that BPA may be a skin sensitiser, but this was based on animal data only.

ANSES (2011, 2013)
In the 2011 ANSES report a study published by Clayton et al. (2011) was evaluated. ANSES considered that no conclusions on effects of BPA on the immune system could be drawn. In the 2013 risk assessment no human data on the effects of BPA on the immune system were discussed.

3.5.1.2. Evaluation of recent human studies on BPA exposure and immune effects

Since the previous EFSA review (2010), six human studies on possible effects of BPA on the immune system were published (Clayton et al., 2011; Savage et al., 2012; Spanier et al., 2012; Vaidya et al., 2012 and Donohue et al., 2013). A detailed description and evaluation of each study is provided separately in Appendix B.

In the study of Clayton et al. (2011), a survey and laboratory data from the 2003–2006 US NHANES were used to evaluate possible associations of urinary BPA levels with serum cytomegalovirus (CMV) antibody levels and diagnosis of allergies or hay fever in US adults and children > 6 years of age. The exposure was assessed by measuring BPA in spot urines. It is not clear at what time points BPA levels, CytomegaloVirus (CMV) titres and allergies were assessed. In analyses adjusted for other possible confounders, in the ≥ 18-year age group, higher urinary BPA levels were associated with higher CMV antibody titers. In the < 18-year age group, lower levels of BPA were associated with higher CMV antibody titers. BPA showed no association with allergy or hay fever diagnosis. The authors do not offer an explanation for these contrasting observations, and conclude that additional studies should be done to further investigate these findings.

Spanier et al. (2012) examined prenatal BPA exposure and childhood wheeze from birth to 3 years of age in 365 mother – child pairs. The exposure was assessed by measuring BPA in spot urines from mothers at 16 and 26 weeks of gestation and at birth. The results were mainly negative: when prenatal BPA exposure was modelled as a continuous variable (mean of three values), BPA was not related

with childhood wheeze. When urinary BPA was categorized above or below the median value, a significant positive relationship with wheeze was found at six months of age, but there was no evidence of a persistent positive association by three years of age. The CEF Panel considers this categorisation of BPA exposure to be questionable and notes that exposure to BPA after birth was not considered.

In a study by Savage et al. (2012), data were obtained from the NHANES study (survey 2005-2006) in which urinary bisphenol A, triclosan, benzophenone-3, propyl, methyl, butyl, and ethyl parabens were correlated with specific aeroallergen and food allergen sensitisation in 860 children aged 6-18 years. The exposure was assessed by measuring in randomly selected urines samples. Serum IgE levels were determined according the Phadia ImmunoCAP system for an array of allergens. A subject was considered to have aeroallergen or food-specific sensitisation if one specific IgE level was 0.35 kU/L or greater. Atopic asthma was defined as having doctor-diagnosed asthma and a positive test for at least one specific IgE. In contrast to triclosan and propyl and butyl parabens, no associations between urinary BPA levels and sensitisation were observed.

In contrast to the study by Savage, but also based on the NHANES database (survey 2005-2006), Vaidya et al. (2012) claimed that urinary BPA is significantly associated with allergic asthma in females. Spot urine samples were used to estimate total BPA concentration of eight phenols (including BPA) and parabens, and were quantified in 2548 survey participants aged 6 years and over. Outcome measures included asthma-related questions, total immunoglobulin E (IgE), and 19 allergen-specific IgE levels. Allergic asthma was defined as a history of ever having asthma, high eosinophil count, and high total IgE or atopy. BPA was associated with a higher likelihood of allergic asthma in females but not in males.

Donohue et al. (2013) examined possible associations of urinary BPA levels with wheeze and asthma in a prospective cohort of 568 low-income mothers and children in inner-city New York. Higher maternal urinary BPA concentration during 3rd trimester of pregnancy was associated with lower occurrence of wheeze at age 5 years, but not with any other outcome measures or time points. However, longitudinal analyses of childhood/postnatal BPA concentrations showed that higher BPA values were associated with increased occurrence of wheeze and/or asthma at several ages.

3.5.1.3. Summary of BPA exposure and immunotoxic effects in humans

For the 2010 EFSA CEF Panel opinion no human studies were available regarding immunotoxic effects of BPA exposure in humans. Since then, five studies (Clayton et al., 2011; Savage et al., 2012; Spanier et al., 2012 ; Vaidya et al., 2012 and Donohue et al., 2013) were published. Clayton et al. (2011) found inconsistent associations between BPA exposure and CMV antibody titres in subjects older than 6 years. In three studies (Clayton et al., 2011; Savage et al., 2012 and Spanier et al., 2012), no associations of either prenatal BPA exposure or exposure at later age stages with allergy, hay fever and wheeze were observed. In contrast, Vaidya et al.(2012) noted an association of urinary BPA levels with allergic asthma, and Donohue et al. (2013) found associations between higher postnatal, but not prenatal, BPA and increased wheeze and asthma. Based on these studies, there are indications that BPA may be linked to immunological outcomes in humans, although in view of the limitations of the studies only limited conclusions can be reached and it cannot be ruled out that the results are confounded by diet or concurrent exposure factors. The associations do not provide sufficient evidence to infer a causal link between BPA exposure during pregnancy or in childhood and immune effects in humans.

3.5.2. Animal studies

3.5.2.1. Summary of previous evaluations

EU-RAR (2003, 2008)
As indicated earlier the EU-RAR (2003, 2008) concluded that BPA is a very weak skin sensitiser, being able to sensitise through the skin of mice only at concentrations higher than 30%. The assessment also concluded that it is unlikely that BPA in foodstuffs poses a risk of skin sensitisation.

EFSA (2006); EFSA CEF Panel (2010)
The 2006 EFSA opinion did not include any study addressing the immune effects of BPA in laboratory animals.

In 2010, EFSA considered that several studies had reported changes in cytokines, changes in T-cell populations and other aspects of immune modulation. However, all the studies suffered from shortcomings in experimental design and reporting. Therefore, the CEF Panel concluded that these studies could not be taken into consideration for derivation of a TDI.

NTP-CERHR (2008)
Only one new animal study (Yoshino et al., 2004) indicating that prenatal exposure to BPA may upregulate immune responses in mice was reviewed in this monograph. However, the study was considered as inadequate for the evaluation process due to weaknesses.

FAO/WHO (2011) noted that in utero exposure or exposure at adult age yielded various indications of immunomodulation in rodents, such as altered cytokine expression, nitric oxide synthesis by macrophages, TNF-α secretion, well as histopathological effects on thymus and spleen, and that these data indicate that BPA may modulate the immune system. Yet, the data were inconsistent and the expert group concluded that more studies need to be performed using standard protocols to conclude on potential adverse immune outcomes of BPA exposure.

ANSES (2011, 2013)
ANSES (2011) considered effects on cytokines, notably a shift in the immune response in the direction of a Th2 phenotype due to proliferation and activation on Th2-cells and production of cytokines as a proven effect. However, it is unknown whether these effects are relevant to humans.

ANSES did not discuss or bring forward immunotoxicity endpoints for risk characterisation in its 2013 risk assessment.

3.5.2.2. Evaluation of recent animal studies on BPA exposure and immune effects

Since the evaluations described above, four animal studies have been published (Lee et al., 2012; Kendziorski et al., 2012; Nakajima et al., 2012; Bauer et al., 2012). A detailed description and evaluation of each study is provided separately in Appendix B.

Lee et al. (2012a) studied adult female mice injected intraperitoneally with 5 mg BPA/kg bw per day for 4 weeks. The treatment resulted in increases in several non-specific inflammatory mediators and total levels of IgE. These effects were diminished or blocked in the presence of a glycoprotein derived from Cudrania tricuspidata Bureau (CTB), investigation of such an inhibitory effect being the main purpose of the study. The CEF Panel noted that only one concentration of BPA was used, no functional endpoints were investigated and the number of animals was relatively small.

Kendziorsky et al. (2012) administered BPA in the diet of CD1 and C57Bl mice (n= 5 per group) at levels of 0, 0.03, 0.3 or 30 mg/kg diet, (estimated to be equivalent to 4.5, 45 or 4500 µg/kg bw per day) from before mating, through gestation, parturition and weaning (in F0 females) and until weeks 19-23 (F0 females). 17α-ethinyl oestradiol (EE; 0.01, 0.1 or 1.3 mg/kg diet) was also administered to separate groups of mice. Reproductive performance was assessed and uterine pathology of the F0

females was investigated following sacrifice at weeks 19-23. The authors observed pyometra, i.e. inflammation in the uterus in a small minority of C57Bl mice receiving 0.3 mg BPA/kg diet, accompanied by changes in uterine morphology. A 5-fold, statistically significantly more pronounced presence of macrophages was observed in the uteri of all C57B1 females at this dose. Pyometra was also observed in the 15µg/kg-d EE treatment group, but no such changes were seen in CD1 mice. The authors concluded that BPA enhances immune responsiveness of the uterus and that heightened responsiveness in C57BL/6 females is related to increased susceptibility to pyometra.

Nakajima et al. (2012) exposed female Balb/c mice to 10 µg/ml BPA in their drinking water from one week prior to gestation until the end of the study on day 25 post partum. Pups were sensitised to ovalbumin at day 4 after birth and challenged at days 18, 19 and 20 after birth. Airway hyperreactivity to methycholine as well as inflammation by evaluating eosinophils in bronchoalveolar lavage were assessed in 22 day old pups. Pups exposed in utero or through mothers' milk in addition to in utero exposure showed increased airway hyperreactivity and increased eosinophil numbers in bronchoalveolar lavage fluid. Pups exposed only post-natally did not show such effects. The authors concluded that prenatal exposure to BPA, followed by postnatal allergic sensitisation and challenge, promoted the development of experimental allergic asthma. They suggested that delayed expression of BPA-metabolising enzymes may explain, at least in part, the enhanced fetal susceptibility.

Bauer et al. (2012) administered BPA by gavage to pregnant C57Bl/6 damns from gestation day 6 until post-natal day 21 to 0, 0.5, 50, or 500 µg BPA/kg/day. To induce allergic inflammation, adult offspring were mucosally sensitized with inhaled ovalbumin containing low dose lipopolysaccharide or intraperitoneally sensitized using ovalbumin with "alum" (not further specified by the authors), followed by ovalbumin aerosol challenge. In the mucosal sensitization model, female offspring maternally exposed to $\geq$ 50 µg BPA/kg/day displayed enhanced lymphocytic and lung inflammation, assessed by histopathology, compared to controls. This effect was evident in females but not males. There were no clear dose-related effects on cell numbers in broncholveolar lavages of the animals. Peritoneally sensitized female but not male mice maternally exposed to $\leq$ 50 but not 500 µg/kg showed a dampened effect on numbers of eosinophils in lung lavage, but there was no clear dose-response relationship. In the peritoneal model, there was no effect on airway hyperresponsiveness in females noted. Unfortunately, this clinical outcome was not measured in the more relevant mucosal model.

3.5.2.3. Summary of the immune effects of BPA in animals

The CEF Panel concluded that in the study of Lee et al. (2012a) non-specific inflammatory mediators and total levels of IgE were affected, but also noted that only one concentration of BPA was used, no functional endpoints were investigated and the number of animals was relatively small.

The CEF Panel notes that in the study of Kendziorski et al. (2012) the relevance of the macrophage infiltration in terms of pyometra is not clear, and the conclusion of the authors that BPA enhances immune responsiveness is speculative. These considerations do not take away from the fact that infiltration of macrophages may be adverse.

The CEF Panel considers that the study by Nakajima et al. (2012) showed enhancement of ovalbumin induced airway hyperreactivity to methycholine as well as increased numbers of eosinophils in bronchoalveolar lavage in pups exposed to BPA in utero and through mothers' milk. The CEF Panel notes that also the study by Bauer et al. (2012) indicated enhancement of ovalbumin-induced allergic responses, notably inflammation, by oral exposure to BPA, and that a dose-dependence was evident. The CEF Panel also noted that in this latter study the inflammation noted was seen in females but not males. It should be mentioned that elevated immune responses in female humans as well as female animals have been reported pereviously, including innate responses, cytokine responses, and vaccine responses (Klein et al., 2010, McLelland and Smith, 2011, Hochstenbach et al., 2012).

Whereas the studies lend support to the notion that immunological effects may be elicited by BPA, all these studies suffered from shortcomings in experimental design and reporting. Therefore, dose-response cannot be confidently established. It is currently not clear whether immunotoxicity is an endpoint of concern for BPA. The CEF Panel noted that this type of effect is insufficiently covered by current testing guidelines, and potential immunotoxicity therefore currently presents an uncertainty area in BPA risk assessment, deserving further consideration.

3.5.3. In vitro studies

One in vitro study (Pisapia et al., 2012) investigated the effect of several substances including BPA on the differentiation of bone marrow dendritic cells isolated from female mice and cultured in hormone-deficient medium. BPA at 10^{-7} M, 10^{-6} M and 10^{-5} M induced the differentiation of 62%, 70% and 91% of the cells to the CD11c+ phenotype, respectively. The CEF Panel noted that due to high BPA concentrations and the specific culture conditions the relevance of this finding for the in vivo situation is not clear.

3.5.4. WoE of immune effects of BPA in humans, animals and in vitro

Whether BPA induces immune effects was considered using a tabular format for weighing different lines of evidence (WoE evaluation). The overall outcome of this WoE evaluation is presented below, while the WoE evaluation tables for these endpoints are presented in full in Appendix C. For interpretation of these tables always refer to Appendix A

Table 10: Overall table on WoE evaluation of immunotoxic effects of BPA in humans and animals

Human studies	
Overall conclusion on the likelihood of association between BPA exposure and developmental immunotoxic effects: There are indications that BPA may be linked to immunological outcomes in humans, although in view of the limitations of the studies only limited conclusions can be reached and it cannot be ruled out that the results are confounded by diet or concurrent exposure factors. The associations do not provide sufficient evidence to infer a causal link between BPA exposure during pregnancy or in childhood and immune effects in humans.	**As likely as not**
Animal studies	
Overall conclusion on the likelihood of immunotoxic effects of BPA in animals: Evidence from the new studies adds to the indications of immunotoxicity of BPA in animals reported in previous reviews.	**From -as likely as not- to likely**

3.5.5. Conclusions on immune effects

Based on recent human studies, there are indications that BPA may be linked to immunological outcomes in humans, although these studies had limitations and confounding factors may have been present. A causal link between BPA exposure during pregnancy or in childhood and immune effects in humans cannot be established.

Studies in animals lend support to the possibility of immunological effects of BPA. Most of these studies suffered from shortcomings in experimental design and reporting. Although dose-responses could not be confidently established in most studies, a dose-related effect was observed in allergic lung inflammation.

Using a WoE approach, the CEF Panel assigned a likelihood level of "-as likely as not- to likely" to immunotoxic effects of BPA. Since the likelihood level for this endpoint is less than "likely" (see Appendix A), this endpoint was not taken forward for assessing the toxicological reference point, but

was taken into account in the evaluation of uncertainty for hazard characterisation and risk characterisation (Section 4.3).

3.6. Cardiovascular effects

3.6.1. Human studies

3.6.1.1. Summary of previous opinions

Studies on obesity and metabolic effects, which may also be linked to cardiovascular outcomes, are evaluated in a separate section (3.7). The number of epidemiological studies on cardiovascular outcomes and BPA exposure in previous reviews has been limited. The outcomes of these reviews is summarised as follows:

EU-RAR (2003, 2008)
No cardiovascular effects linked to BPA exposure were reported in the EU-RAR

EFSA (2006, 2008, 2010)
No cardiovascular effects linked to BPA exposure were reported in the EFSA opinions of 2006 or 2008.

In its 2010 opinion EFSA evaluated two human studies from the National Health and Examination Survey (NHANES), which reported associations between BPA exposure (urine) and cardiovascular effects (Lang et al., 2008; Melzer et al., 2010). Lang et al. used NHANES data from 2003/04 and found that higher BPA concentrations in urine were associated with diabetes and cardiovascular diagnoses, but not with other common diseases. Melzer et al. used NHANES data from 2005/06, and found that in those years, BPA levels were lower than they had been in 2003/04. Both studies were cross sectional, were based on single spot urine samples and self-reported disease. The CEF Panel concluded that further (prospective and/or animal) studies would be needed to demonstrate the biological plausibility of these findings and to explain the potential underlying mechanism of action. A minority opinion stated that "Human studies on the relation between BPA and cardiovascular diseases (Lang et al., 2008 and Melzer et al. 2010) provide some indication of possible relevance of metabolic effects to humans."

FAO/WHO (2011)
The FAO/WHO report from the expert meeting held in 2010 also evaluated two studies regarding BPA exposure and cardiovascular effects (Lang et al., 2008; Melzer et al., 2010) and stated that it was not possible to draw any conclusions based on these cross-sectional analyses.

ANSES (2011, 2013)
The 2011 ANSES report considered the observed correlations between the highest urinary levels of BPA and cardiovascular pathologies (coronary diseases) and diabetes to be indicative of a *suspected* effect, based on the cross-sectional study by Melzer at al. (2010). ANSES did not however take this endpoint forward in its risk assessment of 2013, as it focussed on those effects which would enable toxicological benchmark doses to be determined, and cardiovascular pathologies were not one of these.

3.6.1.2. Evaluation of recent human studies on BPA exposure and cardiovascular effects

Since the previous EFSA review (2010), nine studies have been evaluated (Lind and Lind, 2011; Melzer et al., 2012a, b; Olsén et al., 2012a; ; Lakind et al., 2012; Teppala et al., 2012; Shankar & Teppala, 2012; Shankar et al., 2012a; Bae et al., 2012). A detailed description and evaluation of each study is provided separately in Appendix B.

The studies have been grouped into BPA effects on 1) coronary artery disease (Lind & Lind., 2011; Melzer et al., 2012a; Olsén et al., 2012a; Melzer et al., 2012b; Lakind et al., 2012), and 2) metabolic

syndrome, hypertension and peripheral artery disease (Teppala et al., 2012; Shankar and Teppala., 2012; Shankar et al., 2012a; Bae et al., 2012), although these endpoints also overlap.

1) BPA effects on coronary artery disease/heart attack

Meltzer et al. (2012a) used a prospective nested case-control design and analysed data for 758 cases and 861 controls from a 10 year follow up of the EPIC-Norfolk cohort in the UK. The results showed that higher urinary BPA concentrations were associated with increased risk of developing coronary artery disease. The longitudinal design increases the weight of the study results as compared to results from cross sectional studies. Furthermore, this study is the first to report an association between BPA exposure and cardiovascular outcome in a European population. However, the case definition only included patients admitted to hospital. Furthermore, confounding by diet was not considered.

In a cross-sectional study among 1016 elderly men and women in Sweden, Lind and Lind (2011) studied associations between serum total BPA and four phthalates and carotid atherosclerosis. Serum BPA concentration was not associated with carotid plaque prevalence or intima-media thickness, but was associated with echogenicity of the plaques and intima-media ($p<0.001$).

In a cross-sectional analysis, Olsén et al. (2012b) examined associations between serum BPA and phthalate concentrations and CVD (cardio-vascular disease) risk factors, defined by the Framingham risk score, in the same study population of elderly men and women as in the study by Lind and Lind (2011). Serum total BPA was not associated with coronary risk defined by the Framingham risk score.

In a cross-sectional study in the UK comprising 591 patients with suspected cardiovascular risk, Melzer et al. (2012b) investigated urinary BPA concentrations and cardiovascular disease based on angiography-defined coronary artery stenosis. Higher BPA urinary concentrations were seen in patients with severe coronary artery stenosis in comparison to those with no vascular disease.

Lakind et al. (2012) conducted a re-analysis of more than 4800 participants across four available NHANES data sets to investigate associations between BPA exposure and chronic diseases using scientifically and clinically supportable exclusion criteria and outcome definitions. Coronary heart disease and/or heart attack were among the outcomes examined in the re-analysis. All analyses were adjusted for creatinine, age, gender, race/ethnicity, education, income, smoking, heavy drinking, BMI, waist circumference, calorie intake, family history of heart attack, hypertension, sedentary time, and total cholesterol. When the *a-priori* selected methods were used to address the research question, no associations were found between urinary BPA and heart disease and diabetes. The authors concluded that the discrepancy between their findings on cardiovascular disease and those reported previously, was in part attributable to differences in exclusion/inclusion criteria.

2) BPA effects on metabolic syndrome, hypertension and peripheral artery disease

Studies reporting metabolic syndrome effects of BPA linked with hypertension and cardiovascular disease have been included in this section rather than (or as well as) under metabolic effects (Section 3.7), since hypertension and other factors included in the definition of the metabolic syndrome are clearly relevant to this section on cardiovascular effects of BPA.

Significant associations between urinary BPA and metabolic syndrome and hypertension have been reported in four studies (Teppala et al., 2012; Shankar and Teppala, 2012; Shankar et al., 2012a; Bae et al., 2012). Shankar and Teppala (2012) and Shankar et al. (2012a) used data from NHANES 2003/4, while Teppala et al. (2012) used data from NHANES 2003-2008. These studies were cross sectional and reported that in comparison to subjects ranked in the lower tertile of urinary BPA, those in the upper tertile had increased risk of metabolic syndrome (Teppala et al. 2012), hypertension (Shankar and Teppala, 2012) and peripheral arterial disease (Shankar et al., 2012a). A fourth study (Bae et al., 2012) was a cross-sectional study in a population of elderly citizens in Seoul, Korea.

Although a significant association between urinary BPA levels and hypertension was described, the clinical/pathological significance is doubtful.

3.6.1.3. Summary of BPA exposure and cardiovascular effects in humans

In their 2010 EFSA opinion, the CEF Panel concluded that the studies available at the time were too limited to draw a conclusion regarding BPA exposure and cardiovascular effects in humans. This conclusion was based on two cross-sectional studies reporting associations between BPA exposure and cardiovascular (and metabolic) outcomes. Since then, several additional studies have examined BPA in relation to cardiovascular effects, but all studies except one, were cross-sectional and thus unsuitable to study exposure-disease associations on their own. The reanalysis carried out by Lakind et al. (2014) does not support the causal inferences suggested in other studies. There are indications from one prospective study that BPA exposure may be associated with cardiovascular effects, but it cannot be ruled out that the effect is confounded by diet or other factors. This association does not provide sufficient evidence to infer a causal link between BPA exposure and cardiovascular effects in humans.

3.6.2. Animal studies

3.6.2.1. Summary of previous opinions

Previous evaluations of BPA (EU-RAR, 2003, 2008; EFSA, 2006; NTP-CERHR, 2008; FAO/WHO, 2011; ANSES, 2011, 2013; EFSA CEF Panel, 2010) have not included any studies on cardiovascular effects or cardiotoxicity of BPA in experimental animals.

3.6.2.2. Evaluation and conclusions of recent animal studies on cardiovascular effects of BPA

One recent study on cardiovascular effects in animals was identified in the literature, undertaken to examine the effects of repeated and acute exposure to BPA on cardio-respiratory reflexes elicited by phenylbiguanide (PBG) (Pant et al., 2012). The authors demonstrated that female adult rats fed BPA in the diet (2 µg/kg body weight, n=6) showed an attenuation of PBG-induced cardiac and respiratory frequency changes (bradycardia, hypotension and tachypnoea) compared with controls. Acute exposure of animals to BPA also attenuated PBG-induced cardiac responses significantly, while the effect on respiratory rate was identical to controls. The attenuation of the PBG reflex responses by BPA in acute experiments was associated with decreased vagal afferent activity. The authors suggested that BPA may attenuate protective cardio-respiratory reflexes due to decreased vagal afferent activity. The CEF Panel considered that the PBG model does not contribute to the understanding of BPA effects in humans, and also noted that in the acute experiment suggesting an influence of BPA on vagal nerve afferent activity an extremely high dose of BPA (35 mg/kg bw) was given intravenously.

3.6.3. In vitro studies

Two recent in vitro studies (Yan et al., 2011; Belcher et al., 2012) were performed with isolated rodent ventricular myocytes to investigate the arrhythmogenic effects of E2 and BPA. Rapid effects of BPA and E2 (10^{-12} – 10^{-6} M) on contractility and Ca2+ signalling were observed in myocytes from female but not in male rats. In addition the effects were shown to be ERβ-dependent. However, in contrast to the effects in isolated myocytes the induction of arrhythmia by BPA or E2 was observed in perfused rat hearts only in the presence of a β-adrenergic agent (isoproterenol). The discrepancy between the in vitro and organ experiments indicate that the BPA induction of arrhythmia in vivo might be relevant only under specific conditions, e.g. stress.

Another study (O'Reilly et al., 2012) investigated BPA (10^{-7} – 10^{-4} M) effects on the voltage gated Na^+ channels. BPA-induced blockage of the channel was observed only at and above 10^{-6} M and, therefore, was not considered to be relevant for risk assessment.

3.6.4. WoE of cardiovascular effects of BPA in humans

Whether BPA induces cardiovascular effects was considered using a tabular format for weighting different lines of evidence (WoE evaluation). The overall outcome of this WoE evaluation is presented below, while the WoE evaluation tables for these endpoints are presented in full in Appendix C. For interpretation of this table always refer to Appendix A. No WoE analysis was carried out for the one animal study that was evaluated by the CEF Panel.

Table 11: Overall table on WoE evaluation of cardiovascular effects of BPA in humans

Human studies	
Overall conclusion on likelihood of cardiovascular effects of BPA in humans: There are indications from one prospective study that BPA exposure may be associated with cardiovascular effects, but it cannot be ruled out that the effect is confounded by diet or other concurrent exposure factors. This association does not provide sufficient evidence to infer a causal link between BPA exposure and cardiovascular effects in humans. Potential effects are considered to be as likely as not.	**As likely as not**

3.6.5. Conclusions on cardiovascular effects

In their 2010 EFSA opinion, the CEF Panel concluded that the studies available at the time were too limited to draw a conclusion regarding BPA exposure and cardiovascular effects in humans. Since then, several additional studies have examined BPA in relation to cardiovascular effects, but all studies except one, were cross-sectional and thus unsuitable to study BPA exposure-disease associations on their own.

There are indications from one prospective study that BPA may be associated with such effects but overall a causal link between BPA exposure and cardiovascular effects in humans cannot be established.

There are insufficient animal data to suggest that BPA has an effect on cardiac function or causes cardiotoxicity.

Using a WoE approach, the CEF Panel assigned a likelihood level of "as likely as not" to cardiovascular effects of BPA. Since this likelihood level is less than "likely", this endpoint was not taken forward for risk characterisation. It cannot be ruled out that the observed association between exposure to BPA and cardiovascular effect in the epidemiological studies is confounded by diet or other concurrent exposure factors. This association does not provide sufficient evidence to infer a causal link between BPA exposure and cardiovascular effects in humans. Since there is also a lack of information on the dose-relationship for this association, and since there were only very limited number of animal studies on this endpoint, this endpoint was not included in the uncertainty analysis.

3.7. Metabolic effects

3.7.1. Human studies

3.7.1.1. Summary of previous opinions

The number of epidemiological studies on hormonal or metabolic outcomes and BPA exposure in previous reviews was limited. Results were reported in studies of cardiovascular outcomes. The outcome of these reviews is summarised as follows:

EU-RAR (2003, 2008)
The EU-RAR reports do not describe human studies addressing metabolic effects or weight gain.

EFSA (2006); EFSA CEF Panel (2010)
No metabolic effects linked to BPA exposure were reported in the EFSA opinions of 2006 or 2008.
The EFSA opinion of 2010 included evaluation of two human studies from the USA National Health and Examination Survey (NHANES), which reported associations between BPA exposure in urine and diabetes and cardiovascular conditions (Lang et al., 2008; Melzer et al., 2010). Lang et al. used NHANES data from 2003/04 and found that higher BPA concentrations in urine were associated with diabetes and cardiovascular conditions, but not with other common diseases. Melzer et al. used NHANES data from 2005/06, and found that in those years, BPA levels were lower than they had been in 2003/04. Regarding diabetes: the association between BPA and diabetes was significant in pooled data (2003-06), but did not reach significance with the data from 2005/06 alone. Both cross-sectional studies were based on single spot urine samples and self-reported diabetes and other outcomes. The CEF Panel concluded that further (prospective and/or animal) studies would be needed to demonstrate the biological plausibility of these findings and to explain the potential underlying mechanism of action.

NTP-CERHR (2008)
The NTP-CEHR (2008) report did not assess human studies addressing metabolic effects or weight gain.

FAO/WHO (2011)
The FAO/WHO report from the expert meeting also evaluated the two studies regarding BPA exposure and metabolic and cardiovascular effects (Lang et al., 2008; Melzer et al., 2010) and stated that it was not possible to draw any conclusions based on these cross-sectional analyses.

ANSES (2011, 2013)
The 2011 ANSES report considered the observed correlations between the highest urinary levels of BPA and cardiovascular pathologies and diabetes reported by Lang et al. (2008) and Melzer et al. (2010) to be indicative of a *suspected* effect. In the 2013 opinion ANSES did not include further human epidemiological studies.

3.7.1.2. Evaluation of recent human studies on BPA exposure and metabolic effects and hormonal disorders

This section provides an overview of the human studies on metabolic effects and hormonal disorders published after July 2010. Although cardiovascular and metabolic outcomes are interrelated, cardiovascular outcomes are evaluated in a separate section together with relevant papers on metabolic syndrome.

Since the previous EFSA review (2010), 24 studies have been evaluated (Galloway et al., 2010; Brucker-Davis et al., 2011; Carwile and Michels. 2011; Chou et al., 2011; Ning et al., 2011; Shankar and Teppala, 2011; Silver et al., 2011; Shankar et al., 2012b; Trasande et al., 2012; Wang et al., 2012a, b, c; Zhao et al., 2012; Bhandari et al., 2013; Eng et al., 2013; Harley et al., 2013b; Kim and Park, 2013; Lakind et al., 2012; Li et al., 2012a, 2013; Mendez and Eftim, 2012; Volberg et al., 2013; You et al., 2011; Teppala et al., 2012). The studies evaluated in this section have been grouped according to 1) obesity, 2) endocrine/hormonal outcomes, 3) diabetes, and 4) other outcomes. Some of these studies addressed more than one metabolic endpoint and/or other toxicological endpoints (e.g. cardiovascular effects). Therefore, they are discussed more than once in the sections below, as well as in other parts of this opinion. A detailed description and evaluation of each study is provided separately in Appendix B.

1) BPA effects on obesity

Ten studies examined associations between BPA exposure and obesity or obesity-related measures as main outcomes, five in adults (Galloway et al., 2010; Carwile and Michels, 2011; Shankar et al., 2012b; Wang et al., 2012a; Zhao et al., 2012;) and six in children and adolescents (Trasande et al.,

2012; Wang et al., 2012b; Bhandari et al., 2013; Eng et al., 2013; Harley et al., 2013b; Li et al., 2013). All the studies were cross-sectional, but Harley et al. (2013b) included both cross-sectional and prospective analyses.

Shankar et al. (2012b) used NHANES data from years 2003- 2008 for 3967 participants aged 20 years or more. The association between urinary levels of BPA and obesity, as defined by BMI and waist circumference, was investigated. A positive association was reported between increasing BPA concentrations (in quartiles) and obesity defined by BMI and waist circumference, independently of confounding factors. Carwile and Michels (2011) used data for 2747 adults from the 2003/04 and 2005/06 NHANES, and Wang et al. (2012a) used data for 3390 Chinese adults in Shanghai. Both studies reported that higher urinary BPA excretion was associated with general and central/abdominal obesity. Notably, both the Shankar et al. (2012b) and Carwile and Michels (2011) studies used NHANES data, thus raising the question as to whether they report the same association or they are independent.

Galloway et al. (2010) examined associations between 24-hour urinary BPA excretion and serum sex hormone concentrations in 715 Italian adults (inCHIANTI cohort). This study reported BPA associations with covariates including parameters indicative of obesity. Higher BPA excretion was associated with increasing waist circumference and weight, but not with overweight or obesity defined by BMI cut-offs as defined by the World Health Organization.

Zhao et al. (2012) examined urinary BPA exposure and body composition, hormone levels and bone mineral density in a cross-sectional study in 246 healthy premenopausal women in Shanghai. BPA exposure was not associated with bone mineral density, but weak associations were reported for BPA and body weight, BMI, fat mass and serum leptin.

Trasande et al. (2012) used NHANES data from years 2003-2008 for 2838 children and adolescents aged 6-19 year. Children/adolescents in the lowest quartile of urinary BPA had lower estimated prevalence of obesity than those in quartiles 2, 3 and 4. Similar patterns of association were found in multivariable analyses when BPA was modelled as a continuous variable and BMI z-scores as the outcome. In stratified analysis, significant associations between urinary BPA and obesity were found among whites, but not among blacks or Hispanics. Bhandari et al. (2013) studied urinary BPA and obesity using the same data as Trasande, but with a slightly lower sample size, comprising 2,200 children and adolescents aged 6-18 years with complete data on all covariates. Obesity was defined as the ≥95th percentile of body mass index specific for age and sex. This study also found that higher urinary BPA was associated with obesity; compared with children in the lowest quartile of BPA, children in the highest quartile had a multivariable or for obesity of 2.55 (95% CI: 1.65, 3.95) (P trend < 0.01). The observed positive association was predominantly present in boys and in non-Hispanic whites. Wang et al. (2012b) used data for 259 children and adolescents in Shanghai and reported significant associations between urinary BPA excretion and obesity. Li et al. (2013) examined urinary BPA and obesity in 1,326 school aged children in Shanghai. The habitual diet was assessed by a food frequency questionnaire, and 4 dietary quality indicator variables ("unhealthy diet", "eating junk foods", "eating vegetables", "eating fruits") were included among confounding variables. Increasing urinary BPA was significantly associated with higher risk of overweight in girls aged 9–12 years only, while no association was seen for BMI for girls or boys. Harley et al. (2013b) examined both prenatal (maternal urine) and postnatal (childhood urine) BPA exposure in relation to body mass index in 311 children aged 5 to 9 years in the CHAMACOS cohort. The cross-sectional analysis at age 9 years showed that higher urinary BPA was associated with increased BMI, waist circumference, fat mass, and overweight/obesity in boys and girls. Contrary to the cross-sectional results, the longitudinal analysis showed that higher prenatal exposure (urinary BPA concentration in mothers during pregnancy) was associated with lower BMI, body fat, body weight and obesity occurrence in their daughters at age 9 years. Among girls, being in the highest tertile of prenatal BPA concentration was associated with decreased BMI Z-score (β = -0.47, 95% CI: -0.87, -0.07) and percent body fat (β = -4.36, 95% CI: -8.37, -0.34) and decreased odds of overweight/obesity (OR) = 0.37, 95% CI: 0.16,

0.91) compared to girls in the lowest tertile. These findings were strongest in pre-pubertal girls. Urinary BPA concentration at age 5 years was not associated with any anthropometric parameters at age 5 or 9 years.

In a cross-sectional analyses using NHANES data from 2003-2010 Eng et al. (2013) examined urinary BPA and BMI in 3370 children and adolescents aged 6-18 years in relation to BMI, waist circumference and body fat. In line with previous cross-sectional studies from NHANES higher urinary BPA concentration was associated with obesity (BMI>95%). The study population is the same as that in Trasande et al. (2013). No associations between urinary BPA and laboratory measures of cardiovascular or diabetes risk were found.

2) BPA effects on endocrine/hormonal outcomes

Five cross-sectional studies reported endocrine/hormonal outcomes (Galloway et al., 2010; Brucker-Davis et al., 2011; Chou et al., 2011; Mendez and Eftim, 2012; Wang et al., 2012c) and one prospective study (Volberg et al., 2013). Four used total urinary BPA and two used cord blood BPA as a measure of the exposure. Galloway et al. (2010) found a weak association between higher urinary BPA excretion and increased free testosterone (but no other sex hormones in men), and no associations with sex hormones in women.

Brucker-Davis et al. (2011) conducted a cross-sectional analysis of unspecified BPA in cord blood and cord blood thyroid tests in 54 male infants in France. BPA was not associated with free thyroxine or free triiodothyronine, but weakly associated (not statistically significant) with lower thyroid stimulating hormone (TSH). Chou et al. (2011) examined the relationship between unspecified BPA in maternal blood and umbilical cord blood in 97 mother-child pairs in Taiwan and that higher BPA was associated with high leptin and low adiponectin in cord blood. The BPA measurement in maternal and/or cord blood is not considered a valid measure due to the pervasive contamination from plastic.

Mendez and Eftim (2012) examined the association between urinary BPA exposure and total thyroxine in 1887 subjects in the 2007-2008 NHANES and found no association.

A study in 28 workers from two epoxy-resin factories, professionally exposed to BPA reported associations between urinary BPA excretion and clinically abnormal thyroid hormone concentrations (Wang et al., 2012c). However, relevant confounding factors such as worker co-exposure to other chemicals cannot be excluded.

Volberg et al. (2013) examined associations between prenatal BPA exposure (maternal urinary BPA concentrations during pregnancy) and plasma leptin and adiponectin concentrations in boys and girls at age 9 years in 188 mother-children pairs in a Mexican-American prospective cohort in Salinas Valleys, USA. Higher maternal BPA concentrations during late pregnancy were associated with increased plasma leptin in boys and with increased plasma adiponectin in girls. Associations were adjusted for relevant confounders including fast food and sweet snack consumption at 9 years. No associations between concurrent BPA concentrations and 9 year old child adiponectin or leptin levels were observed.

3) BPA effects on diabetes outcomes

Five studies examined urinary BPA and diabetes outcomes (Ning et al., 2011; Shankar and Teppala, 2011; Silver et al., 2011; Lakind et al., 2012; Kim and Park, 2013). All were cross sectional by design and relied on spot urine BPA exposure assessment. The study by Wang et al. (2012a) mentioned above found that in addition to being associated with increased prevalence of obesity, higher urinary BPA was also associated with increased prevalence of insulin resistance in 3390 Chinese adults aged 40 years or older. Ning et al. (2011) studied 3423 Chinese adults and defined type-2 diabetes from fasting- and 2-h glucose tolerance test and serum insulin levels. Increased risk of type-2 diabetes was

seen for participants in the second and fourth BPA quartiles, but not in the third. A study in 1210 nationally representative Korean adults aged 40-69 years found no association between urinary BPA and self-reported type-2 diabetes (Kim and Park, 2013).

Two cross-sectional studies used NHANES data Shankar and Teppala, 2011; Silver et al., 2011). Shankar and Teppala (2011) examined 3967 adults in pooled data from 2003 to 2008 and examined type-2 diabetes diagnosed by fasting glucose levels and glycosylated haemoglobin according to the latest American Diabetes Associations guidelines. The risk of type-2 diabetes increased with increasing quartiles of BPA in a dose-dependent manner.

Silver et al. (2011) examined 4389 adults and also used pooled data from 2003 to 2008, but defined diabetes 2 as glycosylated haemoglobin ≥6.5% or if participants used diabetic medication. A weak association between BPA and type-2 diabetes mellitus (T2DM) was seen in 2003-08 pooled data. Breaking down by year, the association was only significant in 2003/04, not 2005/06 or 2007/08. Results were similar when glycosylated haemoglobin (HbA1c) was used as a continuous outcome.

It is unclear whether the studies by Silver et al. (2011) and Shankar and Teppala (2011) report the same association or are independent studies.Both studies used a population in which the association was already described before by Lang et al. (2008) and Melzer et al. (2012). Lakind et al. (2012) conducted a re-analysis of the associations between BPA exposure and chronic disease outcomes, including diabetes, using four available NHANES data sets, including the same data used in the studies above. Scientifically and clinically supportable exclusion criteria and outcome definitions were applied. All analyses were adjusted for creatinine, age, gender, race/ethnicity, education, income, smoking, heavy drinking, BMI, waist circumference, calorie intake, family history of heart attack, hypertension, sedentary time, and total cholesterol. When the a-priori selected methods were used to address the research question, no associations were found between urinary BPA and diabetes. The authors concluded that the discrepancy between their findings with regard to diabetes and those reported previously (Lang et al., 2008; Melzer et al., 2010) was largely explained by the choice of case definition. The Lakind et al. (2012) study did not support the associations and causal inferences that were suggested in the previous studies, and highlighted that data from cross-sectional studies like NHANES surveys are inappropriate for drawing conclusions about relations between short-lived environmental chemicals and chronic diseases.

4) BPA effects on other outcomes

Li et al. (2012a) examined associations between urinary BPA and low grade albuminuria in the same Chinese population that was used to examine obesity (Wang et al., 2012a) and diabetes (Ning et al., 2011). A weak association for low grade albuminuria with higher urinary BPA was reported but the clinical relevance of this is not clear. A study in the NHANES examined whether urinary excretion of BPA differed by renal function in subjects without known renal dysfunction (You et al., 2011). Urinary excretion of BPA decreased with decreasing renal function, but the association was weak and the clinical relevance unclear. Also using cross-sectional data from NHANES 2003-2008, Teppala et al. (2012) found that in comparison with subjects ranked in the lower tertile of urinary BPA, those in the upper tertile had increased risk of having metabolic syndrome. Metabolic syndrome was defined by the presence of at least 3 of 5 criteria; abdominal obesity, hypertension, elevated serum triglycerids, glucose intolerance and reduced HDL.

3.7.1.3. Summary of BPA exposure and metabolic and hormonal effects in humans

In their 2010 EFSA opinion, the CEF Panel concluded that the studies available at the time were too limited to draw a conclusion regarding BPA exposure and metabolic and hormonal effects. This conclusion was based on two cross-sectional studies reporting associations between BPA exposure and metabolic (and cardiovascular) outcomes. Since then, several additional studies have examined BPA in relation to metabolic and hormonal effects, but the majority of all new studies are cross-sectional and thus not suitable to study exposure-disease associations. The metabolic disorders

associated with BPA exposure are suggested to be causally linked to poor diets – usually too much sugar, fat and processed food. As diet is the main source of BPA, an obvious possibility is that poorer diets are associated with higher exposure to BPA. One prospective study found that higher BPA concentration in maternal urine during pregnancy was associated with lower measures of obesity in their daughters, and in a second study within the same study population maternal urinary BPA was also associated with plasma adiponectin levels in 9-year old boys and girls, corroborating the BMI findings. In view of the limitations of using urinary BPA concentrations as a surrogate of exposure, the problems of interrelated dietary exposures, mostly cross-sectional designs and inconsistency of the results between cross-sectional and prospective studies, the conclusions that can be drawn concerning the relationship of BPA exposure and the reported findings are limited. Notwithstanding, there are indications from cross-sectional studies that higher BPA may be associated with increased body mass in children, and indication from a prospective study that prenatal BPA exposure may be associated with reduced body mass and lower plasma adiponectin levels in girls and with higher plasma leptin levels in boys. There are no indications of note for other hormonal or metabolic endpoints. A systematic literature review of the epidemiological literature on the relation of BPA with obesity and markers of glucose metabolism and diabetes concluded that assertions about a causal link between BPA and obesity or diabetes are unsubstantiated (Lakind et al., 2014).

3.7.2. Animal studies

3.7.2.1. Summary of previous opinions

EU-RAR (2003, 2008)

The EU-RAR of 2003, updated in 2008 (EU-RAR, 2003, 2008) did not report any information on metabolic effects or obesogenic effects of BPA in animals. Rather, a reduction in weight gain was identified with a LOAEL of 650 mg/kg bw per day in the 2-years NTP study with BPA in rats, although no such effect was reported in mice. In the EU-RAR (2008) in the 2-generation study (Tyl et al., 2005) a dose of 50 mg/kg bw per daywas set as the NOEL based on several endpoints, among them being reduced body weight gain.

EFSA (2006); EFSA CEF Panel (2010)

In 2006, EFSA did not report any studies showing effects of BPA on the metabolism of experimental animals. In 2010, EFSA reviewed a number of studies showing, variously, effects of BPA on insulin secretion in mice (Ropero et al., 2008), increased adipogenesis in the female offspring of rats exposed prenatally to BPA (mean oral dose 70 µg/kg bw per day) (Somm et al., 2009) and aggravated insulin resistance in mice during pregnancy at s.c. doses of 10 or 100 µg/kg/day (Alonso-Magdalena et al., 2010). EFSA also reviewed the study by Miyawaki et al. (2007) and concluded that the small sample size (n=3) invalidated the study. EFSA suggested that the metabolic effects of BPA could be due to interactions with peptide hormonal pathways as well as steroid metabolism and function. EFSA noted however that the study of Ryan et al. (2010b) showed no indications of increased susceptibility to high-fat diet-induced obesity and glucose intolerance in adult mice exposed prenatally to BPA at an oral dose of 0.25 µg/kg bw per day.

NTP-CERHR (2008)

The NTP-CERHR monograph cited 10 studies in which weight gain was not observed following exposure to BPA and five studies in which growth reduction was reported. It reviewed two studies in which endpoints related to carbohydrate or lipid regulation were evaluated, those of Alonso-Magdalena et al. (2006) and Miyawaki et al. (2007) and concluded that "*the data are currently too limited to conclude that developmental exposure to bisphenol A causes diabetes or other metabolic disorders later in life."* NTP-CERHR concluded however that BPA did not have an effect on obesity in experimental animals at doses less than 5000 µg/kg bw per day.

FAO/WHO (2011)

The FAO/WHO opinion reviewed the studies of Miyawaki et al. (2007), Somm et al. (2009), Alonso-Magdalena et al. (2010) and Ryan et al. (2010b). The Expert meeting reported that *"Findings from*

these studies include reports of glucose intolerance and hyperinsulinaemia in the 6-month-old male offspring of OF-1 mice treated with BPA at 0.01 or 0.1 mg/kg bw per day by subcutaneous injection from gestational day (GD) 9 to GD 16 (Alonso-Magdalena et al., 2010); adipocyte hypertrophy and increased mass of parametrial white adipose and brown adipose tissue on PND 21 in female offspring of Sprague-Dawley rats orally treated with BPA at 0 or approximately 0.07 mg/kg bw per day in drinking-water from GD 6 to PND 21 (Somm et al., 2009); and increased cholesterol on PND 31 in female offspring of ICR mice orally treated with BPA at approximately 0.26 or 2.6 mg/kg bw per day in drinking-water from GD 10 to weaning via the dam and then after weaning with the same drinking-water treatment as the dam (Miyawaki et al., 2007)". The opinion concluded that the available data suggest that further assessment of the potential effects of BPA on adiposity, glucose or insulin regulation, lipids and other end-points related to diabetes or metabolic syndrome is warranted.

ANSES (2011; 2013)

In 2011, the ANSES experts reviewed the same studies considered by FAO/WHO and EFSA in 2010 and also the study of Rubin et al. (2001), which had shown obesity in the offspring of Sprague-Dawley female exposed via drinking water, at approximately 0.1 mg BPA/kg bw per day (low dose) or 1.2 mg BPA/kg bw per /day (high dose) from GD6 throughout the period of lactation, persisting into adulthood. On this basis, they concluded that effects of BPA on lipogenesis in experimental animals (including adipocyte hypertrophy, predisposition to obesity, elevated cholesterol levels and triglyceride levels and overexpression of lipogenic proteins following pre-, peri-natal or adult exposure were proven. These effects, together with others, were considered as critical and were taken forward for risk assessment.

In the ANSES risk assessment of 2013, the increase in body weight in experimental animal studies together with increases in plasma lipids (such as cholesterol and triglycerides) and lipogenesis were retained as the critical effects. The ANSES opinion considered the Miyawaki et al. (2007) study in ICR mice to be the pivotal study for risk assessment, and derived a LOAEL of 0.26 mg/kg bw per day for BPA based on an increase in body weight and cholesterolemia in females.

3.7.2.2. Evaluation of animal studies on effects of BPA on metabolism (lipogenesis, obesity) or effects related to glucose or insulin regulation (diabetes)

Since the EFSA opinion of 2010, the WHO Expert meeting of 2010 and the ANSES report of 2011, several additional experimental studies have reported metabolic effects of BPA (including effects on body weight/obesity, lipogenesis or adipogenesis) and/or effects related to glucose or insulin regulation. These studies are summarised below, under the various endpoints, together with summaries of the relevant earlier studies (Miyawaki et al., 2007; Somm et al., 2009) that were considered by the CEF Panel to add to the overall body of evidence for effects in this emerging area. A detailed description and evaluation of each study is provided separately in Appendix B

Studies involving prenatal exposure

Increased body weight/body weight gain:

a) *Studies with BPA exposure alone*

Miyawaki et al. (2007) exposed mice to BPA in drinking water with doses corresponding to 0, 0.26, 2.72 mg/kg bw per day from GD 10 to PND 21. Body weights of female offspring were increased at the low and high dose group, body weights of the males at the high dose group. Adipose tissue weight was increased significantly in females at the low dose and in males at the high dose group. In the study of Somm et al. (2009) in rats receiving a dose of approximately 70 µg/kg bw per day from GD 6 until PND 21, body weight on PND 1 was increased in males and females whereas body weight and parametrial white fat tissue was increased only in females.

Anderson et al. (2013) exposed mice 2 weeks before mating, during gestation and lactation (PND 21) to 0, 50 ng, 50 µg or 50 mg of BPA/kg diet, corresponding to 0, 10.75 ng, 10.75 µg, and 10.75 mg/kg bw per day. One male and one female/litter were followed until 10 months of age, and were given standard diet or diets containing BPA at the same levels as administered to the dams. Increased oxygen consumption and carbon dioxide production was found in all BPA-treated animals. The CEF Panel noted however that the dose-response relationship was inconsistent. Spontaneous activity was increased only in females. Food consumption in females was significantly reduced but without a clear dose-response, whereas in males the reduction of food intake did not reach statistical significance. Body weight and body fat was not statistically different from control in either sex. In the study of Angle et al. (2013) pregnant mice were exposed to five BPA doses (5, 50, 500, 5 000 and 50 000 µg/kg bw per day) from GD 8 to GD 18. The multiple parameters measured showed an inconsistent pattern, with many effects seen on one or more of the parameters at a certain dose, without a corresponding effect on a second, pathophysiologically-related parameter. The interpretation of the results is not clear, in particular a unifying mode of action is lacking.

In the study of US FDA/NCTR (2013) Sprague-Dawley rats were treated with BPA administered by oral gavage from gestation day 6 through the start of labor. BPA was then given directly to pups from PND 1 until termination at PND 90 ± 5 at doses of 2.5, 8, 25, 80, 260, 840, 2 700, 100 000, and 300 000 µg/kg bw per day. The number of litters per dose group was 18-23. At the dose of 300 mg/kg bw per day several effects were noted which were similar to those of the positive control EE_2, such as preweaning body weight reduction (12 – 16% and 9 – 12% in females and males, respectively), reduced retroperitoneal fat pad (females only) on PND 90, and reduced body weight on day 90,

b) *Studies with BPA exposure combined with further intervention*

The publication of Somm et al. (2009) reported on a further treatment modality. Two groups of rats in this study were exposed to BPA from GD 6 until PND 21 and were then fed either with a normal diet or with a high fat diet from week 4 until week 14. The body weights were higher in both sexes than in the controls. However, the weeks in which body weights were higher were not identical in both sexes.

Xu et al. (2011b) hypothesised that obesity might be due to an increased preference of adult rats for a sweet taste, linked to prenatal and postnatal exposure to BPA. Female Sprague Dawley rats were exposed to BPA in drinking water at doses of 0.01, 0.1 and 1.0 mg/l from GD 11 to PND 21. All females including controls showed a preference for saccharin-containing drinking water compared with plain water, without a BPA treatment-related effect, whereas male offspring showed an increased preference for only 0.25% (but not for 0.5%) saccharin, and for 15% sucrose, compared with male controls. Male offspring from dams receiving 0.1 mg/l BPA and administered 15% sucrose in their drinking water postnatally also showed increased body weight gain compared with controls, the percentage of body fat was higher, as was their tail blood pressure. The drinking water consumption was not reported and hence no clear information on the BPA dose received is available. Further methodological flaws of the study are to be noted: the litter effects were not fully taken into consideration, the response to saccharin is inconsistent and it is unclear why only the mid dose group of BPA-exposed pups was chosen for the sucrose preference test and why only in this group the body weight was tested. The flaws limit the conclusions that can be drawn from this study.

Wei et al. (2011) administered doses of 0, 50, 250 or 1250 µg BPA/kg bw per day orally by gavage in corn oil to pregnant Wistar rats from GD 0 to PND 2. The offspring were maintained on either a normal or a high fat diet for 16 weeks. The authors only showed the full data set of results for the 50 µg/kg bw per day dose. Some data for the other doses were reported in the supplemental information. Offspring exposed prenatally to 50 µg BPA/kg bw per day and maintained on a normal diet showed increased weight gain from week 17 (females) or week 19 (males). Effects were more evident in animals fed a high fat diet. No effects of BPA were observed at doses of 250 or 1250 µg BPA/kg bw/ day. The amount of fat fed to the animals was twice the normal dietary intake of fat. As in humans the recommended intake of fat amounts to 40-45% of the total intake in newborns and 25-30% in adults

and adolescents, doubbling the intake would mean that newborns would take only 10-20% food other than fat and adults and adolescents would take only 40 -50 % of their daily food as non fat food. It is obvious that this is itself a very unhealthy diet. The statistical analysis was flawed; in particular, the choice to consider the litter size as a covariate in the ANCOVA analysis was not properly justified. The effect is seen only at the lowest dose.

In addition, there is no convincing evidence that BPA is obesogenic later in life in studies with intrauterine and subsequent long-term dosing.

In the study by MacKay et al. (2013) a normal or high-fat diet was given in adult life to the offspring of CD mice exposed from GD 1 and until PND 21 to diets containing 0, 1 or 20 µg BPA/kg feed, (equivalent to an average of 0.19 and 3.49 µg/kg bw per day prenatally and 0.36 and 7.2 µg/kg bw per day of BPA postnatally). Female offspring of dams receiving 20 µg BPA/kg feed which were fed a high fat diet as adults showed increased body weight gain compared with controls and also the DES positive control, and also ate more. Male offspring showed no similar BPA-linked effect on body weight gain. Males at both levels of BPA showed a dose-related increase in weight in the retroperitoneal and intrascapular brown adipose fat pads compared with control and DES-exposed mice, and similar effects were seen in female offspring at the higher dose but not at the lower level of BPA. The CEF Panel noted however that the magnitude of the effects reported was small and that the very high fat content of the feed (60% of the calories by fat) renders the interpretation of the results difficult.

Further endpoints:

Insulin
In the study of Wei et al. (2011) offspring exposed prenatally to 50 µg BPA/kg bw per day and maintained on a normal diet showed higher serum insulin levels at week 15 for males and week 26 for females but not at doses of 250 and 1 250 µg/kg bw per day. The effect at 50 µg/kg bw per day was even more pronounced in animals fed with a high fat diet.

In contrast, in the study of Anderson et al. (2013) no effects were seen on insulin release when offspring were exposed via their dams to doses between 10.75 ng, 10.75 µg, and 10.75 mg BPA/kg bw per day throughout gestation and via breast milk, and thereafter by diet until month 10. In the study of Angle et al. (2013) with doses of 5, 50, 500, 5 000 and 50 000 µg/kg bw per day, insulin in serum was higher than in controls only in the 5 µg/kg bw per day BPA group but not for 50, 500, and 50 000 µg/kg bw per day. Results for the 5 000 µg/kg bw per day group were not given. In the insulin tolerance test the glucose AUC was higher than in the controls in the 5 and 5000 µg/kg bw per day group, indicating impaired regulation.

No effects on insulin were observed in the US FDA/NCTR study (2013) with BPA doses of 2.5, 8, 25, 80, 260, 840, 2 700, 100 000 and 300 000 µg/kg bw per day.

Serum leptin
In the study of Miyawaki et al. (2007) serum leptin was increased only in females of the low dose group (0.26 mg/kg bw per day =260 µg/kg bw per day)).

Wei et al. (2011) administered doses of 0, 50, 250 or 1 250 µg BPA/kg bw per day orally by gavage in corn oil to pregnant Wistar rats from GD0 to PND 2. The offspring were maintained on either a normal or a high fat diet for 16 weeks. Serum leptin was elevated in the 50 µg BPA/kg bw animals compared with controls at week 26, but not in the groups with higher BPA doses. In the study of MacKay et al. (2013), with a BPA dose of 3.49 µg/kg bw per day prenatally and 7.2 µg/kg bw per day postnatally, females on a high-fat diet postnatally had increased leptin concentrations with reduced proopiomelanocortin mRNA expression in the arcuate nucleus and oestrogen receptor α expression patterns. Serum leptin in the study of Angle et al. (2013) was increased at 500 µg/kg bw per day but

lower than control at 50 and 50 000 µg/kg bw per day. A reduced serum leptin concentration was also measured in the US FDA/NCTR study (2013) at the highest dose (300 000 µg/kg bw per day).

It is to be noted that the CEF Panel in its assessment looked at the changes reported in every study; in none of them the observed changes have been discussed related to the results former study and under a unifying hyphothesis.

The CEF Panel considered that, based on the different dose-response of the observed effects in the various studies, the effect of BPA on serum leptin is unclear.

Glucose/Glucose tolerance

In the subgroups of male rats in the Somm et al. (2009) study that were exposed to BPA (70 µg/kg bw per day from GD6 to PND 21) and then fed either with a normal diet or with a high fat diet from week 4 until week 14, no effect of BPA exposure on glucose and glucose metabolism was found at week 14 with normal diet and also with high caloric fat diet. In the study by MacKay et al. (2013) male mice exposed to a dose of BPA of 3.49 µg/kg bw per day prenatally and 7.2 µg/kg bw per day of BPA postnatally showed impaired glucose tolerance on normal or high-fat diet.

In contrast, in the study of Anderson et al. (2013) in mice no effects were seen on glucose tolerance when offspring was exposed via their dams to doses between 10.75 ng, 10.75 µg, and 10.75 mg/kg bw per day BPA throughtout gestation and via breast milk, and thereafter by diet until month 10. In the study of Angle et al. (2013) in mice glucose tolerance test was impaired in all the doses (5, 50, 500, 50 000 µg/kg bw per day) with the exception of the highest dose (500 000 µg/kg bw per day).

In the study of US FDA/NCTR (2013) in Sprague-Dawley rats no effect of BPA on glucose was observed with doses of 2.5, 8, 25, 80, 260, 840, 2,700, 100,000, and 300,000 µg/kg bw per day from GD 6 until PND 90 by direct gavaging from PND 1.

In summary, the CEF Panel considered that the results of studies in rats indicated no effect of BPA on glucose/glucose tolerance whereas in mice some effects were seen in studies which had methodological deficiencies and hence did not demonstrate a convincing effect of BPA on this endpoint.

<u>Studies in adult mice and rats</u>

Increased body weight/body weight gain:

Marmugi et al. (2012) administered BPA in the diet to male CD1 mice for 28 days, dosing (estimated by the authors) was equivalent to 0, 5, 50, 500 and 5000 µg/kg bw per day. No effect was seen on body weight gain and relative liver weight, but perigonadic white adipose tissue (pWAT weight) was significantly increased only in the 50 µg/kg bw per day group.

In the study of Hassan et al. (2012) exploring mechanistic aspects of BPA effects in the liver, rats received BPA (0.1, 1, 10, 50 mg/kg/day) via gavage for four weeks The final body weights in the 0.1 mg/kg bw per day group showed a significant decrease and the 10 mg/kg bw per day group a significant increase compared to the control group.

In the study of Rönn et al. (2013) intakes of BPA, given in drinking water to female F-344 rats, were between 4.6 (week 9) and 5.6 (week 2) µg/kg bw per day at the lowest dose, between 46.3 (week 6) and 61.6 (week 3) µg/kg bw per day at the mid dose and 400.3 (week 9) and 595.3 (week 2) µg/kg bw per day at the highest dose, according to the authors. Dosing was from five to 15 weeks of age.There were no significant effects of BPA on body weight or weight of the perirenal fat pad and no differences were seen in total or visceral adipose tissue volumes between the groups. Liver fat content was significantly higher in rats receiving the two higher doses of BPA compared with controls ($p = 0.04$).

Further endpoints

Insulin

In the study of Marmugi et al. (2012) in mice, plasma insulin levels were significantly increased following oral exposure to 5, 50, and 500 µg BPA/kg bw per day, with the greatest effect (threefold increase above the control) being seen at the lowest dose. In the study of Batista et al.(2012), 3-month old mice administered a total of 100 µg BPA/kg bw daily by subcutaneous injection (in two injections) for 8 days showed higher plasma insulin concentrations in the fed state and increased glucose-stimulated insulin secretion in isolated pancreatic islet of Langerhans. In the studies of D'Cruz et al. (2012b), in male rats with BPA doses of 0.005, 0.5, 50 and 500 µg/kg bw per day by oral gavage for 45 days, plasma insulin was increased and testicular insulin was significantly decreased down to the lowest level of BPA exposure of 5 ng/kg bw per day. Jayashree and co-workers (Jayashree et al., 2013; Indumathi et al., 2013) in a study in adult male rats found that serum insulin was significantly increased in a dose-related manner at oral BPA doses of 20 mg/kg bw per day and 200 mg/kg bw per day for 30 days.

Glucose and Glucose tolerance

In the study of Marmugi et al. (2012) in mice, no significant effect was apparent on plasma glucose and total, LDL- or HDL-cholesterol. In the studies of D'Cruz et al. (2012a), in rats, levels of plasma glucose were significantly increased across all doses from 500 µg/kg bw per day down to 5 ng/kg bw per day, whereas the testicular glucose level significantly decreased, again at all dose levels. In the study of Batista et al. (2012), glucose tolerance testing showed that BPA-treated mice were insulin resistant and had increased glucose-stimulated insulin release.

Other effects

In the study of Marmugi et al. (2012) the group of mice exposed to 500 µg BPA/kg bw per day showed a significant increase in plasma triglyceride levels. Furthermore, the results of the microarray assays showed a stimulatory effect of BPA on expression of key enzymes involved in lipogenesis, cholesterol biosynthesis and, to a lesser extent, enzymes involved in glucose metabolism as well as master transcriptional regulators of hepatic lipid and glucose homeostasis with a complex dose-response pattern.The dose-response relationship is different between the endpoints, even if they are biologically related. Hence, the CEF Panel considered that it is difficult to understand the underlying mechanism.

D'Cruz et al. (2012b), in a study in male rats with BPA doses of 0.005, 0.5, 50 and 500 µg/kg bw per day by oral gavage for 45 days reported that various insulin signalling molecules were significantly decreased in testis in a dose-related manner at all dose levels. Similarly, a dose-dependent and significant decrease in testicular superoxide dismutase and catalase activities was measured at all doses, and lipid peroxidation was increased, together with decreases in testicular marker proteins and key enzymes of steroidogenesis. There was loss of germ cells and decrease in the spermatids in rats treated with 500 µg/kg bw per day BPA. The statistics were not properly reported as a one-way ANOVA was followed by Tukey's post test, but the results of the overall ANOVA were not given. The use of this statistical approach with such a small sample size is questionable. The reported changes in testicular pathology cannot be related to functional deficits.

The study of Batista et al. (2012) in mice administered a total of 100 µg BPA/kg bw daily by subcutaneous injection (in two injections) for 8 days reported that whole-body energy homeostasis, as assessed by reduced food intake, reduced locomotor behavior and decreased energy expenditure during night, was reduced, although respiratory exchange ratio was unchanged. Changes in a number of insulin-signalling pathways were also reported in the study.

In the studies of Jayashree and co-workers (2013) glucose oxidation was reduced at dose levels of 20 mg BPA/kg bw per day and 200 mg BPA/kg bw per day, both in liver and in skeletal muscle, and

glycogen content of the liver was also reduced. In skeletal muscle, treatment with BPA significantly decreased the insulin receptor, protein kinase B and glucose transporter-4 levels (both plasma membrane and cytosolic fraction), but did not affect the mRNA levels for these proteins. In the liver both mRNA and protein levels were significantly decreased at the highest BPA dose.

Study in a specific mouse strain

Bodin and co-workers (2013) investigated possible effects of BPA, administered at 0, 1 and100 mg/l BPA in the drinking water of non-obese pre-diabetic (NOD) mice (n = 6-10 per group for different parameters) on the development of type 1 diabetes (T1DM). The authors estimated that these levels corresponded to intakes of 0, 150 or 15000 µg/kg bw per day in non-diabetic mice. The incidence and degree of insulitis in the pancreas was comparable between groups at week 7, but was markedly increased compared with controls in 12-weeks-old female mice exposed to 1 mg/l BPA in drinking water. Insulitis was less severe in the female animals receiving 100 mg/l and was decreased in male mice exposed to BPA compared with controls. Serum glucose levels were increased in the 1 mg/ml BPA group, indicating an accelerated onset of T1DM, but this was not seen in the animals exposed to 100 mg/l BPA. Insulin levels did not differ significantly between the groups and while T4 levels increased slightly with increasing BPA intake, this was not statistically significant. Serum levels of cytokines and autoantibodies also did not differ between the groups.

3.7.2.3. Summary of metabolic effects of BPA in animals

A number of studies in both prenatally- and postnatally exposed rats and mice report effects of BPA exposure on metabolic function in terms of glucose or insulin regulation or lipogenesis, and body weight. In some of the studies effects were only seen at one dose level which was interpreted by the authors as being an evidence for non-monotonicity of the dose-response curve. However dose-response curves in which effects of different size are present at two low dose levels and a smaller effect size at a higher dose level than the two low doses were not observed. Hence, the assumption of non-monotonicity is not supported by the data.

Although some isolated effects may have been assessed as likely effects in evaluating whether the effects are clinically relevant for humans an overall assessment has to be performed. It has to be noted that the effects observed in the different studies are contradictory and in some of the studies may be associated with high fat feed intake which cannot be considered as a good model for human health assessment. In addition, there is no convincing evidence that BPA is obesogenic later in life in studies with intrauterine and subsequent long-term dosing. In adult animals, body weight was not influenced by BPA in the two studies in which it was measured, while fat pad weight was not changed compared with controls in one study and increased in the other. Levels of serum glucose were increased in one study and unchanged in the other whereas glucose in testis was decreased in one study. Insulin plasma/serum levels were increased in BPA-treated animals in two studies in mice and two studies in rats over a range of doses from 0.005 µg/kg bw per day up to 30 000 µg/kg bw per day across studies. Changes in insulin signalling are reported in several studies, which point at possible mechanisms of action for the elevated insulin and might explain the impaired glucose tolerance described in one study. However, no long term study confirms the presence of diabetes in the animals. The finding of insulin resistance, if existing, seems to be a reversible effect and seems not to indicate the first step in developing diabetes mellitus type 2.

3.7.3. In vitro studies

Several in vitro studies conducted after 2010 examined the effects of BPA on insulin secretion, mitochondrial morphology and function and gene expression in different cell types.

Insulin secretion stimulated by glucose levels above the normal value in fasting humans (8-17.7 mM in the experiments versus 4-5.5 mM) was further increased by treatment with BPA concentrations (10^{-10} M, 10^{-9} M and $2x10^{-9}$ M) in mouse and human islets, in primary rat islet cells and in a rat insulinoma cell line (Soriano et al., 2012; Song et al., 2012; Lin et al., 2013). Increase was less than

twofold with concentrations up to a concentration of $2x10^{-9}$. In the presence of 3 mM glucose there was either no BPA effect (Soriano et al. 2012) or the insulin secretion was induced at BPA concentrations greatly exceeding the concentration which could be expected from human exposure to BPA (Song et al., 2012). It remains open whether the results at high glucose levels can be regarded as adverse because the increase in insulin secretion is modest even at BPA concentrations in the medium of about 100 fold the in vivo human serum concentration. Results from a study using ERβ-/-mice in comparison with wild-type mice suggest that BPA´s effects on insulin secretion, KATP channel activity and glucose-induced [Ca2+] oscillations in pancreatic β-cells is linked to the presence of ERβ. BPA-induced toxicity and apoptosis was associated with changes in the morphology and the membrane potential of mitochondria in pancreatic β-cells and insulinoma cells.

In the human hepatic cell line HepG2 mitochondrial dysfunction along with signs of oxidative stress were induced by BPA concentrations of 10^{-12} M - 10^{-8} M (Huc et al., 2012).

In human adipose tissue isolated from children and in preadipocyte/adipocyte cells, BPA at 10^{-8} M increased the expression of 11β-hydroxysteroid-dehydrogenase, PPARγ and lipoprotein lipase and, in addition, induced lipid droplet accumulation in adipocytes at terminal differention (Wang et al., 2012a, b). These data suggest that concentrations of BPA which are more than 1000 fold above human concentrations promote adipogenesis in vitro.

Using transfection gene reporter assays with monkey kidney cells, Sheng et al. (2012) observed a BPA (10^{-9} M to 10^{-7} M)-induced suppression of thyroid hormone receptor transcription through a non-genomic pathway. However, the concentrations are beyond the range which could be expected from human exposure to BPA, and the relevance of the complex in vitro-transfection data for the in vivo situation is unclear.

3.7.3.1. Summary of metabolic effects of BPA in vitro

Three studies demonstrated an increase of glucose-stimulated insulin secretion by BPA concentrations of 10^{-10} M – $2x10^{-9}$ M in pancreatic cells/tissue. This is a concentration range which is reached with an oral dose of 100 µg/kg bw per day in mice (C_{max} $1.8x10^{-10}$ M in in Doerge et al., 2011b) and in rats (C_{max} $3.6x10^{-10}$ M in Doerge et al., 2010a). Thus, it is likely that nanomolar concentrations of BPA can affect insulin secretion in vitro. However, considering the limitations of in vitro studies (e.g. substrate and hormone concentrations which often differ from the in vivo situation) the relevance of the above mentioned observations for the function of pancreatic β-cells in vivo is currently unclear.

3.7.4. WoE of metabolic effects in humans, animals and in vitro

Whether BPA induces metabolic effects was considered using a tabular format for weighting different lines of evidence (WoE evaluation). The overall outcome of this WoE evaluation is presented below, while the WoE evaluation tables for these endpoints are presented in full in Appendix C. For interpretation of these tables always refer to Appendix A.

Table 12: Overall table on WoE evaluation of metabolic effects of BPA in humans and animals

Human studies	
Overall conclusion on likelihood of associations between BPA and obesity in humans There are indications from a prospective study that prenatal BPA exposure (maternal urinary BPA concentrations) may be associated with reduced body mass in girls, while cross-sectional studies indicate associations between childhood BPA exposure and obesity. It cannot be ruled out that the results are confounded by diet or concurrent exposure factors. The associations do not provide sufficient evidence to infer a causal link between BPA exposure and obesity in humans. Potential effects are considered to be as likely as not".	**As likely as not**

Human studies	
Overall conclusion on likelihood of associations between BPA and hormonal effects in humans There are indications from one prospective study that maternal BPA exposure may be associated with adipokine expression in 9 year old children, but it cannot be ruled out that the result is confounded by diet or concurrent exposure factors. The association does not provide sufficient evidence to infer a causal link between BPA exposure and hormonal effects in humans. Potential effects are considered to be as likely as not."	**As likely as not**
Overall conclusion on likelihood of associations between BPA and diabetes effects in humans: The indications that BPA may be associated with diabetes in humans is unlikely.	**Unlikely**
Overall conclusion on likelihood of associations between BPA and metabolic syndrome in humans: The indication that BPA may be associated with metabolic syndrome in humans is unlikely.	**Unlikely**
Overall conclusion on likelihood of associations between BPA and renal effects in humans: The indication that BPA may be associated with renal function in humans is unlikely.	**Unlikely**
Animal studies	
Overall conclusion on likelihood of metabolic effects in animals exposed postnatally Evidence for associations between BPA exposure and metabolic effects in animals exposed postnatally is inconsistent. There is reasonable evidence for effects on glucose or insulin regulation and/or effects on pancreatic morphology and/or function in shorter term studies, but no evidence that BPA is causing diabetes, insulin resistance and increases in weight (obesogenic) longer-term.	**As likely as not**
Overall conclusion on likelihood of metabolic effects in animals exposed prenatally : Since the initial reports that BPA had potential effects on adiposity, glucose or insulin regulation, lipids and other end-points related to diabetes or metabolic syndrome in animals exposed prenatally, several new studies have been published. There is reasonable evidence for effects on glucose or insulin regulation and/or effects on pancreatic morphology and/or function in shorter term studies, but no evidence that BPA is causing diabetes, insulin resistance and increases in weight (obesogenic) longer-term.	**As likely as not**

3.7.5. Conclusions on metabolic effects

Of the reviewed human studies on metabolic effects only two were prospective while 22 were cross-sectional and thus not suitable on their own to study exposure-disease associations. Inconsistent with the results of cross-sectional studies one prospective study found that a higher BPA concentration in maternal urine during pregnancy was associated with a lower level of obesity in daughters. A causal link between BPA exposure and metabolic effects in humans cannot be established.

A number of studies in pre- and postnatally exposed rats and mice indicate that BPA exposure could have an effect on metabolic function as evidenced by effects on glucose or insulin regulation or lipogenesis, and body weight gain (short-term studies). Based on the results from other studies with a longer duration (e.g. 90 days) there is no convincing evidence that BPA is obesogenic after intrauterine exposure or in longer-term studies.

Using a WoE approach, the CEF Panel assigned a likelihood level of "as likely as not" to metabolic effects of BPA. Since the likelihood level for this endpoint is less than "likely" (see Appendix A), this endpoint was not taken forward for assessing the toxicological reference point, but was taken into account in the evaluation of uncertainty for hazard characterisation and risk characterisation (Section 4.3).

3.8. Genotoxicity

3.8.1. Summary of previous opinions on BPA genotoxicity

The genotoxicity of BPA has been reviewed on a number of occasions (Haighton et al., 2002, EU, 2003; EFSA, 2006; NTP-CERHR, 2008; EFSA CEF Panel, 2010; FAO/WHO, 2011). BPA has been tested in a range of in vitro assays including gene mutation assays in bacteria, yeast and mammalian cells, chromosome aberration tests, sister chromatid exchange, cell transformation assays and cell-free systems including DNA binding and microtubule disruption. In vivo studies have included micronucleus formation, chromosome aberration studies, dominant lethal assay and DNA adduct formation.

EU-RAR (2003 and/or 2008)

The EU Risk Assessment Report (EU RAR), in reviewing studies published up to 2002 concluded that in vitro BPA did not induce gene mutations or structural chromosome aberrations in bacteria, fungi or mammalian cells in vitro, but had some aneugenic potential as evidenced by positive findings in an in vitro micronucleus test in Chinese hamster V79 cells and in an aneuploidy assay in Syrian hamster embryo cells (EU, 2003). The EU RAR noted that the potential of BPA to produce aneuploidy was supported by the demonstration of microtubule disruption in the presence of BPA in cell-free and cellular systems and also noted that BPA has been reported to produce DNA adducts in a post-labelling assay with isolated DNA (EU-RAR, 2003). The EU RAR concluded that the potential of BPA to produce aneugenicity in vitro was not expressed in vivo, based on negative findings in a guideline mouse micronucleus test supported by a negative result in an inadequately-reported dominant lethal study. The authors of the EU RAR further concluded that the finding of DNA adduct spots in a postlabelling assay in rats in vivo was unlikely to be of concern, given the lack of evidence for mutagenicity and clastogenicity of BPA in cultured mammalian cells.

NTP-CERHR (2008)

The NTP-CERHR monograph reviewed the above database and also studies published between 2002 and 2008 (NTP-CERHR, 2008). The authors noted more recent in vitro studies providing evidence of an effect of BPA on meiotic and mitotic cell division, but not induction of aneuploidy. These studies included effects on maturation of mouse oocytes, increased frequency of mitotic cells with aberrant spindles, and effects on cellular and nuclear division in fertilized sea urchin eggs. NTP-CERHR summarised the results of two in vivo studies demonstrating an increase in hyperploid (aneuploid) metaphase II oocytes following treatment of peripubertal or pregnant mice with 0.020 mg BPA/kg bw per day, without a significant increase in aneuploid embryos. These findings were not however reproduced in two subsequent in vivo studies using a similar design. NTP-CERHR concluded that "*since no impact of such effects on reproduction is reported in animal breeding studies, the significance of these findings with regard to human health hazards is not clear"* (NTP-CERHR, 2008).

EFSA (2006 and 2010)

EFSA in 2006 noted that BPA is not considered to be genotoxic in bacteria and in mammalian cells, based on previous reviews of BPA genotoxicity (EC, 2002; EU-RAR, 2003; Haighton et al., 2002, as cited by EFSA, 2006). In the EFSA opinion of 2010, the CEF Panel noted that "*Naik and Vijayalaxmi (2009) reported that oral administration of BPA as single (10, 50, 100 mg/kg bw) or repeated doses (5x10 mg/kg bw) did not increase the incidence of structural chromosomal aberrations or micronuclei in bone marrow of Swiss albino mice. Administration of BPA, however, was associated with an increased incidence of achromatic lesions (gaps) which cannot be considered as an evidence of a clastogenic potential in the absence of a concurrent increase in structural chromosomal aberrations"*. The CEF Panel concluded therefore that the findings of this study did not alter the 2006 EFSA conclusion that BPA has no clastogenic potential in vivo. The CEF Panel noted however that the authors also reported that BPA had an effect on spindle structure, which could be interpreted as an indication of aneuploidy. The CEF Panel concluded however, "*considering the thresholded mechanism for aneuploidy induction, the large margin between the doses tested negative in the*

micronucleus test and the TDI provided adequate reassurance on the lack of aneugenic effects" (EFSA CEF Panel, 2010). The 2010 EFSA opinion also summarised the study of Muhlhauser et al. (2009), providing data showing a borderline effect of BPA on chromosome alignment or spindle abnormalities. The CEF Panel noted that effects of BPA on meiotic spindle can be modulated by the amount of phytoestrogens present in the diet, and concluded that *"the consequences of these cytological effects on chromosome segregation are unknown and therefore these effects cannot be considered as markers of aneuploidy"*. The CEF Panel also noted the in vivo studies reviewed by NTP-CERHR and concluded overall that *"these data have no impact on the Panel´s previous conclusion on the lack of aneugenic activity of BPA in mouse germ cells*."

FAO/WHO (2011)
The report of the 2010 Joint FAO/WHO Expert Meeting on Toxicological and Health Aspects of Bisphenol A concluded that "BPA is not a mutagen in in vitro test systems, nor does it induce cell transformation. BPA has been shown to affect chromosomal structure in dividing cells in in vitro studies, but evidence for this effect in in vivo studies is inconsistent and inconclusive. BPA is not likely to pose a genotoxic hazard to humans." (FAO/WHO, 2011).

ANSES (2011; 2013)
No mention of genotoxic effects by BPA was present in either of the two ANSES reports.

3.8.2. Evaluation of studies on genotoxicity of BPA (2006-2013)

This section provides an overview of the *in vitro* and *in vivo* studies on genotoxicity published after the EFSA opinion from 2006 (since this endpoint was not specifically dealt with in the 2010 EFSA opinion), that the CEF Panel considered as the most relevant to this evaluation.

The detailed description and evaluation of each study are provided separately in Appendix B.

3.8.2.1. In vitro studies

Masuda et al. (2005) reported negative mutagenicity results of BPA in a bacterial reverse mutation assay (Ames test) both in the absence and presence of S9 metabolic activation, using a limited battery of tester strains (TA98 and TA100) and a single concentration (1mM),

In the study by Tiwari et al. (2012) mutagenicity of BPA was determined in an Ames assay using tester strains of *S. typhimurium* TA 98, TA 100 and TA 102 in the presence and absence of S9 metabolic activation. Negative results were observed at concentrations up to 200 µg/plate, where toxicity was observed.

In the study by Iso et al. (2006), the authors aimed to assess potential DNA damage induced by BPA (10 nM-0.1 mM) using the alkaline comet assay and the detection of phosphorylated histone γ-H2AX in two non-isogenic human cell lines (MCF-7 and MDA-MB-231) positive and negative for oestrogen receptors (ER) respectively. Results reported indicate that BPA was able to induce DNA breakage as shown by significant increases in tail length in the alkaline comet assay and significant induction of phosphorylated histone γ-H2AX, a marker for induction of DNA double strand breaks. These effects were reported to be more pronounced in the ER-positive MCF-7 cells compared to the ER-negative MDA-MB-231 ones.

In the study by Johnson and Parry (2008) the aneugenicity of BPA was investigated in the cytokinesis blocked micronucleus assay (CBMA) in human (AHH-1) lymphoblastoid cells over a very narrow range of low concentrations (1.5, 3.1, 6.2, 7.7, 9.2, 10.8, 12.3, 18.5, 24.6, and 37.0 µg/ml). For mechanistic evaluation of the aneugenic effects of BPA fluorescently labelled antibodies for α and γ-tubulin were used to visualize the microtubules and the microtubule organizing centers (MTOCs) in a V79 Chinese hamster cell line. Results obtained indicated dose-related and statistically significant increases of binucleate-micronucleated cells from 12.3 µg/ml and above, with a threshold for

induction of micronuclei between 10.8 and 12.3 µg/ml. Induction of aberrations in the mitotic machinery, in the form of multiple spindle poles at 8.4 µg/ml BPA and above was also observed. Aberrant mitotic divisions were hypothesized to be the mechanism for the generation of micronuclei via chromosome loss, thus confirming a threshold mechanism of action for the induction of aneuploidy by BPA.

Tayama et al. (2008) reported positive results for induction of sister chromatid exchanges (SCE's), chromosome aberrations (CA), DNA strand breaks (evaluated by alkaline comet assay) and colchicine-mitosis-like (c-mitoses) figures, a marker for spindle disrupting effects in a CHO-K1 cell line *in vitro* following treatment with BPA at dose-levels of 0.1-0.7 mM. Positive findings reported in this study for DNA strand breaks (evaluated by alkaline comet assay), chromosomal aberrations and SCE were only observed at the highest concentration employed in the presence of a marked cytotoxicity. The induction of c-mitoses, which appears to be not influenced by cytotoxicity and methods applied, can be considered as a further evidence of a spindle disrupting effect of BPA.

In the study by Izzotti et al. (2009), BPA was reported to induce dose-related increases of DNA adducts as detected by ^{32}P-postlabelling in an acellular sytem constituted by a mixture of calf thymus DNA and an exogenous metabolising system containing 10% liver S12 fraction derived from Aroclor 1254-pre-treated Sprague–Dawley rats. In this investigation, chemical characterisation of DNA adducts was not performed.

In the study by De Flora et al. (2011), BPA was investigated by ^{32}P-postlabelling for induction of DNA adducts in two human prostate (PNT1and PC3) cell lines. Results obtained showed formation of DNA adducts (4.2 and 2.7 fold increases over control in PNT1 and PC3 cells respectively) following metabolic conversion of BPA by PNT1 and PC3 human prostate cell lines. The CEF Panel noted that metabolic competence for these cell lines has not been demonstrated and that chemical characterisation of the DNA adducts has not been performed.

In the study by Audebert et al. (2011), BPA was shown to be negative for induction of phosphorylated histone γ-H2AX, a marker for induction of DNA double strand breaks in the human cell lines HepG2 (human hepatocellular carcinoma cells) and LS174T (human epithelial colorectal adenocarcinoma cells). The CEF Panel noted that the H2AX assay is not a validated genotoxicity test.

In the study by Fic et al. (2013) BPA was assessed for its mutagenic and genotoxic potential using the Ames test (*Salmonella typhimurium* strains TA98 and TA 100) in the absence and presence of S9 metabolic activation and the alkaline comet assay in HepG2 cells at concentrations of 0.1, 1.0 and 10.0 µM for 4 and 24 hours. BPA was not mutagenic in the Ames test, while in the comet assay it induced statistically significant increases in DNA damage only after 24 hours exposure at any of the concentrations used. These increases were, however, not concentration-related.

3.8.2.2. Summary of in vitro studies

BPA did not induce gene mutation or chromosomal aberrations in bacteria, yeast and mammary cells (EFSA, 2006; Masuda et al., 2005; EFSA CEF Panel, 2010; Tiwari et al., 2012). The potential of BPA to affect spindle apparatus inducing aneuploidy was clearly demonstrated in a number of reliable studies (Johnson and Parry, 2008; Tayama et al., 2008). The compound was shown to induce DNA adducts in acellular systems (Izzotti, 2009), in hamster and human cell lines (EFSA, 2006, De Flora et al., 2011) and DNA damage in non-isogenic human cell lines (MCF-7 and MDA-MB-231) (Iso et al., 2006).

3.8.2.3. In vivo studies

The study by Masuda et al. (2005), designed to investigate potential genotoxicity from the reaction of BPA and nitrite under acidic conditions to simulate the stomach environment, showed that when BPA was administered alone at 228 mg/kg bw by oral gavage to male ICR mice it did not induce

micronuclei in peripheral blood reticulocytes at the 24, 48 and 72 hour sampling times. Although only one dose-level was assessed the CEF Panel considered the study useful for the evaluation of genotoxicity.

Pacchierotti et al. (2008) evaluated potential aneugenic effects of BPA on mouse female germ cells following a single treatment at 0.2 and 20 mg/kg bw, or seven daily administrations at 0.04 mg/kg bw by oral gavage or administration for seven weeks in drinking water at 0.5 mg/l. The authors also examined effects of BPA on male germ and somatic cells (as evidenced by induction of micronuclei in bone-marrow cells following six daily administrations of BPA at 0.002, 0.02 and 0.2 mg/kg bw by oral gavage). Results obtained for female animals indicated no significant induction of hyperploidy or polyploidy in oocytes and zygotes at any dose-level and treatment condition employed. Significant increases in the number of metaphase II oocytes with prematurely separated chromatids were observed, however these proved to be of no consequences in terms of fidelity of chromosome segregation during the second meiotic division as shown by normal chromosome complements of zygotes obtained under the same experimental conditions. Similarly, no induction of hyperploidy or polyploidy in epydidimal sperms, were observed in male mice. Furthermore, negative results for induction of micronuclei in bone marrow cells of male mice were also observed.

In the study by Izzotti et al. (2009) BPA was investigated for its capability to cause DNA adducts, detected by ^{32}P-postlabelling in both liver and mammary cells of female CD-1 mice receiving BPA in their drinking water (equivalent to 200 mg/kg bw per day) for 8 consecutive days. Results obtained indicated the formation of bulky DNA adducts (two major DNA adducts) in the liver (3.4 fold increase over control level) as well as in the mammary cells (4.7 fold increase over control level). The authors attributed the formation of adducts to the reactive metabolite BPA-3,4-quinone (BPAQ), formed by metabolism of BPA in humans and in experimental animals.

Naik and Vijayalaxmi (2009) evaluated potential genotoxic effects of BPA by analyses of chromosomal aberrations and micronuclei in bone marrow cells of Swiss albino mice following a single administration at 10, 50 and 100 mg/kg bw or five daily administrations at 10 mg/kg bw by oral gavage. To further assess for potential interference of BPA with the mitotic spindle apparatus, induction of c-mitoses was also evaluated following single administration of BPA by oral gavage at 10, 50 and 100 mg/kg bw. No significant increases of chromosomal aberrations or micronuclei were induced at any dose-level and sampling time used. On the other hand, dose-related and statistically significant increases in the frequencies of gaps were observed at all dose-levels assayed at the 48 and 72 hour sampling time and at the two higher dose-levels (50 and 100 mg/kg bw) at the 24 hour sampling time. In addition, BPA also induced c-mitotic effects as shown by the increase of mitotic indices and decrease in anaphase at the two higher dose-levels (50 and 100 mg/kg bw) at 24, 48 and 72 hour sampling times. Despite some methodological deficiencies of the study, the CEF Panel concluded that BPA under the reported experimental conditions was not clastogenic and did not elicit micronuclei induction which would be indicative of a clastogenic and/or aneugenic potential at dose-levels employed. Furthermore, the CEF Panel noted that gaps, significantly increased in the chromosomal aberration assay, are usually not considered relevant for the evaluation of genotoxicity.

In the study by De Flora et al. (2011), BPA was assessed for induction of micronuclei in bone marrow cells and evaluation of the degree of DNA breakage by means of alkaline comet assay in peripheral blood following *in vivo* treatment of male Sprague-Dawley rats *via* drinking water for a calculated daily exposure to 200 mg/kg bw for 10 consecutive days. Despite some methodological deficiencies of the study, the CEF Panel considered the study useful for the evaluation of genotoxicity.

In the study by Ulutaş et al. (2011) BPA was assessed for its potential genotoxicity in peripheral blood nucleated cells of rats by means of the alkaline comet assay following oral administration at 125 and 250 mg/kg bw per day for four weeks. Results obtained showed statistically significant increases of both tail length and tail moment for BPA at the highest dose-level (250 mg/kg bw per day) which were, however, not marked. No effect was observed at the lower dose-level (125 mg/kg

bw per day). Given methodological deficiencies, the CEF Panel considered that the results obtained are of limited value.

Dobrzyńska and Radzikowska (2013) investigated the effects of BPA alone or in combination with X-rays for induction of DNA strand breaks by means of DNA tail moment in the alkaline comet assay in somatic and germ cells of male mice following administration in drinking water for two weeks. Levels in drinking water were designed to achieve BPA intakes of 0, 5, 10, 20 or 40 mg/kg bw per day. Two additional groups received either 5 or 10 mg BPA/kg bw per day via drinking water in combination with daily radiation doses of 0.05 Gy or 0.10 Gy of X-rays which were not considered in this evaluation. BPA induced statistically significant increases of DNA breakage in male germ cells at 24 hours and 5 weeks from last administration of test compound and in bone marrow, spleen, kidney and lung cells at 24 hours from last administration. However, the increases observed were not dose-related and were obtained following collection of organs/tissues at 24 hours or 5 weeks from last administration. The CEF Panel considered that this experimental design is inadequate, since potential induced damage may rapidly be repaired and thus may not persist for a long time. Given this, and also noting other methodological deficiencies, the CEF Panel considered that no conclusion could be drawn from this study.

In the study by Tiwari et al. (2012) BPA was investigated for induction of micronuclei and structural chromosome aberrations in bone marrow cells and primary DNA damage in blood lymphocytes using single cell gel electrophoresis (Comet assay). Furthermore, plasma concentrations of 8-hydroxydeoxyguanosine (8-OHdG), lipid peroxidation and glutathione activity were also evaluated to assess potential induction of oxidative DNA damage in rats following oral administration of BPA once a day for 6 consecutive days at dose-levels of 2.4 µg, 10 µg, 5 mg and 50 mg/kg bw per day. Results obtained showed marked and dose-related increases of both micronuclei and structural chromosome aberrations in bone marrow cells of male and female rats exposed to BPA. The observed increases achieved statistical significance at dose-levels as low as 10 µg/kg bw per day. Similarly, the analysis of primary DNA damage evaluated by comet assay in isolated peripheral blood lymphocytes showed marked and dose-related increases which were statistically significant at dose-levels as low as 10 µg/kg bw per day. The CEF Panel considered that study has major shortcomings including the observation of chromosomal aberration incidences which are not compatible with aberrations induced by known chemical clastogens and high DNA damage in controls in the absence of evaluation of cytotoxicity in the comet assay.

In the study by Tiwari and Vanage (2013), BPA was investigated for the induction of dominant lethal mutations in the different stages of spermatogenesis in the rat. Furthermore, effects of BPA on male reproductive functions and potential DNA damage induced in epydidimal sperm, assessed by the alkaline comet assay were also investigated. The authors concluded that BPA induced dominant lethal mutations during the fourth and sixth weeks after BPA exposure, thus indicating its sensitivity to mid-spermatid and spermatocyte stages of spermatogenesis, at the highest dose-level employed (5 mg/kg bw) and that the positive findings obtained were corroborated by DNA damage observed in the epydidimal sperm cells by the alkaline comet assay. Overall, the CEF Panel noted that the conclusion raised by the authors are not supported by their experimental data due to experimental shortcomings which include a limited number of male animals employed and an inadequate selection of dose-levels (only two dose levels with a very large difference between the high and the low dose). In addition, negative historical control data were not reported. Thus, overall the result cannot be considered reliable.

3.8.2.4. Summary of in vivo studies

BPA did not induce chromosomal damage in rodents, evaluated as micronuclei frequency and as chromosomal aberrations (Masuda et al., 2005; Pacchierotti et al., 2008; Naik and Vijayalaxmi, 2009; De Flora et al., 2011). The potential of BPA to affect the spindle apparatus was shown by the increases of c-like metaphases in bone marrow of male mice Naik and Vijayalaxmi, 2009) and of the number of metaphase II oocytes with prematurely separated chromatids in female mice after single or

multiple treatment with BPA (Pacchierotti et al., 2008). No induction of hyperploidy or polyploidy was observed in somatic as well in germinal cells. BPA was shown to induce DNA adducts in liver and mammary gland of female mice (Izzotti et al., 2009).

3.8.3. WoE of the genotoxicity of BPA in vitro and in vivo

The genotoxicity of BPA was considered using a tabular format for weighting different lines of evidence (WoE evaluation). The overall outcome of this WoE evaluation is presented below, while the WoE evaluation tables for these endpoints are presented in full in Appendix C. For interpretation of these tables always refer to Appendix A. The outcome of the WoE evaluation of in vivo BPA genotoxicity is also included in the subsequent carcinogenicity section, given the possible relevance of this endpoint in cancer development.

Table 13: Overall table on WoE evaluation of genotoxicity

In vitro studies	
Overall conclusion based on in vitro studies – via non thresholded mechanism: BPA has not been shown to induce gene mutations nor chromosomal aberrations in bacteria and mammalian cells.	**Unlikely**
Overall conclusion based on in vitro studies – via thresholded mechanism: BPA has been clearly shown to be aneugenic through induction of micronuclei caused by spindle disrupting effects of BPA identified by the use of fluorescently labelled antibodies for α and γ-tubulin to visualize the microtubules and the microtubule organizing centers of the mitotic spindles (Johnson and Parry 2008). Further evidence for spindle disrupting effects of BPA have been also indicated by Tayama et al. (2008) who showed significant increases of colchicine-like metaphases (c-metaphases) in CHO-K1 cells.	**Very likely**
Animal studies	
Overall conclusion based on in vivo studies – via non-thresholded mechanism: BPA has not been shown to be clastogenic in vivo (micronuclei and chromosomal aberrations)	**Unlikely**
Overall conclusion based on in vivo studies - via thresholded mechanism: The potential of BPA to produce aneuploidy in vitro was not expressed in vivo, based on negative findings obtained for induction of aneuploidy in female and male mouse germ cells (Pacchierotti et al., 2008) and for induction of micronuclei in somatic bone marrow cells (Masuda et al., 2005; Pacchierotti et al., 2008; Naik and Vijayalaxmi , 2009; De Flora et al., 2011). However, BPA induced dose-related increases of c-like metaphases in mouse bone-marrow cells (Naik and Vijayalaxmi , 2009) and significant increases in the number of metaphase II oocytes with prematurely separated chromatids (Pacchierotti et al., 2008), pointing to potential mitotic spindle disrupting effects of BPA in vivo.	**As likely as not**

3.8.4. Conclusions on genotoxicity of BPA

The genotoxicity of BPA has been reviewed on a number of occasions (Haighton et al., 2002; EU-RER, 2003; EFSA, 2006; NTP-CERHR, 2008; EFSA CEF Panel, 2010; FAO/WHO, 2011). In the present evaluation an overview of the in vitro and in vivo studies on genotoxicity of BPA published from 2006-2012 that the CEF Panel considered as the most relevant to the human risk assessment has been performed.

In a number of these studies judged by the CEF Panel as reliable although with limitations, BPA did not induce gene mutation in bacteria (Masuda et al., 2005; Tiwari et al., 2012), micronuclei (Masuda et al., 2005; Pacchierotti et al., 2008; Naik and Vijayalaxmi , 2009; De Flora et al., 2011) and chromosomal aberrations in erythropoietic cells of rodents treated in vivo with BPA (Naik and Vijayalaxmi , 2009).

On the other hand, BPA has been clearly shown to be aneugenic in an in vitro study in mammalian cells by Johnson and Parry (2008) who demonstrated induction of micronuclei as a consequence of spindle disrupting effects of BPA. Further evidence for spindle disrupting effects of BPA have also been indicated by induction of colchicine-like metaphases (C-metaphases) in mammalian cells in vitro

(Tayama et al., 2008) and in vivo by induction of prematurely separated chromatids in metaphase II of mouse oocytes (Pacchierotti et al., 2008) and c-metaphases in mouse bone marrow cells in vivo (Naik and Vijayalaxmi , 2009).

Overall, these results point to the fact that BPA interacts with mitotic machinery through a mitotic spindle disrupting effect for which a threshold mechanism of action is expected, since induction of aneuploidy predicted for spindle poisons needs to disable multiple targets of the mitotic machinery before a quantitative response can be detected (COM Guidance on a Strategy for Testing of Chemicals for Mutagenicity, Department of Health, 2000).

In addition the CEF Panel concluded that the positive finding in the postlabelling assays in vitro and in vivo was unlikely to be of concern, given the lack of mutagenicity and clastogenicity of BPA in vitro and in vivo. BPA is not mutagenic (in bacteria or mammalian cells), nor clastogenic (micronuclei and chromosomal aberrations). The potential of BPA to produce aneuploidy in vitro was not expressed in vivo.

Using a WoE approach, the CEF Panel assigned a likelihood level of "unlikely" to BPA genotoxicity.

3.9. Carcinogenicity

3.9.1. Human studies

3.9.1.1. Summary of previous opinions

EU-RAR (2003, 2008)
The EU-RAR stated that there are no human data that can contribute to the assessment of whether or not BPA is carcinogenic.

EFSA (2006); EFSA CEF Panel (2010)
For the 2006 EFSA opinion, the AFC Panel did not identify any human data relevant to the assessment of BPA carcinogenicity.

In the EFSA opinion of 2010, the CEF Panel described the cross-sectional study of Yang et al. (2009) in Korean women affected by breast cancer as having several methodological shortcomings and insufficient reporting, preventing any conclusion to be drawn on the association between BPA exposure and breast cancer. Also, no association between cancer and BPA exposure was reported in the Lang study (2008).

NTP-CERHR (2008)
The NTP monograph reviewed the results of a study by Hiroi et al. (2004) suggesting that patients with endometrial cancer and complex endometrial hyperplasia had lower blood levels of BPA than healthy women and women with simple endometrial hyperplasia. Among the strengths and weaknesses of the study, the NTP noted that *"Because this was a small, cross-sectional study, it is not possible to determine whether this association preceded disease, or could have been associated with the disease process."*

FAO/WHO (2011)
The Expert Meeting noted that no studies of carcinogenicity of BPA in humans have been identified in the literature.

ANSES (2011; 2013)
In 2011, ANSES concluded that there have been no epidemiological studies published to date investigating a possible association between exposure to BPA and prostate disease. The only epidemiological study available on the association between BPA exposure and breast cancer, i.e. Yang et al. (2009), was considered by ANSES as having major methodological limitations and

therefore unsuitable to draw any conclusion. No additional human studies were reviewed by ANSES in its 2013 report.

3.9.1.2. Evaluation of recent human studies on BPA exposure and carcinogenic effects

Only one new human case-control study has been published since 2010, reporting a positive association between meningioma and urinary bisphenol A levels in Chinese adults (Duan et al., 2012). The study is very small and there are uncertainties about selection of patients and controls. Urinary BPA levels were determined at the time of diagnosis of meningioma and a causal association cannot therefore be identified. Confounding factors such as age, gender, body mass index (BMI) and hormone replacement therapy (HRT) cannot be excluded; the CEF Panel noted that a higher risk of meningioma was observed among current users of oral contraceptives than never users in a large European cohort study (Hazard Ratio, 3.61) (Michaud et al., 2010).

Some of the cases of meningioma had received therapeutic intervention but no details were provided in the publication. The results of this small case-control study do not provide significant new information about the carcinogenicity of BPA in humans.

3.9.1.3. Summary of the evidence for carcinogenicity of BPA in humans

The very few epidemiological studies published to date, investigating a possible association between exposure to BPA and incidence of certain cancers, specifically breast cancer (Yang et al., 2009) and meningioma (Duan et al., 2012), do not allow any conclusion to be drawn regarding the carcinogenicity of BPA in humans.

3.9.2. Animal studies

3.9.2.1. Summary of previous reviews of the carcinogenicity of BPA

The carcinogenicity of BPA has been reviewed on a number of occasions (EU-RER, 2003; EFSA, 2006; NTP-CERHR, 2008; EFSA CEF Panel, 2010; FAO/WHO, 2011; ANSES, 2011, 2013). BPA has been tested for carcinogenic potential in two NTP guideline carcinogenicity studies in rats and mice and in a number of experimental carcinogenicity models, together with shorter term rodent studies investigating effects in mammary and prostate glands. The outcome of these reviews is summarised as follows.

<u>EU-RAR (2003 and/or 2008)</u>
The EU RAR concluded that BPA did not have carcinogenic potential, based on the available evidence at that time, including two oral carcinogenicity bioassays in rats and mice conducted by the NTP.

<u>NTP-CERHR (2008)</u>
The NTP-CERHR monograph reviewed the overall database on carcinogenicity of BPA, including a number of studies showing that perinatal exposure of rodents to low doses of BPA via the subcutaneous route caused proliferative changes in the mammary gland. The report concluded that while the findings were not sufficient to conclude that bisphenol A is a rodent mammary gland carcinogen or that bisphenol A presents a breast cancer hazard to humans, exposure of rats to BPA during gestation may lead to the development of mammary changes in adulthood that could potentially progress to tumours. NTP concluded that there was *minimal concern* for exposures of fetuses, infants, and children to BPA, based on the reported effects. NTP-CERHR also concluded that there was some concern that perinatal exposure to bisphenol A in rodents may alter prostate and urinary tract development, but that the evidence was not sufficient to conclude that bisphenol A is a rodent prostate gland carcinogen or that bisphenol A presents a prostate cancer hazard to humans.

EFSA (2006); EFSA CEF Panel (2010)
In 2006, EFSA reported on several studies not reviewed in the EU RAR, examining the effect of BPA on tumour induction in experimental carcinogenicity systems. EFSA did not consider that the findings reported were indicative of a carcinogenic or a tumour-promoting potential of BPA.

EFSA in its 2010 opinion reviewed a number of additional studies on proliferative changes in the mammary gland following administration of BPA and published subsequent to the NTP-CERHR monograph, notably those of Moral et al. (2008), Jenkins et al. (2009) and Betancourt et al. (2010) involving the oral route of administration. EFSA concluded that the data reported by these authors suggested that either lactational or in utero exposure to BPA may increase the susceptibility of the rat mammary gland to cancer induction by experimental carcinogens such as 7,12-dimethylbenz(*a*)anthracene (DMBA). EFSA noted that this could be linked to an enhanced cell proliferation/apoptosis ratio, as reported by the authors, and indicated that the effects deserved further consideration.

FAO/WHO (2011)
The FAO/WHO Expert Meeting concluded that *"BPA has been studied in rodent carcinogenicity studies with dosing beginning in young adulthood. The studies, although suggestive of increases in certain tumour types, were considered not to provide convincing evidence of carcinogenicity. BPA exposure during the perinatal period has been reported to alter both prostate and mammary gland development in ways that may render these organs more susceptible to the development of neoplasia or preneoplastic conditions with subsequent exposures to strong tumour-initiating or tumour-promoting regimens. In the absence of additional studies addressing identified deficiencies, there is currently insufficient evidence on which to judge the carcinogenic potential of BPA."*

The Expert Meeting also reviewed the body of evidence demonstrating proliferative changes in the mammary gland and changes in the prostate gland following perinatal exposures to BPA and concluded that the studies had deficiencies in design or execution that prevented a definitive evaluation of BPA's carcinogenic potential, including lack of consideration of litter effects, small numbers of animals, study duration and/or additional treatment with a strong initiating or additional promoting agent(s). The meeting concluded that there was currently insufficient evidence to judge the carcinogenic potential of BPA for the mammary gland, prostate or other organs.

ANSES 2011, 2013
In 2011, ANSES considered, in relation to the carcinogenicity of BPA in rodents, that there were "proven" effects of BPA on acceleration of structural maturation of the mammary glands in adult rodents associated with prenatal or perinatal exposure (Markey et al., 2001; Munoz-de-Toro et al., 2005; Murray et al., 2007; Moral et al., 2008; Vandenberg et al., 2008); "proven" effects of BPA on the development of intraductal hyperplastic lesions in adult animals after pre- or perinatal exposure (Durando et al., 2007; Murray et al., 2007); "proven" effects of BPA on the development of intraductal hyperplastic lesions in adult animals after pre- or perinatal exposure (Durando et al., 2007; Murray et al., 2007); a suspected effect of BPA on the development of neoplastic lesions (intraductal carcinoma in situ) after perinatal exposure; a "suspected" effect of BPA on enhanced susceptibility of the mammary glands to tumour development later in life (after exposure to a known carcinogenic agent) due to pre- or perinatal exposure based on the studies by Jenkins et al. (2009) and Betancourt et al. (2010).

Additionally, ANSES concluded that reported effects of BPA on the prostate in animals were "controversial".

In 2013, ANSES concluded that "*the studies showing the development of neoplastic-type lesions (ductal carcinoma) or even an increase in the likelihood of mammary glands subsequently developing mammary tumours (during co-exposures to a carcinogenic agent) were to be considered for risk assessment.* In particular, ANSES used the architectural changes of the mammary gland (Moral et al.,

2008, oral NOAEL of 25 µg/kg bw per day in prenatally-exposed rats (by the oral route) and ductal hyperplasia (Murray et al., 2007; sc LOAEL of 2.5 µg/kg bw per day (no NOAEL could be identified) as points of departure for its risk assessment.

3.9.2.2. Overview of specific animal studies on effects of BPA on cell proliferation and other endpoints considered relevant to carcinogenicity after oral or subcutaneous exposure to BPA, published before 2010

The WoE approach that has been taken in the current opinion has necessitated the inclusion of a number of key/pivotal studies on the proliferative effects of BPA, particularly on the mammary gland, and effects on other endpoints considered relevant to carcinogenicity, already evaluated in the previous risk assessments summarised above. These include studies carried out using the oral route of exposure (e.g. Moral et al. 2008; Jenkins et al., 2009; Betancourt et al., 2010) and also studies using the subcutaneous route of exposure, that were not previously considered in the risk assessments carried out by EFSA in 2006 and 2010. These studies have been briefly summarised here and also included in the WoE tables presented in Appendix C.

Proliferative changes in mammary gland

In reviewing the Moral et al. (2008) study, EFSA CEF Panel (2010) noted that the effects of prenatal BPA exposure (25 and 250 µg/kg bw per day applied by gavage on days 10-21 post-conception) on mammary gland morphology, proliferation and modification of gene expression were investigated in Sprague-Dawley CD rats. The architectural modifications induced by the higher dose of BPA in mammary glands of female offspring were transient increases in the total number of epithelial structures (day 21 only), (terminal end buds (TEBs), terminal ducts (TDs), alveolar buds (Abs), and type 1 lobules (Lob 1) (days 21 and 100, but not at days 35 and 50) and lobule type 1 (day 35 only). The proliferative index in the epithelial structures was not affected by BPA treatments. Time- and dose-dependent modifications in gene expression profiles were observed after treatment with both doses of BPA: modulated (mainly up-regulated) genes related to cell proliferation, apoptosis and differentiation, cell communication, signal transduction, immunity, protein metabolism and modification.

In a study examining the effect of lactational exposure to BPA on dimethylbenzanthracene (DMBA)-induced mammary cancer in female offspring, Jenkins et al. (2009) gavaged nursing Sprague-Dawley rats with BPA (0, 25 or 250 µg/kg b.w./day) from lactation day 2 to 20. Increased cell proliferation and reduced apoptosis in the mammary gland of female offspring were observed at the high dose group at 50 days of age but not at 21 days of age. Consistent with increased proliferation and reduced apoptosis, the authors reported changes in expression of a number of proteins linked with apoptosis and also changes in progesterone receptor (PR)-A, steroid receptor activator (SRC) 1 to 3, and erbB3. The expression of oestrogen receptor (ER)-α was slightly reduced. At 50 days of age, one female offspring from each litter of each treatment group was given a single gavage dose of DMBA (30 mg/kg). BPA-treatment increased the number of tumours (2.84 ± 0.31, 3.82 ± 0.43, and 5.00 ± 0.88 for control, low and high BPA groups, respectively) with the effect at the high dose group being statistically significant. Tumour latency was also reduced (65, 53, 56.5 days for control, low and high BPA groups, respectively) with statistically significance at the high dose group. The CEF Panel noted however that the study had limitations, as documented in Appendix B.

In the study by Betancourt et al. (2010), involving prenatal BPA exposure of female Sprague-Dawley rats to 0, 25 or 250 µg BPA/ kg bw per day, administered by gavage on GD 10-21, the high BPA dose (250 µg BPA/ kg bw per day, GD 10-21) was reported to enhance cell proliferation in mammary glands of the offspring (whereas apoptosis was not affected), associated with an increased cancer susceptibility and shift of the window for susceptibility for DMBA-induced tumourigenesis in rat mammary gland from PND50 to PND100. However, the study revealed similar shortcomings in design and reporting as the study by Jenkins et al. (2009), and the CEF Panel concluded at that time that these data cannot be taken into consideration for derivation of a TDI for BPA.

In relation to studies using the subcutaneous route (s.c.) of administration, EFSA (CEF Panel, 2010) had previously noted that "*Studies using s.c. application of BPA also indicated that prenatal BPA exposure results in an increased cell proliferation/apoptosis ratio in normal tissue as well as preneoplastic lesions of rat mammary gland (Durando et al., 2007; Murray et al., 2007; Vandenberg et al., 2007; 2008).*" The CEF Panel has re-evaluated these studies, and has included them in its WoE analysis in reaching a conclusion regarding possible proliferative effects of BPA in the mammary gland. Summaries of the design and findings of these studies are provided in Appendix B. Additionally, the CEF Panel noted the findings of a number of earlier s.c. studies ((Markey et al., 2001, 2005; Munoz-de-Toro et al., 2005; Nikaido et al. 2004., 2005; Rubin et al., 2006) on the same endpoint, as summarised in Annex 2 of the EFSA opinion of 2006 (EFSA, 2006), and has similarly included them in its WoE analysis.

3.9.2.3. Evaluation of recent animal studies related to potential carcinogenic or proliferative effects and/or morphological changes due to BPA

This section provides an overview of the experimental animal studies relevant to the potential carcinogenic effects of BPA or effects on cell proliferation in certain organs that could be related to the development of cancer, published after 1st July 2010. A more detailed description and evaluation of each study is provided in Appendix B.

Mammary gland

Since the previous EFSA review (2010), further studies (Jones et al., 2010; Ayyanan et al., 2011; Jenkins et al., 2011; Weber Lozada and Keri, 2011; Kass et al., 2012; Tharp et al., 2012; Acevedo et al., 2013; Vandenberg et al., 2013c; US FDA/NCTR, 2013 and Delclos et al., 2014) have reported proliferative effects on mammary tissue and/or effects on mammary tumour growth following administration of BPA. These studies mainly employed pre- or perinatal administration, with the exception of the studies using transgenic mouse models by Jones et al. (2010) and Jenkins and colleagues (2011), in which dosing took place during postnatal/adult life.

The study by Jones et al. (2010) used an adult knockout mouse model of mammary neoplasia that is believed to reproduce human susceptibility gene 1 (BRCA1*)-related breast cancer. The results indicated that exposure to a low dose of BPA (250 ng/kg bw per day) for 4 weeks using osmotic pumps increased mammary epithelial cell proliferation and hyperplasia in adult BRCA1* knockout mouse mammary glands compared with wild type mice exposed to vehicle (dimethyl sulphoxide) only. However, the CEF Panel noted that the phenotype of the transgenic mice is likely to involve morphological and histological changes, making it difficult to compare directly the effect of BPA between wild-type and adult Brca1 knockout mouse since the development stage of the mammary gland in the transgenic mouse may not be similar to that of an adult mouse. These in vivo results were complemented by in vitro mechanistic investigations in MCF-7 cells, supporting the hypothesis that loss of BRCA1* function in mammary cells can enhance BPA-induced cell proliferation via interference with the ERα signalling pathway.

The study of Jenkins et al. (2011) examined the susceptibility of female transgenic MMTV-erbB2/neu mice to the development of mammary carcinomas after oral exposure to BPA at levels 0, 2.5, 25, 250 or 2500 µg BPA/l in drinking water during adulthood (PND 56-252), estimated by the authors to be equivalent to 0, 0.5, 5, 50 and 500 µg BPA/kg bw per day. The aim of the study was to evaluate the effect of chronic administration of low doses of BPA to a strain of mice susceptible to mammary carcinoma. The treatment schedule was reported to result in a decreased tumour latency and increased tumour multiplicity, enhanced tumour volume and higher incidence of lung metastasis. These effects were observed at least in one of the two lower doses but not at levels of 250 or 2500 µg BPA/l drinking water. This was considered by the authors to be indicative of a non-monotonic dose-response. Conversely, an increase was reported in the cell proliferation index of mammary epithelial cells evaluated on PND 112, statistically significant from a level of 25 µg BPA/l drinking water, but without any further increase at higher dose levels (i.e. 250 and 2500 µg BPA/l in drinking water). The mammary epithelial apoptotic index increased at higher doses and achieved a statistical significance

only at the top dose of 2500 µg BPA/l in drinking water (equivalent to 500 µg BPA/kg bw per day). According to the authors the cell proliferation-to-apoptosis ratio displayed a non-monotonic dose-response curve that closely mimicked the tumourigenic response, although statistical analysis showed that only the BPA dose of 25 µg BPA/l in drinking water (equivalent to 5 µg /kg bw per day) produced a significantly greater cell proliferation-to-apoptosis ratio than in controls while the effects on mammary tumours (i.e. increase of tumour numbers per mouse, the reduction of tumour latency) were already statistically significant at a tenfold lower dose level.

This study by Jenkins et al. (2011) in transgenic mice addressed similar toxicity endpoints to those previously evaluated by the same research group in the DMBA mammary tumour rat model after lactational (Jenkins et al., 2009) or prenatal (Betancourt et al., 2010) BPA exposure. These earlier findings were reviewed by the CEF Panel in 2010 (EFSA CEF Panel, 2010), and were then considered to deserve further consideration. In contrast to the 2011 study, the 2009 Jenkins study in the DMBA mammary tumour rat model summarised above did not show a non-monotonic dose-response for any of the parameters tested. Many of the shortcomings that EFSA had noted in 2010 concerning study design and reporting of the Jenkins et al. (2009) and Betancourt et al. (2010) studies (EFSA CEF Panel, 2010), also apply to the Jenkins et al. (2011) paper, as summarised in the comments of the CEF Panel to the more detailed summary of the study provided in Appendix B. The time of necropsy of individual animals was not clearly reported; they were only described to be at 252 days of age or when tumour burden exceeded 10% of body weight. Additionally, there was no indication of animal randomisation, which the CEF Panel considered to be of particular importance when animals are derived from small transgenic colonies. The CEF Panel also noted that although BPA was administered in drinking water, the daily intake of water was only measured in preliminary studies and the daily exposure to BPA in the published study was therefore based on estimations.

The study conducted by Weber Lozada and Keri (2011) used the DMBA mammary tumour mouse model to assess the effects of fetal exposure to BPA (via oral gavage of the dams at dose levels of 0, 25 or 250 µg BPA/kg bw per day) on mammary tumour development in adults. A dose-response in the reduction in latency of mammary tumour development was observed in mice treated with BPA before birth. Reduced tumour latency after prenatal BPA exposure is in line with the findings of Jenkins et al. (2009), although the Jenkins study showed no dose-response relationship for this effect. The CEF Panel noted that no information was given on the tumour incidence, or on the number of animals that died of other causes than mammary cancer. There are also some concerns about methodological issues (incomplete histological evaluation, etc.). Moreover, the CEF Panel noted some uncertainties related to the histopathological examination of the induced tumours, which indicated that they were all squamous carcinomas and not the characteristic mammary adenocarcinomas found in this model. Overall, the CEF Panel considered that the work by Weber Lozada and Keri (2011) has limitations that hamper the clear interpretation of the data.

Two other new rodent studies reviewed used complex protocols in which the morphology, cell proliferation and other characteristics of mammary tissue were studied in offspring of mothers treated with BPA (Ayyanan et al., 2011; Kass et al., 2012). The CEF Panel considered that the morphological endpoints examined in these studies have no clear link to the development of mammary cancer in adult rodents or humans, although they provide some support for the hypothesis that BPA causes proliferative changes in the mammary gland. However the complexity of these studies, the limited numbers of animals, the likely experimental and inter-animal variability as well as lack of any exposure data in these studies hamper the clear interpretation of the data.

The study by Tharp and colleagues (2012) on BPA-related changes in the mammary gland of the monkey is notable as it studied mammary gland morphology in five control neonate rhesus monkeys and four neonates from mothers given orally 400 µg of BPA per kg of body weight daily from gestational day 100 to term. This regimen resulted in 0.68 ± 0.312 ng of unconjugated BPA per ml of maternal serum (range: 0.22–1.88 ng/ml), and 39.09 ± 15.71 ng/ml of conjugated BPA (range: 11.42–94.82 ng/ml), as assessed in a toxicokinetic experiment using deuterated BPA. Morphometric analysis

of the mammary glands removed from female offspring at birth showed that there was a statistically significant difference between treated and controls in the number of buds/ductal mammary units per unit area. Although the CEF Panel acknowledged the value of a study in a primate model, it also noted that animal numbers and mammary gland sampling were limited (as expected for a study involving primates) and therefore possibly unrepresentative.

Vandenberg et al. (2013c) concluded that BPA induces proliferative changes in the mammary gland of the male CD-1 mouse. BPA was given to pregnant and lactating mice at doses of 0, 0.25, 2.5, 25 or 250 µg/bw per day via osmotic mini-pumps and mammary glands were examined at several time points (3-4, 7-9 and 12-16 months) in the adult offspring. The authors reported that the mammary glands of male offspring treated with BPA showed changes in ductal area and branching points compared with controls. The authors concluded that their results indicated a non-monotonic dose-response to BPA, since at 3-4 months animals exposed to 0.25 or 2.5 showed more advanced mammary gland development than the controls, but animals receiving 25 or 250 µg/bw per day were statistically indistinguishable from controls. Similar effects were seen at later time periods, but the pattern of dose-responses changed to monotonic dose curves at 12-16 months. A NOAEL was not identified. The CEF Panel noted that the study used few animals per group and limited sampling for measurement of the mammary gland development, while this phenomenon demonstrated considerable individual variability. Furthermore, the conclusions of the authors were based on slight, but statistically significant differences between the groups (for morphological measurements), with considerable individual variability in the measured effects as reflected in large standard errors around the mean (SEM). In some cases where no visible mammary gland was seen, another sample was collected from a litter mate, which the CEF Panel considered as inappropriate.

In a recent study that examined proliferative changes and development of neoplasia in the mammary glands of rats, BPA (0; 0.25; 2.5 or 250 µg/kg bw per day) was administered prenatally only (GD 9 – GD 23) or both pre- and perinatally (GD 9 – PND 21) to Sprague Dawley rats via subcutaneously-implanted osmotic pumps (Acevedo et al., 2013). Mammary gland tissue was collected at PND 50, PND 90, PND 140 and PND 200 for histopathological evaluation of proliferative and neoplastic changes. Levels of total and unconjugated BPA were measured in the sera of dams, fetuses and nursing pups. Mean unconjugated internal dose levels of BPA of 1.25 mg/ml serum were reported in dams at the highest dose applied compared to no detectable levels in the controls. No statistically significant increase in the mean unconjugated serum levels was observed in fetuses after gestational exposure and pups after gestational and lactational exposure with the highest dose of BPA. At PND50 atypical ductal hyperplasia (ADH) was reported in a varying number of BPA-treated animals in all treatment groups (n=5 per group, incidence ranging from 0-60%) without a dose-effect relationship. Incidence of ADH was highest at the lowest BPA dose (0.25 µg/kg bw per day) after gestational exposure, whereas the same dose group exposed during gestation and lactation did not develop ADH. One animal (out of five) had a ductal carcinoma in situ (DCIS) at PND 50. ADH was also evident at PND 90, 140 or 200 following gestational or gestational + lactational exposure (n=23-35) and isolated mammary adenocarcinomas were observed in most groups, except in controls. One adenocarcinoma was observed at PND90 in the 2.5BPA group. However, the incidences of proliferative lesions and tumours were not statistically significantly increased in treated animals compared with controls. On the basis of these results, the authors concluded that BPA can act as a complete mammary gland carcinogen in the rat. The CEF Panel did not agree with this conclusion, noting that a small number of rats per group were examined at PND50; that the mean free BPA serum levels in fetuses were not significantly increased and those of pups were not detectable (<LOD) even at the highest BPA dose given; that overall the incidence of mammary lesions and mammary tumours was low, and data for the historical incidence of these lesions was not provided.

The US National Center for Toxicological Research (US FDA/NCTR) has recently completed a subchronic (90-day) toxicity study, conducted under the auspices of the NTP, involving pre- and post-natal administration of BPA to Sprague Dawley rats (US FDA/NCTR, 2013 and Delclos et al., 2014). The study was conducted as a range-finding study for a planned chronic toxicity/carcinogenicity

study. At the time of release of the EFSA draft opinion on BPA for consultation, the latter study has been started, but is in the very early phases and no results from the study will be available until 2016. As indicated in the report of the study, "*The major focus of the study was on reproductive tract development, and the rationale for concerns regarding potential effects on the prostate and mammary gland have been discussed thoroughly in the NTP Brief on BPA (Shelby, 2008); however, evaluation of other endpoints were included, including those related to cardiovascular toxicity and obesity."* The F1 rats were exposed throughout development in utero (from gestation day 6 up to parturition) and pups were directly dosed by gavage from PND 1 up to PND 90±5. Exposure of the mothers was stopped after PND 0. BPA doses of 2.5; 8; 25; 80; 260; 860 and 2 700 µg/kg bw per day were considered "low dose BPA" and the 100,000 and 300,000 µg/kg bw per day groups were considered "high dose BPA". Vehicle (0.3% carboxymethylcellulose) and naïve control groups were included as well as two doses (0.5 and 5.0 µg/kg bw per day) of ethinyl oestradiol (EE_2) as an oestrogen reference control. Additional groups were exposed from GD 6 to PND 21 for histopathological examination of the mammary glands. As is typical for NTP-sponsored studies, all pathological observations were subjected to extensive quality control procedures, including a re-reading of slides by a second internal pathologist, followed by a second set of review by outside pathologists, and finally a Pathology Working Group was convened during which consensus was reached on all diagnoses prior to release of the Final Pathology Report.

Mammary gland duct hyperplasia of minimal severity was reported in the female groups examined at PND 21. The incidence of hyperplastic lesions was statistically significant by at least one of the three statistical methods used when compared with the vehicle control group in the 2 700 and 100 000 µg/kg bw per day groups, but not in the 300 000 µg/kg bw per day group. This observation was considered possibly treatment-related by the study authors but not by the original study pathologist. Mammary gland duct hyperplasia was also reported in the high dose female BPA groups examined at PND 90. Using the Poly-k test, the increase in minimal severity mammary gland duct hyperplasia was statistically significant in the 300 000 µg/kg bw per day group compared with vehicle controls. A significant increase in incidence of mammary gland duct hyperplasia compared with vehicle control was seen in the 2 700, 100 000 and 300 000 µg/kg bw per day groups when analysis was carried out using the JT/SW or RTE statistical tests. Both of these tests incorporate lesion severity, but only the RTE method does not explicitly assume a monotonic dose-response curve (CFSAN, 2013). This increase was considered as a possible treatment-related effect by the study authors. BPA did not cause duct hyperplasia in the mammary glands of male rats, while conversely the reference oestrogen EE_2 induced hyperplasia in the male but not the female mammary gland. A single mammary gland ductal adenocarcinoma (1 out of 260 female rats in the entire study) was seen in the 2.5 µg BPA/kg bw per day dose group at PND 90.

The CEF Panel concluded that the observation of mammary hyperplasia in female rats in this study, albeit of minimal severity, was relevant for the risk assessment of BPA, given the findings in other studies reported above.

Prostate gland effects

EFSA CEF Panel (2010) had previously noted work linking BPA to trans-generational and developmental epigenetic changes in rodents, including aberrant expression of growth regulatory genes in rat prostate. In the one relevant study reviewed in the current opinion (Prins et al., 2011), the Sprague-Dawley rat model of prostate neoplasia was used to study the effects of dosing 10µg/kg bw BPA orally or subcutaneously on post-natal days 1, 3 and 5 on the development of prostate cancer in rats subsequently given both testosterone and ooestradiol-17β for 16 weeks from postnatal day 90 to drive prostatic intra-epithelial neoplasia (PIN) lesions in the prostate. Whilst microscopic evaluation suggested that BPA increased the incidence of prostate intraepithelial neoplasia, and (atypical) hyperplasia, the histopathology was confounded by the presence of prostatic inflammation. Moreover, the degree of cytological pleomorphism (atypia) reported in this study was insufficient to confirm the presence of intraepithelial neoplasia.

Effects in testes

In a study in which pregnant and lactating Long-Evans rats were given BPA via gavage from gestational day 12 to postpartum day 21, Leydig cell division was stimulated in the prepubertal period and increased Leydig cell numbers were shown in the testes of adult male rats at 90 days (Nanjappa et al., 2012). The CEF Panel noted that rats are quantitatively far more sensitive to the development of Leydig cell tumours than men, since Leydig cell luteinizing hormone releasing hormone (gonadotropin-releasing hormone) receptors are unique to rats and also have over 10 times more luteinizing hormone receptors than men (Cook et al., 1999).

Effects on liver

In the study from Weinhouse et al (2014) mice (10 males and 10 females/group) were treated perinatally through maternal diets with 0, 50 ng, 50 ug or 50 mg BPA/kg diet (corresponding to 0, 10 ng, 10 ug or 10 mg BPA/kg bw per day (calculation based on dam wt) up to weaning (PND 22). Pups were maintained on standard phytoestrogen-free control diet for 10 months.

In the females of the top dose the incidence of combined adenomas and carcinomas with or without preneoplastic lesions was significantly increased, but no significant effect was seen in males.

Liver tumours were also seen in male control mice. Moreover, historical incidence of liver adenomas and carcinomas in female was not reported for the isogenic mice.

3.9.2.4. Summary of the evidence for carcinogenicity, effects on cell proliferation and morphological changes induced by BPA in animals

In their 2010 EFSA opinion, the CEF Panel concluded that the studies then available suggested possible enhanced susceptibility to mammary tumours in rodents exposed to BPA during development, which deserved further attention. These conclusions were based mainly on the review of the studies by Jenkins et al. (2009) and Betancourt et al. (2010) in the rat model of DMBA-induced mammary carcinogenesis after either lactational or in utero BPA exposure. The CEF Panel considered at that time that these studies had several shortcomings that precluded their use for the derivation of a new TDI, and the data had unclear relevance for human health.

Since then, a number of laboratory animal studies have been reported to show effects on mammary tissue (mammary tumour induction, enhancement of mammary tumour growth and/or proliferative changes in mammary gland) after prenatal, perinatal and adult exposure to BPA. In relation to possible carcinogenic effects of BPA in animals when exposed postnatally/during their adult life, the CEF Panel noted that while the study of Jenkins et al. (2011) showed an increased susceptibility of adult female transgenic MMTV-erbB2 mice to the development of mammary carcinomas after oral exposure to BPA, the study had a number of deficiencies as discussed above, and the result is at variance with the lack of any tumorigenic effect in female mice in the 2 year NTP study at high BPA doses. The relevance of the findings in this sensitive transgenic mouse model is also uncertain. The observation that BPA induces prostatic "intraepithelial neoplasia" in the prostate of rats exposed in the immediate postnatal period (Prins et al. 2011) is also of uncertain relevance given the background inflammatory changes occurring in the animals and judged deficiencies in the histopathological examination. Overall the CEF Panel concluded that these studies do not provide convincing evidence that BPA is carcinogenic in animals when exposed postnatally/during their adult life.

In relation to possible carcinogenic effects of BPA in animals when exposed prenatally, several studies including the new study of Weber Lozada and Keri (2011), and the earlier studies of Jenkins et al. (2009) and Betancourt (2010) used the DMBA mammary tumour mouse model to assess the effects of fetal exposure to BPA on mammary tumour development in adults. The authors reported an increased susceptibility to developments of mammary cancer, decreased tumour latency and increased tumour multiplicity. The study of Acevedo et al. (2013) reported proliferative changes and development of neoplasia within a relatively short time period (up to PND 200) in the mammary glands of rats exposed to BPA prenatally or both pre- and perinatally. In contrast the study of Ayyanan et al. (2011) did not demonstrate an increased incidence of mammary tumours following exposure to BPA over a 1-year period. Overall, however, the CEF Panel concluded that based on the

WoE evaluation and the experimental deficiencies in the studies, the findings in these studies do not provide convincing evidence that BPA is carcinogenic in animals when exposed pre- or perinatally.

The other new studies (Jones et al., 2010; Kass et al., 2012; Vandenberg et al., 2013c) examined the effects of BPA exposure on mammary gland proliferation in rodents as a possible indicator of potential tumourigenesis in this organ. One study (Tharp et al., 2012) reported advancement of developmental parameters in the mammary gland of rhesus monkeys, with increased epithelial density of terminal endbuds. The US FDA/NCTR subchronic toxicity study provided some evidence of a BPA-related effect in the mammary gland of female rats.

Overall, the CEF Panel concluded that although there were methodological weaknesses in all these studies with the exception of the US FDA/NCTR subchronic toxicity study, which was a detailed guideline study conducted in accordance with GLP, taken together they provide further evidence that BPA may enhance mammary epithelial proliferation in animal models.

3.9.3. In vitro studies related to carcinogenesis/cell proliferation

None of the new publications on in vitro-effects of BPA include experimental models for the screening of carcinogens, e.g. cell transformation assays. The reported findings rather support recent evidence of BPA-mediated induction of cell proliferation and inhibition of apoptotic pathways. A reduction of rapamycin- or tamoxifen-induced apoptosis was observed in non-malignant human breast epithelial cells at low nanomolar BPA concentrations along with BPA-induced transcriptional changes (e.g. increased expression of total and phosphorylated AKT1, down-regulation of p53) which are related to the induction of cell growth and are resembling those changes observed in carcinogenic progression (Goodson et al., 2011; Dairkee et al., 2013). In a human epithelial breast cell line (HBL-100) proliferation was induced at 10^{-10} M BPA (Wu et al., 2012). Increased proliferation was also observed in cell cultures of normal human mammary epithelial cells treated with a high nanomolar BPA concentration (10^{-7} M) and changes in DNA methylation associated with tumor development (e.g. CDKN2A hypermethylation) were reported at 10^{-8} M BPA in these cells (Qin et al., 2012a).

The in vitro studies in mammary epithelial cells suggest that BPA at nanomolar concentrations can modulate proliferation-associated signalling pathways. Whilst these changes may also be crucial for tumorigenesis, the in vitro findings do not allow for a clear interpretation of the complex concentration- and time-dependent pattern of the molecular changes in vivo and thus the relevance of in vitro models using artificial culture conditions (e.g. hormone or oxygen concentrations) to the in vivo situation is still unclear. In human epithelial ovarian cancer cells (BG-1) BPA (10^{-9} M – 10^{-7} M) induced growth and the expression of the stromal cell derived factor-1 (CXCL12) (Hall et al., 2012). Both the BPA induction of growth and that of CXCL12 were inhibited by the ER antagonist ICI 182,780 or by transfection of cells with CXCL12 siRNAs indicating that the ER-CXCL12-CXCR4 signalling pathway is important for the BPA effects in these cells. In breast cancer cells and cancer-associated fibroblasts, BPA (at 10^{-7} M and higher) induced proliferation and migration via a G protein-coupled receptor pathway (GPR30/GPER) (Pupo et al., 2012). Other in vitro studies using human breast (Jung et al., 2011; Lee et al., 2012b; Zhang et al., 2011; Tilghman et al., 2012) and ovarian (Hwang et al., 2011) cancer cells reported effects only at high concentrations of BPA (at 10^{-7} M and above)) which are out of the inclusion criteria (see Appendix A).

3.9.4. WoE of the possible carcinogenicity of BPA in humans and animals and its potential to cause proliferative changes or advancement of developmental parameters in tissues

Whether BPA induces and/or promotes carcinogenicity in organs such as mammary gland, prostate gland or testis, or causes proliferative changes or advancement of developmental parameters in these organs or in vitro that could potentially be linked to development of cancer was considered using a tabular format for weighting different lines of evidence (WoE evaluation). The outcome of the WoE evaluation of BPA genotoxicity is also included, given the relevance of genotoxicity in the evaluation

of the possible carcinogenicity of BPA. The overall outcome of this WoE evaluation is presented below, while the WoE evaluation tables for these endpoints are presented in full in Appendix C. For interpretation of these tables refer to Appendices A and C.

Table 14: Overall table on WoE evaluation of genotoxicity, carcinogenicity and cell proliferation/morphological changes of BPA

GENOTOXICITY	
Overall conclusion on in vivo genotoxicity studies – via non-thresholded mechanism: BPA has not been shown to be clastogenic in vivo (micronuclei and chromosomal aberrations).	**Unlikely**
Overall conclusion on in vivo genotoxicity studies - via thresholded mechanism: The potential of BPA to produce aneuploidy in vitro was not expressed in vivo, based on negative findings obtained for induction of aneuploidy in female and male mouse germ cells (Pacchierotti et al., 2008) and for induction of micronuclei in somatic bone marrow cells (Masuda et al., 2005; Pacchierotti et al., 2008; Naik and Vijayalaxmi , 2009; De Flora et al., 2011). However, BPA proved to induce dose-related increases of c-like metaphases in mouse bone-marrow cells (Naik and Vijayalaxmi , 2009) and significant increases in the number of metaphase II oocytes with prematurely separated chromatids (Pacchierotti et al., 2008) pointing to potential mitotic spindle disrupting effects of BPA in vivo.	**As likely as not**
CARCINOGENICITY	
Overall conclusion on carcinogenicity of BPA in humans: The very few epidemiological studies published to date, investigating a possible association between exposure to BPA and incidence of certain cancers, specifically breast cancer (Yang et al., 2009) and meningioma (Duan et al., 2012), do not allow any conclusion to be drawn regarding the carcinogenicity of BPA in humans.	**Insufficient data for application of WoE approach**
Overall conclusion on carcinogenicity of BPA in animals when exposed during their adult life (post-pubertal) only: Effects on mammary gland growth demonstrated in a transgenic mouse model with post-pubertal exposure (Jenkins et al., 2011) or on reported prostatic "intraepithelial neoplasia" in rats exposed to BPA in the immediate postnatal period (Prins et al., 2011) do not provide convincing evidence that BPA is carcinogenic in animals when exposed postnatally/during their adult life.	**Unlikely to as likely as not**
Overall conclusion on the carcinogenicity of BPA in animals exposed during pre- and post-natal (during lactation) development: Recent studies **(**Jenkins et al., 2009, Betancourt et al., 2010; Weber Lozada and Keri, 2011; Acevedo et al, 2013) investigated the potential carcinogenic effects of fetal or perinatal exposure to BPA. Given the various weaknesses identified in these studies, they do not provide convincing evidence that BPA is carcinogenic in animals when exposed during pre- and post-natal (during lactation) development.	**Unlikely to as likely as not**
CELL PROLIFERATION	
Overall conclusion on proliferative changes of BPA in animals when exposed postnatally/ during their adult life: Both Jones et al (2010) and Jenkins (2011) reported an effect on mammary gland proliferation in sensitive transgenic or gene knock-out models as well as wild-type mice (Jones et al. only) given BPA during adulthood, although proliferative effects in these mouse models are inconsistent with the lack of any proliferative effect on mammary tissue in mice in the two year NTP carcinogenicity study. However, the CEF Panel concluded overall that there is some indication that BPA can cause proliferative changes in the mammary gland of genetically modified animals when exposed postnatally/in adult life. The CEF Panel considered that evidence for proliferative changes induced by BPA in other organs (i.e. prostate) is currently too weak to reach a conclusion.	**As likely as not (for mammary gland proliferation)**

CELL PROLIFERATION	
Overall conclusion on BPA- induced proliferative changes/ developmental advancement in the mammary gland of animals exposed during pre- and/or post-natal (during lactation) development or up to PND 90 (gavage): The EFSA opinion of 2010 noted potential proliferative effects of fetal or perinatal exposure to BPA. Since 2010 additional studies including a study in non-human primates (Ayyanan et al., 2011, Tharp, 2012, Vandenberg, 2013c, Acevedo, 2013, US FDA/NCTR, 2013 and Delclos et al., 2014) have also suggested that BPA can have proliferative effects on mammary tissues and strengthen the evidence for an effect of BPA on mammary gland proliferation in animals exposed during pre- and post-natal development. The CEF Panel considered that evidence for proliferative changes induced by BPA in other organs (e.g. testis: Nanjappa et al., 2012) is currently too weak to reach a definite conclusion.	**Likely (for mammary gland proliferation)**

3.9.5. Conclusion on carcinogenicity of BPA and proliferative/morphological changes in tissues induced by BPA based on evidence from human, animal and in vitro studies

In summary, BPA is not mutagenic (in bacteria or mammalian cells), or clastogenic (micronuclei and chromosomal aberrations). The potential of BPA to produce aneuploidy in vitro was not expressed in vivo. The positive finding in the postlabelling assays in vitro and in vivo is unlikely to be of concern, given the lack of mutagenicity and clastogenicity of BPA in vitro and in vivo.

The CEF Panel concluded that very few epidemiological studies published to date have investigated a possible association between exposure to BPA and incidence of certain cancers, specifically breast cancer and meningioma. These studies do not allow any conclusion to be drawn regarding the carcinogenicity of BPA in humans.

BPA was not carcinogenic in two standard oral cancer bioassays in rats and mice exposed (from 6-8 weeks of age) for 2 years. New results do not provide convincing evidence that BPA is carcinogenic in animals when exposed during their adult life or when exposed perinatally.

Using a WoE approach, the CEF Panel assigned a likelihood level of "unlikely to - as likely as not -" to carcinogenic effects of BPA. Since the likelihood level for this endpoint is less than "as likely as not" (see Appendix A), this endpoint was not taken into account in the evaluation of uncertainty for hazard characterisation and risk characterisation.

Earlier evidence for BPA effects on cell proliferation and differentiation in the mammary gland and other tissues has been supported by recent studies, e.g. a subchronic rat study with prenatal exposure to BPA. The proliferative changes in the mammary gland reported in these new studies, including a non-human primate study, are insufficient to conclude that there is a link to cancer development in later life. However, there might be a possible role of BPA in increasing the susceptibility to mammary gland carcinogenesis.

The proliferative responses and possibly enhanced sensitivity to mammary gland carcinogens seen in animal studies might be of relevance for human health and are therefore included in the risk assessment. An ongoing long-term study on BPA in rats[13], including in utero exposure, may help to clarify whether these proliferative changes or changes in differentiation result in an increased incidence of tumours in this species.

[13] [13] See at: http://ntp.niehs.nih.gov/testing/status/agents/ts-10034-y.html

Using a WoE approach, the CEF Panel assigned a likelihood level of "likely" to BPA induced proliferative changes in the mammary gland. Therefore, this endpoint was brought forward for hazard characterisation and for uncertainty analysis (Section 4.3).

The CEF Panel considered that the evidence for proliferative changes induced by BPA in other organs (e.g. prostate or testis) is currently too limited to reach a conclusion.

3.9.6. Hazard characterisation (dose-response relationship) for effects of BPA on the mammary gland of animals

The above analysis indicates, based on a WoE approach, that while there is no convincing evidence that BPA is carcinogenic in animals when exposed as adults or during pre- and post-natal (during lactation) development, a number of the animal studies reviewed above suggest that BPA can have a proliferative/developmental advancement effect on mammary tissue, prostate epithelium and Leydig cells and may also have an effect on tumour growth in animal models, particularly in sensitive transgenic models or when followed by a treatment with a complete carcinogen. Effects in many of these studies are seen at dose levels well below the current NOAEL of 5 mg/kg bw per day BPA, although in the recent robust study of US FDA/NTCR (2013) and Delcos et al. (2014), proliferative changes were primarily seen at the high dose levels of BPA (100 000 and 300 000 µg/kg bw per day). On the basis of the WoE analysis as summarised in table 14 and described in more detail in Appendix C, the proliferative effect of BPA on the mammary gland is considered to be a "likely" effect and is taken forward for risk characterisation (see Section 5), given the consistency of the effect in a number of studies. The proliferative effects reported in the prostate and the testis in several studies were not taken forward for hazard/risk characterisation as the CEF Panel considered that the evidence for such effects was currently too weak to be used in risk assessment.

A prerequisite for the risk characterisation step is hazard characterisation, involving examination of a possible dose-response relationship for the effect under consideration and identification of a dose level at which the effect is not anticipated to occur (NOAEL) or a dose level at which the incidence of the effect is considered to be low (LOAEL or BMDL). The CEF Panel has considered the evidence for a dose-response relationship for mammary gland proliferation in the studies showing such an effect and reviewed in this opinion (Markey et al., 2001; Munoz-de-Toro et al., 2005, Durando et al., 2007; Murray et al., 2007; Vandenberg et al., 2007; 2008; Moral et al., 2008; Jenkins et al, 2009; Betancourt et al., 2010; Jones et al., 2010; Ayyanan et al., 2011; Jenkins et al. 2011; Tharp et al., 2012; Acevedo et al., 2013; US FDA/NCTR, 2013 and Delclos et al., 2014; Vandenberg et al., 2013c). Many of these studies showed effects at low doses of BPA, but some of them were single dose studies while other studies with multiple doses showed increases in proliferation without a clear dose-relationship. Several of the studies (e.g. Markey et al., 2001; Murray et al., 2007; Ayyanan et al. 2011; Vandenberg et al., 2013c) were reported to show a non-monotonic dose-response curve for proliferation (Section 4.3). The CEF Panel considered the effects nonetheless supportive of a BPA-induced effect on the mammary gland. As shown in table 15 the lowest doses (LOAELs) at which the proliferative effects on the mammary gland were reported ranged from 25 ng BPA/kg bw per day (Munoz-de-Toro et al., 2005) to 2.7 mg/kg bw per day (US FDA/NCTR, 2013 and Delclos et al., 2014).

Table 15: BPA-induced proliferative changes in the mammary gland of various species

Study	Administration, animal species	LOAEL/NOAEL
Acevedo et al., 2013	0, 0.25, 2.5, 25 and 250 µg BPA/kg bw per day subcutaneously from GD9 to GD23 to Sprague Dawley rats	Atypical ductal hyperplasia (ADH) was reported in a few animals in all treatment groups without reaching statistical significance

Study	Administration, animal species	LOAEL/NOAEL
Ayyanan et al., 2011	2.5 µg/L to 5000 µg/L, 0.6, 3, 6, 12, 120, 600 and 1200 µg BPA/kg bw per day in drinking water of C57Bl/6 mice.	NOAEL for mammary cell number 3 µg BPA/kg bw per day, but this was a non-monotonic LOAEL for increase in terminal end buds.
Betancourt et al., 2010	0, 25 or 250 µg BPA/ kg bw per day (GD 10-21) to Sprague-Dawley CD rats rat, cell proliferation and gene expression measured in high dose and controls only	Cell proliferation as measured by Ki-67 expression significantly increased compared with control at 250 ug/kg b.w. per day, but 25 ug/kg bw dose not examined.
Durando et al., 2007	25 µg BPA/kg bw per day administered sc by mini-pump from from GD 8 to GD 23 in Wistar rats.	LOAEL 25 µg BPA/kg bw per day.
US FDA/NCTR, 2013 and Delclos et al., 2014	2.5, 8, 25, 80, 260, 840, 2700, 100 000, 300 000 µg BPA/kg bw per day by gavage to F0 female Sprague-Dawley rats from GD 6 up to labour onset and pups from PND 1 until tissue harvesting, up to PND 90	LOAEL for ductal hyperplasia 2,700 µg/kg bw
Jenkins et al., 2009	0, 25 or 250 µg BPA/kg bw per day by gavage to nursing Sprague-Dawley rats from lactation day 2 to 20	Cell proliferation as measured by Ki-67 expression significantly increased compared with control at 250 ug/kg b.w. per day, but 25 ug/kg bw dose not examined.
Jenkins et al., 2011	0, 2.5, 25, 250, 2500 µg BPA/L given in drinking water to young adult female MMTV-erbB2 mice (PND 56-252), estimated to be 0, 0.5, 5, 50 and 500 µg BPA/kg bw per day.	Ratio of cell proliferation index to apoptotic index was significantly increased at the 5 µg BPA/kg bw per day dose level only.
Jones et al., 2010	0.25 µg BPA/kg bw per day for 4 weeks using osmotic pumps in adult BRCA* knockout mice compared to wild type mice	Increased epithelial cell proliferation at 0.25 µg BPA/kg b.w./day
Markey et al., 2001	0, 25 and 250 ng BPA/kg bw per day administered sc by mini-pump to CD-1 mice from GD 9 through postnatal day 4.	LOAEL 25 µg BPA/kg bw per day; No dose-response, the reported effect being slightly greater at 25 µg BPA/kg bw per day
Moral et al., 2008	25 and 250 ug BPA/kg bw per day administered to Sprague-Dawley rats from day 10 post-conception to delivery.	NOAEL 25 µg BPA/kg bw per day (for morphological changes)
Munoz-de-Toro et al., 2005	25 and 250 ng BPA/kg bw per day administered sc by mini-pump to ovarectomised and intact CD-1 mice from day 9 of pregnancy through postnatal day 4.	LOAEL 25 ng BPA/kg bw per day
Murray et al., 2007	2.5, 25, 250 and 1000 µg BPA/kg bw per day administered sc by mini-pump from GD 9 until postnatal day (PND) 1 in Wistar-Furth rats.	LOAEL 2.5 µg BPA/kg bw per day
Tharp et al., 2012	Rhesus monkeys given orally 400 µg of BPA per kg of body weight daily from gestational day 100 to term.	LOAEL 400 µg/kg bw per day.
Vandenberg et al., 2007, 2008	250 ng BPA/kg bw per day administered sc by mini-pump from GD 9 to day 18 in CD-1 mice	LOAEL 250 ng BPA/kg bw per day.
Vandenberg et al., 2013c	0, 0.25, 2.5, 25 and 250 µg BPA/kg bw per day subcutaneously from day 9 of pregnancy for 14 days until day 16 of lactation in male CD-1 mice	LOAEL 25 µg/kg bw

The CEF Panel considered that none of these studies was sufficiently robust methodologically or showed a consistent dose-response to be used as the basis of a revised TDI. In particular the CEF Panel noted that the results of the two studies carried out by Nikaido et al. (Nikaido et al., 2004, 2005) reported on scores for tissue differentiation. The CEF Panel concluded however that the studies cited could be used in the WoE approach to support the conclusion that it is "likely" that pre- and postnatal (during lactation) exposure to BPA results in proliferative changes in the female mammary gland in animal models, including the Tharp et al. (2012) study in rhesus monkeys, a species that is considered to have particular relevance for human health risk assessment (Tab. 14, Appendix C).

The 2013 US FDA/NCTR subchronic toxicity study, involving prenatal exposure of adequate numbers of rats to a very wide range of BPA doses was considered by the CEF Panel to be a detailed and methodologically robust study, conducted in accordance with GLP that could be used on its own for risk assessment purposes. The CEF Panel noted, however, that the design of the study involved two high dose levels of BPA (100 mg/kg bw per day and 300 mg/kg bw per day) and seven "low" dose levels, ranging from 2.5 to 2700 µg/kg bw per day, together with a vehicle and a naïve control, with a very wide spacing between the high and low dose ranges. The CEF Panel noted also that the study was a range-finding study for a subsequent chronic toxicity/carcinogenicity study, and that it was not designed for the purpose of establishing a health-based guidance value.

Nevertheless, the CEF Panel considered that the study provided some evidence for a BPA-related effect in the mammary gland of female rats at 100 000 and 300 000 µg/kg bw per day, and possibly also at the 2 700 µg/kg bw per day dose level (at PND 21). The table below shows the dose-response for these data. The CEF Panel also noted that results of hormone analyses in the high dose BPA female rats showed that oestradiol and prolactin levels were significantly higher than vehicle controls, effects that could possibly be linked to the mammary gland proliferation seen in these animals.

Table 16: Dose-response information for mammary duct hyperplasia in BPA exposed rats (data from FDA/NCTR, 2013; Delclos et al, 2014)

Dose µg/kg bw per day	**Summary data on incidence of mammary duct hyperplasia in female rats at PND 21 (**US FDA/NCTR, 2013 and Delclos et al., 2014)		**Summary data on incidence of mammary duct hyperplasia in female rats at PND 90 (**US FDA/NCTR, 2013 and Delclos et al., 2014)	
	Incidence	**Group size**	**Incidence**	**Group size**
0.0	0	16	7	20
2.5	2	19	11	23
8	1	13	6	18
25	4	19	11	21
80	1	20	8	20
260	1	13	8	20
840	2	18	9	20
2 700	5 ($p < 0.05$)	17	11	20
100 000	6 ($p < 0.01$)	17	13	20
300 000	3	12	14	19 ($p < 0.01$)

Note: results were not significant compared to vehicle control (poly-k test) except where stated

The data shown in table 16 were therefore subjected to statistical dose-response modelling in an attempt to calculate the BMDL for mammary duct hyperplasia. The report in Appendix V shows the outcome of this modelling. Following detailed analysis of the results, the CEF Panel concluded that the data could not be used to provide such a BMDL, since the outcome of modelling contained considerable uncertainty, shown by relative large differences in the BMDLs calculated from the different models, and a wide confidence interval (more than 10 fold difference between the BMD and BMDL) for some models (see Section 4).

3.9.7. Conclusions on hazard characterisation for effects on the mammary gland in animal models

While the CEF Panel concludes that this endpoint should be considered in the risk assessment of BPA since it has been concluded that it is "likely" that prenatal exposure to BPA results in effects on the female mammary gland in animal models, the CEF Panel considered that none of these studies were sufficiently robust methodologically or showed a consistent dose-response that could be used to compare with the current TDI or as the basis of a revised TDI.

3.10. Mechanisms of action of BPA including epigenetic effects

3.10.1. Summary of previous reviews on endocrine-mediated action of BPA

Numerous *in vitro* and *in vivo* studies have investigated the mechanisms of action of BPA and have been reviewed on a number of occasions (e.g. EFSA, 2006; EFSA CEF Panel, 2010; FAO/WHO, 2011). Many effects induced by BPA appear to be tissue-, sex- and concentration-specific. For several BPA-induced effects "windows of exposure" have been reported. Due to the complexity of BPA´s interaction with different hormone receptors and signalling pathways it is challenging to establish which specific endocrine mechanism triggers a certain in vivo effect of BPA. As an additional mode of action of BPA epigenetic effects have been reported, i.e. changes in DNA methylation, histone modification and miRNA expression patterns. Overviews of mechanistic studies aimed at identifying the mode of action of BPA have been included in a number of previous evaluations of BPA and are summarised as follows.

EU-RAR (2003, 2008)
The EU-RAR noted that BPA has oestrogenic activity in vitro and in vivo, its activity being generally 3-5 orders of magnitude less than that of 17β-oestradiol, and that there was also limited evidence for anti-androgenic activity and stimulation of progesterone activity, as well as an increase in prolactin release.

EFSA (2006); EFSA CEF Panel (2010)
In 2006, the EFSA AFC Panel described several studies showing altered gene expression in target organs for BPA, in particular in oestrogen-responsive genes, and also discussed the weak oestrogenicity of BPA (and its higher binding affinity for the beta oestrogen receptor as compared to the alpha receptor) along with the weak antagonism to thyroid hormone receptors and the weak interference with different steps in androgen receptor function in vitro. The AFC Panel noted that the in vitro observations in specific cellular systems had an unclear relevance for the risk assessment of adverse in vivo effects of BPA.

In its 2010 opinion, the EFSA CEF Panel considered that effects induced by low BPA concentrations (<5 mg/kg bw per day) may be independent of the classical hormone receptor pathway and may be alternatively induced by cell membrane-triggered signalling pathway via protein kinases. No conclusion could be reached on the implications of the observed biochemical and molecular changes and their potential impact on human health.

In its 2010 opinion EFSA also noted that according to several study reports BPA has been "*linked to transgenerational and developmental epigenetic changes*" in different rodent tissues, *e.g.* mammary glands, prostate, forebrain and reproductive tract. BPA-induced epigenetic alterations were associated with histopathological changes in rat prostate (Ho et al., 2006) and functional criteria such as RNA and protein expression, and cell turnover in rat prostate and the reproductive tract in mice (Ho et al., 2006; Bromer et al., 2010). EFSA noted at this time that "*a conclusion cannot be reached on the implications of the observed biochemical and molecular changes and to establish whether they have any impact on human health*."

NTP-CERHR (2008)

The NTP-CERHR monograph noted that a growing number of cellular targets for BPA have been identified, including non-nuclear oestrogen receptors such as ncmER, oestrogen-related receptor gamma ERR-γ and GPR30, and also the aryl hydrocarbon receptor (AhR). NTP-CERHR noted that these receptor interactions "*may help explain toxicological effects that are not considered oestrogenic or predicted simply based on the lower potency of bisphenol A compared to estradiol."*

FAO/WHO (2011)

The FAO/WHO Expert Meeting report includes an extensive review of the biological activities of BPA. The report concluded that "*available data show that BPA's biochemical and molecular interactions are complex, involving classic estrogen receptors as well as a variety of other receptor systems and molecular targets."* The FAO/WHO experts concluded in their review that BPA may exert pleiotropic cellular responses and tissue-type specific effects and that at cellular and intracellular levels BPA could exhibit non-monotonic dose-responses. At half-maximal activity concentration (AC_{50}) values below 10 µmol/l three main gene targets were mentioned, i.e. the oestrogen receptor 1 (ESR1, also referred to as oestrogen receptor alpha), xenobiotic sensing and metabolizing CYP enzymes and genes involved in the down-regulation of inflammatory responses. At higher AC_{50} values in excess of 100 µmol/l indications of cell toxicity were generally observed. It was concluded that dose-response analyses may be useful to identify the involvement of multiple receptor/signalling pathways.

While noting the oestrogenic activity of BPA, the meeting considered that it should not be considered to act only as an estrogen, or even as a selective estrogen receptor modulator (SERM), and concluded that "*The complexity of BPA's interactions and concentration ranges at which the observations have been made make it challenging to conclude whether a given in vivo finding is biologically plausible based on consistency and potency of a response compared with estrogens alone."* The FAO/WHO Expert Meeting additionally concluded that "*exposure to BPA in utero …has been shown to affect the methylation status and expression of several differentially methylated promoters, raising the possibility that BPA also acts through mechanisms resulting in alteration of CpG methylation."*

ANSES (2011; 2013)

In line with other evaluations, ANSES (2011) reported that in addition to its oestrogenic activity, BPA interacts with other cell receptors, including androgen, thyroid hormone and aromatic hydrocarbon receptors, the transmembrane oestrogen and GPR30 receptors and can induce expression of the nuclear peroxisome proliferation-activated receptor PPARγ. ANSES concluded that "*an interpretation of the effects of BPA only from the angle of an oestrogeno-mimetic effect would be simplistic. The involvement of several of these systems during an exposure to BPA could explain certain effects observed at low doses, owing to a possible synergy of action, but also the non-monotonic dose-response relationships reported in certain studies."*

3.10.2. Evaluation of recent mechanistic studies relevant to an understanding of the mode or modes of action of BPA

This section provides an overview of a number of in vitro mechanistic studies published after 1st July 2010 that contribute to the further identification and understanding of the mode or modes of action of BPA and hence are relevant to the asssessment of its risks for humans, also in light of the CEF Panel's previous evaluation of BPA in 2010 (EFSA CEF Panel, 2010).

A more detailed description and evaluation of each study is provided separately in Appendix B.

An overview on the interaction of BPA with classical ERs and other receptors is given in the Background Paper on mechanisms of action of BPA prepared for the FAO/WHO Meeting 2010 by Thayer and Belcher (2010). In a recent study by Li et al. (2012b) the cell type-specific BPA-induced activation of cell signalling via ERα and ERβ was investigated using three different human cell lines, i.e. HeLa cells (cervix epitheloid carcinoma, HepG2 cells (hepatocellular carcinoma) and Ishikawa cells (endometrial adenocarcinoma). In the nanomolar range (10^{-9} M and 10^{-8} M) BPA increased the

ERα activity only in HeLa cells while higher BPA concentrations were needed to induce the ERα acitivity in the other two cell lines or the ERβ activity in HeLa cells. Both BPA concentrations inhibited the E2-mediated activity via the ERα in Ishikawa cells but not in HeLa cells. Using also two other oestrogenic compounds, i.e. a fluorinated BPA and the mycotoxin zearalenone, the authors observed compound-specific dose-response curves via both ERs.

Using transfected Vero (African green monkey kidney) cells Sun and coworkers (2012) reported a significant activation of the ERα at and above 4.4x 10^{-7} M BPA and a significant anti-androgenic or anti-thyroidal activity only at 10-fold higher concentrations, confirming the weaker interference of BPA with the latter receptors. While studies on the stimulation of growth at high concentrations of BPA (>10^{-7} M) in breast cancer cells (e.g. Lee et al. 2012b) and at lower concentrations (10^{-12} M and above) in spermatogonial GC-1 cells (Sheng and Zhu, 2011) suggest that BPA´s proliferative effect is more closely related to ERα than to ERβ, other studies on the contractile effects of BPA (10^{-9} M) in isolated myocytes (Belcher et al., 2012) or on the function of β-cells and islets of Langerhans (Soriano et al., 2012) were apparently mediated via the ERβ. A study by Tanabe et al. (2012) indicates that BPA´s effect on spinogenesis may be at least partly mediated via the ERγ. In ERα expressing cells growth stimulation by BPA (10^{-9} M) may be associated with the activation of cGMP-dependent protein kinase PKG and EGFR-ERK pathways (Sheng and Zhu, 2011) while in cells lacking the classical oestrogen receptors an induction of ERK1/2 phosphorylation was only observed at high BPA concentrations (≥10^{-7} M) (Pupo et al., 2012). Li and coworkers (2012) proposed, based on their findings in HepG2 cells, that the p44/42 MAPK activation by BPA is ERα dependent and the src pathway is involved in rapid action of BPA. An additional pathway, i.e. the mammalian target of rapamycin (mTOR), was studied in human breast epithelial cells treated with BPA (10^{-10} M to 10^{-7} M) along with a reduction of the tamoxifen- and rapamycin-induced apoptosis (Goodson et al., 2011).

Based on gene expression experiments in various cell types the EFSA CEF Panel, 2010 opinion concluded that particularly at lower BPA concentrations (in the nanomolar range) the BPA-induced changes "*did not correlate to the estrogenic effects of BPA*". Similarly the Thayer and Belcher describe in the FAO/WHO Background Paper (2010) a limited number of "overlapping" expressed genes after BPA and E2/EE treatment, indicating substance-specific responsiveness of gene expression to oestrogenic substances. This conclusion is further confimed by a study on gene expression in human foreskin fibroblasts derived from young patients affected by hypospadia (Qin et al., 2012b). The authors report that only a small subset of BPA (10^{-8} M)-induced genes was also affected by E2. Peretz and coworkers (2012) reported that BPA-induced growth inhibition and follicle atresia in mouse antral follicles were not inhibited by the ER antagonist ICI 182,780 or increased by ER-overexpressing follicles and they therefore concluded that the BPA effects were not mediated via the genomic oestrogen signalling pathway.

For the evaluation of the impact of potential oestrogen-independent signalling pathways in the action of BPA, the CEF Panel considered that it may be useful to consider also dose-response analyses. Two recent in vitro studies indicate the involvement of PPARγ activation in 3T3-L1 cells after treatment with 20 µM BPA (Taxvig et al, 2012) and an increased PPARγ expression in BPA (10^{-8} M - 8x 10^{-5} M)-treated adipose tissue of children (Wang et al., 2013). The CEF Panel noted that the relevance of these observations at high BPA concentrations for risk assessment is questionable.

In several cell models an increased production of reactive oxygen species (ROS) and/or a hyperpolarisation of mitochondrial membranes were observed at BPA concentrations in the nanomolar range. Huc et al. (2012) reported on an induction of mitochondrial ROS production by BPA (10^{-12} M to 10^{-4} M) in HepG2 cells with a maximum at 10^{-9} M after a 72 hour treatment. In rat insulinoma cells (immortalized pancreatic cell line) an increase in early apoptotic cells was observed at and above 2x10^{-8} M BPA (48 h) along with a reduction of the mitochondrial mass, disturbed mitochondrial membrane potential, increased cytochrome c release and a reduced ATP concentration. Western blot analysis of Bax and Bcl-2 expression suggested that apoptosis is mediated via caspase-dependent mitochondrial pathway (Lin et al., 2013). Song et al. (2012) reported on a BPA-induced

decrease in islet viability ($\geq$1.1x10^{-8} M) primary rat pancreatic islet cells and toxic effects on mitochondria at 1.1x10^{-7} M BPA (swollen morphology and a loss of structural integrity) along with a reduction of the cytosolic ATP content.

3.10.3. Epigenetic effects of BPA

Epigenetic effects of BPA were examined in studies using the Agouti mouse model with pre-gestational, gestational and lactational BPA exposure (Dolinoy et al., 2007; Anderson et al. 2012). In the Agouti mouse model epigenetic changes are correlated with changes in the Agouti gene expression which cause a wide variation in coat color ranging from yellow (unmethylated) to brown (methylated) and which may also induce other effects including obesity, diabetes and tumorigenesis. Maternal BPA exposure resulted in a dose-dependent shift in coat color distribution by decreasing methylation at specific CpG sites in the A^{vy} allele. The methylation status found in tail tissue correlated with that in liver, kidney and brain of the same individuals, suggesting that BPA-induced epigenetic alterations occur in embryonic stem cells. Notably, these BPA-effects could be antagonised by supply of methyl-donors via the feed, providing functional support to biochemical data. However, in a recent study by Rosenfeld et al. (2013) exposure of A^{vy}/a conceptuses to BPA and genistein through maternal diet did not cause any consistent shift in offspring coat color relative to controls. A number of different experimental and analytical differences limit the ability to compare the Rosenfeld et al. 2013 study to previous available studies using Agouti mouse model (Dolinoy et al. 2007 and Anderson et al. 2012): mainly a different coat color categorization associated with a different statistical analysis and the use of multiparity where the dam age could impair the evaluation of the BPA treatment effect. The CEF Panel noted, that for the time being, the non-consistent results concerning BPA-effects on coat color distribution in the Agouti mouse model cannot be explained but require further research.As to the human relevance of the agouti gene mutation Avy (viable yellow; having an intracisternal A particle (IAP) inserted in the PS1A region), which is the most commonly employed in epigenetic studies, it should be emphasised that no comparable retroviral insert is present in the human genome and therefore, effects identified in these mice might not translate to humans (Rosenfeld, 2010). However, despite these limitations, the CEF Panel concluded that the current data on the Agouti mouse model should not be neglected but considered as an indication that BPA in principle has the potential to alter the epigenome.

In different rodent models the subcutaneous or intraperitoneal route of BPA administration were used. Ho et al (2006) analysed the prostate upon neonatal BPA exposure (10 µg/kg bw, subcutaneously) and provided evidence that BPA can cause epigenetic alterations of genes involved in signal-transduction, e.g. a continously enhanced expression of PDE4D4, which may be associated with an increased susceptibility to prostate cancer with aging. Notably, this alteration became manifest before histopathological changes in the prostate. Using the same experimental model for investigating the prostatic epigenome, Tang et al. (2012) reported hypomethylation of the nucleosome binding protein-1 (Nsbp1)-promoter whereas the physiological, age-related demethylation of Hippocalcin-like 1 (Hpcal1) was blocked by neonatal BPA exposure. Further evidence suggesting epigenetic effects of BPA was provided by Bromer et al. (2010) reporting Hoxa10-hypomethylation (along with a weak increase in RNA-expression) in the uterus of offspring upon maternal exposure (5 mg BPA/kg, intraperitoneal). Doherty et al. (2010) reported an increased mammary histone H3 trimethylation in mice exposed to BPA (maternal dose: 5 mg BPA/kg, i.p. on gestation day 9-26), associated with an increased expression of EZH2 protein. A recent study on behavioural effects of low BPA doses (2, 20, 200 µg/kg bw/ day in utero) in mice showed that BPA affected also DNA methyltransferase expression (Dnmt1 and Dnmt3a), DNA methylation of ERα (Esr1 exon A) and gene expression of ERs including Esr1 in a dose-dependent (mainly non-monotonic way), brain region-specific and sex-specific manner in juvenile offspring (Kundakovic et al., 2013). Surprisingly, reduction of Esr1 expression correlated with hypomethylation of Esr1 in the hypothalamus of female mice, while it was associated with hypermethylation in the male prefrontal cortex. This may indicate that additional factors (*e.g.* local histone modifications or levels of transcription factors as suggested by the authors) may contribute to the specific Esr1 expression. The DNA methylation status of this gene was further affected by age (neonatal vs. adult hypothalamus) and maternal care which masked some of the BPA-

induced methylation effects. Overall, these data support the hypothesis that BPA may affect the epigenome in several tissues however the results may be critically dependent on the study design and further unknown cellular factors.

The in vivo observations suggesting that BPA cause epigenetic alterations are supported by results from cell cultures studies with human cancer cells (Avissar-Whiting et al., 2010; Doherty et al., 2010; Weng et al., 2010; Qin et al., 2012a) and rodent cell lines (Ho et al., 2006; Tang et al., 2012). DNA methylation levels of genes related to development of most or all tumor types, such as *BRCA1, CCNA1, CDKN2A (p16), THBS1, TNFRS F10C* and *TNFRS F10D*, were increased in BPA-exposed HMEC. Avissar-Whiting et al. (2010) investigated the effect of BPA (0.25 to 25 ng/µl of BPA for six days (medium refreshed on day 2 and 4) on microRNAs (miRNAs) in human placental cells. Microarray analysis revealed several miRNAs to be significantly altered in response to BPA treatment in two cell lines (3A and HR-8). Real-time PCR results confirmed that *miR-146a* was particularly strongly induced and its overexpression in cells led to slower proliferation as well as higher sensitivity to the DNA damaging agent, bleomycin. BPA-induced epigenetic changes were also studied in breast epithelial cells using mammospheres as a model (Weng et al., 2010). The mammospheres were treated with low-dose BPA ($4x10^{-9}$ M); as a result of exposure to BPA, for instance, the expression of lysosomal-associated membrane protein 3 (LAMP3) became epigenetically silenced in breast epithelial cells.

3.10.4. Conclusions on mechanistic studies with BPA including epigenetic effects

Mechanistic studies published since 2010 continue to support the conclusion that BPA affects a number of receptor-dependent and independent signalling pathways, resulting in effects on hormone homeostasis and gene expression as well as cytogenetic and epigenetic effects.

The CEF Panel confirmed its conclusion (EFSA CEF Panel, 2010), that no single clearly defined mode of action of BPA can be identified that can contribute substantially to the understanding of the potential effects of BPA in humans. However, given that BPA appears to have multiple modes of action at the cellular level, and at least some of these MoAs involve cellular responses that are highly conserved across species (e.g. binding to oestrogen or androgen receptors), the relevance for humans of the variety of effects that have been reported for BPA in mechanistic studies cannot be totally discounted. On the other hand, many studies show effects at concentrations that are inappropriately high compared with human exposures. They cannot therefore be used in risk assessment. Also, whether these in vitro mechanistic studies have in vivo relevance is unclear.

4. Hazard characterisation: health-based guidance value

4.1. Critical endpoints

Section 3 provides a re-evaluation of the potential health hazards of BPA, taking into account the scientific literature 2010 – 2012, (see Appendix A) published since the last evaluation of this chemical by EFSA (EFSA CEF Panel, 2010) and also the comprehensive reviews carried out by risk assessment bodies worldwide (SCF, 2002; EU-RAR, 2003, 2008; EFSA, 2006, 2008; AIST, 2007, 2011; NTP-CERHR, 2007, 2008; Health Canada, 2008; EFSA CEF Panel 2010; US FDA, 2010a; ANSES, 2011, 2013; FAO/WHO, 2011). Reflecting the key endpoints identified in those reviews, the hazard identification phase for BPA included evaluation of the following:

- General toxicity
- Reproductive and developmental effects
- Neurological, neurodevelopmental and neuroendocrine effects
- Effects on the immune system
- Cardiovascular effects
- Metabolic effects
- Genotoxicity

- Carcinogenicity, effects on the mammary gland and cell proliferative effects

The health-based guidance value (TDI) for BPA established by EFSA in 2006 and reconfirmed in 2008 and 2010 is based on general toxicity in two multi-generation reproductive toxicity studies in rodents, in which the critical effects were changes in body and organ weights in adult and offspring rats and liver and kidney effects in adult mice, respectively (Tyl et al., 2002; 2008). The TDI of 50 µg/kg bw per day was derived by application of an uncertainty factor of 100 to the NOAEL of 5 mg/kg bw per day identified in both studies. In the current re-evaluation, the CEF Panel has considered whether any of the studies in the recent scientific literature challenge the validity of this TDI and/or provide an alternative basis for derivation of a new TDI.

The possibility that exposure to BPA is linked to one or more of the effects listed above, following pre- and/or postnatal exposure, was evaluated in the current opinion following consideration of the results of studies in vitro, in experimental animals and in humans (Section 3). The critical toxicological effects (at least "likely effects") for BPA were identified using a WoE approach.

The CEF Panel considered that the “likely” effects indicative of general toxicity in rats and mice that were already described in the EFSA CEF Panel, 2010 opinion should be maintained as a critical endpoint for risk assessment of BPA. Additionally the CEF Panel concluded that BPA-induced effects on the mammary gland of rats, mice or monkeys exposed pre- or perinatally were “likely” effects”. The conclusion on mammary gland effects resulted from the CEF Panel’s evaluation of new evidence published since EFSA’s previous risk assessment in 2010 and earlier studies using the subcutaneous route of administration (not considered in the EFSA CEF Panel, 2010 opinion). Sections 3.2.5 and 3.9.6 describe the hazard characterisation step for these two “likely” endpoints, providing an analysis of the dose-response relationship and identification of a reference point for the purposes of deriving a health-based guidance value.

The CEF Panel also considered that recent scientific literature has provided additional indications (compared with its 2010 evaluation) of reproductive and developmental effects at doses of BPA below the NOAEL for general toxicity, and also neurological/ neurodevelopmental/ neuroendocrine, immune-modulatory and metabolic effects, as described in Section 3. In light of the methodological shortcomings identified in the evaluated studies, the CEF Panel considered that none of these effects could be considered as “likely”, following application of a WoE approach. The uncertainty evaluation of these effects is described in Section 4.3 below.

4.2. Outcome of hazard characterisation and derivation of a point of departure for general toxicity

As indicated in sections 3.2.5 and 3.9.6, the CEF Panel has carried out dose-response modelling on the data for general toxicity (Tyl et al., 2002, 2008) and proliferative changes in the mammary gland (mammary gland duct hyperplasia) (US FDA/NCTR, 2013 and Delclos et al., 2014), following the guidance of the Opinion of the EFSA Scientific Committee on the use of the Benchmark Dose (BMD) approach in Risk Assessment (EFSA Scientific Committee 2011). Since the public consultation, the individual data from Tyl et al. 2008 have become available, and new BMD calculations on the changes in organ weights were performed based on individual data. The outcomes of these analyses are shown in table 7 in sections 3.2.5 and in more detail in Appendix E.

The CEF Panel conducted a detailed analysis of the results on proliferative changes in the mammary gland reported in the subchronic (90-day) toxicity study involving pre- and post-natal administration of BPA to Sprague Dawley rats (US FDA/NCTR, 2013 and Delclos et al., 2014). The analysis revealed large differences in the BMD estimates obtained with the various models and the wide BMD confidence intervals, obtained with some of the models (Appendix E). The CEF Panel concluded that the dose-response relationship in the US FDA/NCTR study (2013) and Delclos et al. (2014) is not suitable to derive with any confidence a reference point for this endpoint. Instead, the CEF Panel

considered this and other studies together when taking account of the possibility of mammary gland effects in the uncertainty evaluation below (see Section 4.3).

The kidney weight changes in the mouse (Tyl et al., 2008) showed a good dose-response relationship, and consistent results were obtained when sex and generation (F0 and F1) were used as covariates. The CEF Panel noted that the modelling for BPA-related kidney weight changes in the mouse (Tyl et al., 2008) gave a lower BMDL than for the liver effects in the rat (Tyl et al., 2002). The consistent BPA-related increase in kidney weight in this species accompanied by nephropathy at the highest dose (Tyl et al., 2008) is considered adverse. Considering that fluctuations in the body weight influence organ weights, and that there was greater consistency in the dose-response relationship between sexes and generations in the relative kidney weight, the CEF Panel decided to use relative rather than absolute kidney weights to derive a reference point. A $BMDL_{10}$ of 8 960 µg/kg bw per day was calculated, based on a 10% increase in the mean relative kidney weight in male mice of the F0 generation (see Section 3.2.5). This $BMDL_{10}$ will be used as reference point for the risk assessment.

After the publication of the 2010 opinion new toxicokinetic data have become available which allow a more accurate substance-specific extrapolation of data from animals to humans, using the human-equivalent dose (HED) approach (see Section 3.1.5 for explanation) and the human-equivalent dose factor (HEDF) of 0.068 for oral exposure of adult mice. As explained in Section 3.1.5, the HED is defined by a common relationship between the external dose given to an animal and the resultant AUC and the external dose given to a human and its AUC. The HED derived from the $BMDL_{10}$ of 8 960 µg/kg bw per day is 609 µg/kg bw per day. The CEF Panel decided to use the HED value of 609 µg/kg bw per day as the reference point for derivation of a health-based guidance value for BPA (table 17).

Table 17: Outcome of the BMD analysis for effects of BPA on mean relative kidney weight in (Tyl et al., 2008) and conversion of the BMDL10 to the HED.

Study	**Species (generation)**	**Route of administration**	**Toxic effect**	**External dose level (µg/kg bw per day)**			**HED* (µg/kg bw per day)**
				BMD_{10}	**$BMDL_{10}$**	**$BMDU_{10}$**	
Tyl et al., 2008	Mice (F0) males, with sex and generation (F0/F1) as covariates	Oral feed	Increased mean relative kidney weight	35 100 – 36 000	8 960	108 900	609

** Derived by multiplication of the $BMDL_{10}$ by the HEDF of 0.068 for oral exposure of adult mice.

4.3. Uncertainty in hazard characterisation of other endpoints

4.3.1. Method for assessing uncertainty

For the reasons outlined in section 3.2.6, the CEF Panel used the HED of the BMDL for increased relative kidney weight in mice as the reference point for establishing a TDI. In setting the TDI, the CEF Panel assessed whether an extra factor (besides the uncertainty factors to account for inter- and intra-species differences) should be included to account for the uncertainty in the database concerning the mammary gland, and the reproductive, neurobehavioural, immune and metabolic systems. According to the outcome of the WoE evaluation, an association between BPA and these hazards was considered "as likely as not"– with the exception of proliferative changes in mammary gland and immunotoxicity, which were respectively assessed as "likely" and from "-as likely as not- to likely". The CEF Panel considered that the uncertainty about these effects contributes to uncertainty in the hazard characterisation for BPA. For each effect a specific set of endpoints and criteria were used for inclusion of animal studies in the uncertainty analysis which were as follows:

- Proliferative changes in the mammary gland: all studies – both before and after 2010, addressing effects on ductal hyperplasia (including terminal end buds and ductal area), intraductal hyperplasia and epithelial cell proliferation.
- Reproductive and developmental toxicity: all publications (new and pre-2010 studies) addressing effects on endometrial hyperplasia, ovarian cysts and anogenital distance (AGD) at levels below the HED of 3.6 mg/kg bw per day. These endpoints were selected because other evaluations regarded these as critical endpoints.
- Neurobehavioural effects: all studies published after 2010 which contribute more than negligibly to the WoE.
- Immunotoxicity: all animal studies published after 2010.
- Metabolic effects: all publications addressing effects on glucose and insulin published after 2010, for which parameters there were clearly indications of effects in in vitro studies.

The WoE evaluation (Appendix C) allowed an estimation of the overall likelihood that BPA is hazardous with respect to certain toxicological endpoints in animal studies, considering the findings for all of the dose levels tested (with the exception of reproductive effects for which a cut-off dose level was taken as a study inclusion criterion). However, to assess whether an extra UF (and its magnitude) in the health-based guidance value would be appropriate to account for uncertainties in the database, the following were considered: (i) the likelihood that BPA exposure is associated with a certain outcome in animals at particular dose intervals (and especially at doses below the HED for the increase in relative mean kidney weight), (ii) the relevance of this effect to humans and (iii) its adversity in humans, if it occurs.

The CEF Panel approached the evaluation of uncertainty in three stages. In the first stage, it carried out the following steps for each of the selected types of effect in turn, to assess the likelihood that BPA might affect a certain endpoint in animals at each dose interval.

- The experts reviewed the studies previously considered in the WoE assessment (see bulleted list above).
- The experts extracted key information from each study and collated this in a graphical format (Section 4.3.2). The graphs summarise, for each study, the life stage of the animals at treatment onset, duration of treatment and sampling time for measurements, the doses tested, whether there was a statistically significant effect at any dose. They also show the number of strengths and weaknesses of the study, as assessed in the WoE assessment but excluding any strengths or weaknesses that did not apply to the effect of interest. In addition, they show the CEF Panel's evaluation of the reliability of the study and its relevance to the effect of interest, taking into account all the preceding information. When assessing reliability and relevance, the strengths and weaknesses were not weighted equally but according to the CEF Panel's evaluation of their relative importance.
- In order to allow studies for different species using different routes of exposure to be plotted on the same dose scale, as well as on the same scale as the reference dose for the increase in relative kidney weight – all doses were converted to HED.

In the next stage of the uncertainty evaluation, the experts met to assess the relevance of each effect to humans, and its adversity in humans, if it occurs.

In the final stage the experts were asked to make judgements about the overall likelihood, in each HED dose interval, that BPA has the inherent ability to cause one or more types of effects in animals and that it is relevant and adverse in humans, based on the group summaries of the judgements that had been made in the earlier stages of the assessment (See Appendix D.III, for details).

4.3.2. Outcome of the uncertainty evaluation

4.3.2.1. Uncertainty in the proliferative changes in mammary gland

The uncertainties related to the induction of proliferative changes in the mammary gland following BPA administration, i.e. intraductal hyperplasia, epithelial cell proliferation and ductal hyperplasia (including increase in the number of TEBs), were evaluated taking into account the reliability of the study results.

As shown in figure 10 below, three studies with subcutaneous, pre- or perinatal BPA exposure (Durando et al., 2007; Murray et al., 2007; Vandenberg et al., 2008) report on intraductal hyperplasia in the mammary gland (i.e. an increase in the relative number of ducts lined by three or more layers of epithelial cells), while in two other studies with perinatal BPA exposure (Acevedo et al., 2013; Delclos et al., 2014) no such lesions were detected.

Whilst epithelial cell proliferation is a normal physiological process in certain life stages (pre-/perinatal period, pregnancy) and per se does not lead to tumour formation it is generally accepted that under certain pathological conditions such as recurrent tissue damage and repair the proliferating tissue becomes more susceptible to tumour development. In studies with rats treated with BPA and, thereafter, with a well known complete carcinogen (Jenkins et al., 2009; Betancourt et al., 2010) as well as in studies with transgenic mice (Jenkins et al. 2011; Jones et al., 2010) increased cell proliferation was reported along with tumour formation. In case of the study by Jenkins et al. (2011) – who observed cell proliferation in transgenic mice which spontaneously develop tumours – the relevance of these findings to whether proliferative changes occur at low BPA doses in normal animals was considered medium, taking into account the increased sensitivity of this mouse model to tumour development due to over-expression of erbB2. Increase in the number of terminal end buds as well as ductal hyperplasia were reported in several studies even at very low BPA doses (e.g. Ayyanan et al., 2011). However, it should be noted that these putative preneoplastic lesions may be reversible and will not in all cases progress to neoplasia. In addition the CEF Panel took into consideration the study reliability (e.g. data reporting, methology) which was considered for all studies on BPA-induced proliferative effects as low or medium. In the CEF Panel´s evaluation of the reported effects, a low study reliability would lead to a high uncertainty, e.g. in case of the low dose effects reported by Ayyannan (2011) and Jones (2010). Based on these considerations and the lower relevance of transgenic mice studies, the CEF Panel considers the proliferative effects at a HED below 1 µg BPA/kg bw per day as "very unlikely" (VU). The statistical significant effects in the HED range between 1 and 100 µg/kg bw per day have to be balanced by the transient nature of the effects (e.g. Munoz-de-Toro et al., 2005; Vandenberg et al., 2008, 2013c) and many negative findings which led to an expert judgement of "unlikely to -as likely as not (U/ALAN). At a HED above 100 µg BPA/kg bw perday the majority of the studies showed statistically significant proliferative effects and therefore they were considered as "-as likely as not- to "likely" (ALAN/L) (Figure 10).

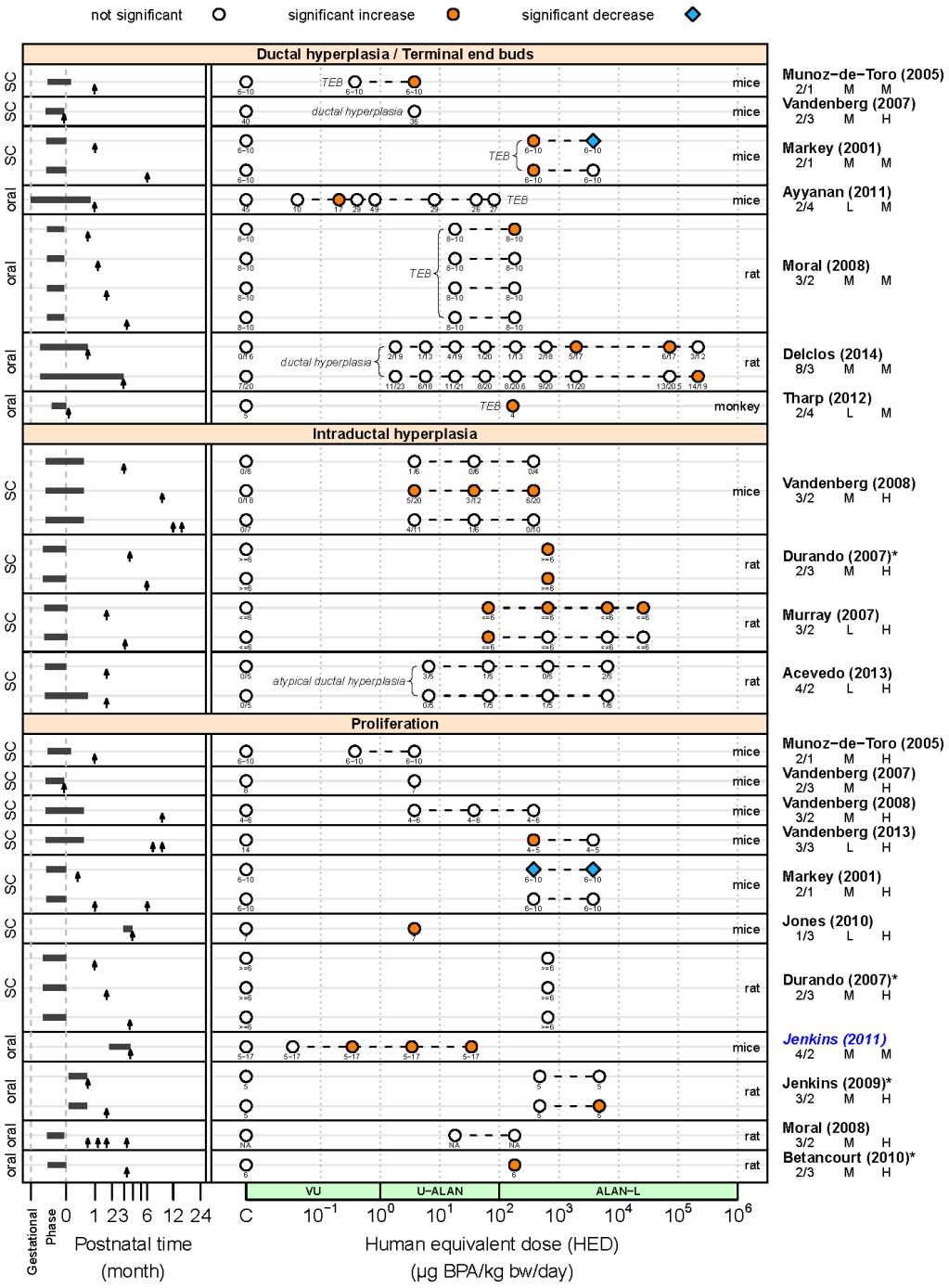


Figure 10: Summary plot for the effects of BPA exposure on the proliferative changes in the mammary gland: Ductal hyperplasia (including TEBs), intraductal hyperplasia (relative number of ducts lined by three or more layers of stratified epithelial cells) and epithelial cell proliferation (proliferative index as measured by BrdU, Ki67or PCNA labelling). The left hand text indicates the exposure route (oral or subcutaneous, SC). The left hand pane shows the experimental design with the horizontal bars showing the timing and duration of exposure (while the up-arrows show when the measurements/assessments were made. The central pane shows the study results , based on the x-axis showing the human equivalent dose (HED) depending upon species, age and route of exposure. The lightly shaded boxes on the x-axis show the uncertainty assessment (very unlikely=VU, unlikely=U, as likely as not=ALAN, likely=L and combinations thereof). The central pane also shows the sex and species of the

animals under consideration (right hand-side of pane) and the nature of the symbols denotes critical information: open circles show no significant effect, red/shaded circles show significant increase and blue/shaded diamonds show significant decrease (see summaries of the studies in Appendix B for details). Numbers under the symbols either denote incidence (x/y) or samples size (n=x). The text in the right hand column shows information for each study (first author, year), the ratio of strengths/weaknesses of the study (taken from Appendix B), the reliability of the study (L=low, M=medium, H=high) and the relevance of the study to the biological variable and question being posed in the WoE assessment (L=low, M=medium, H=high) (see Appendix C). An asterisk by the study year indicates a study with a carcinogen treatment following BPA administration; an author´s name in blue italics indicates a study with transgenic mice.

Relevance and adversity to humans for mammary gland proliferation

Intraductal hyperplasia is observed in humans and is considered as a precursor of ductal carcinoma both in rodents and in humans. Therefore this lesion is of high relevance to predict cancer in the human and animal mammary gland and is considered as adverse.

Ductal hyperplasia and an increase of the number of TEBs may be regarded as supporting evidence for tumour formation along with an increase in the proliferation of epithelial cells. These effects in experimental animals are dependent on the study design (e.g. the type of the diet, the administration and doses of the substances, the exposure time, the sampling time point). The CEF Panel noted that ductal hyperplasia may not always progress to neoplastic lesions but may be reversible. Therefore, the relevance of these hyperplastic lesions – in the absence of intraductal hyperplasia – is questionable for humans and the level of adversity of these findings is unknown.

Increased epithelial cell proliferation in the mammary gland of rodents is linked to prolactin which is also associated with an increased breast cancer risk in women (Harvey, 2012). Thus, an increase in prolactin levels constitutes an underlying mechanism in the induction of cell proliferation which may be indicative and therefore relevant for tumour promotion in both the human and rodent mammary gland.

4.3.2.2. Uncertainties in reprotoxicity

As shown in Figure 11 below, four studies are included in the assessment of endometrial hyperplasia and ovarian cysts. Declos et al. (2014) and Newbold et al. (2009) found no significant effect of BPA on the incidence of endometrial hyperplasia at doses ≤3.6 mg/kg bw per day HED while Signorile et al. (2010) and Newbold et al. (2007) reported significant increases in the incidences of endometrial hyperplasia. The Newbold et al. (2009) study also reported an increase in incidence of ovarian cysts at a single dose of BPA with a P-value of 0.05 (marginal significance). Signorile et al. (2010) and Newbold et al. (2007) were the weakest of the four studies, with more weaknesses than strengths and used the fewest number of doses, with the smallest dose range of BPA. The CEF Panel also noted that the duration of BPA exposure in Newbold et al. (2007) was extremely short.

Six studies are included in the assessment of AGD. Four studies found no significant effect of BPA on AGD (de Catanzaro et al., 2013; Delclos et al., 2014; Ferguson et al., 2012; LaRocca et al., 2011). However, of the significant effects reported at BPA ≤3.6 mg/kg bw per day HED, only the Christianen et al. (2014) reported a clear adverse effect: reduced AGD in male rats. Both Kobayashi et al. (2012) and Christiansen et al. (2014) reported a similar significant decline in AGD in females and in Kobayashi et al. (2012) the effect was no longer seen in the second, older, age group.

Taken together these studies show some contradictory and variable results with only Christiansen et al. (2014) showing any dose-response relationship although very slight. Taking the WoE overall, combined with the uncertainty evaluation process the CEF Panel concluded that up to 1 mg/kg bw per day HED (covering doses ≤3.6 mg/kg bw per day HED), the likelihood of BPA inducing negative

effects ranged from very unlikely-unlikely to very unlikely-as likely as not. Above 1 mg/kg bw per day the likelihood that BPA had an adverse effect climbed to very unlikely-likely and then likely-very likely (see Figure 11). The latter conclusion agrees with the opinion of the CEF Panel that at doses >3.6 mg/kg bw per day BPA is more likely to have adverse effects on reproductive development and function.

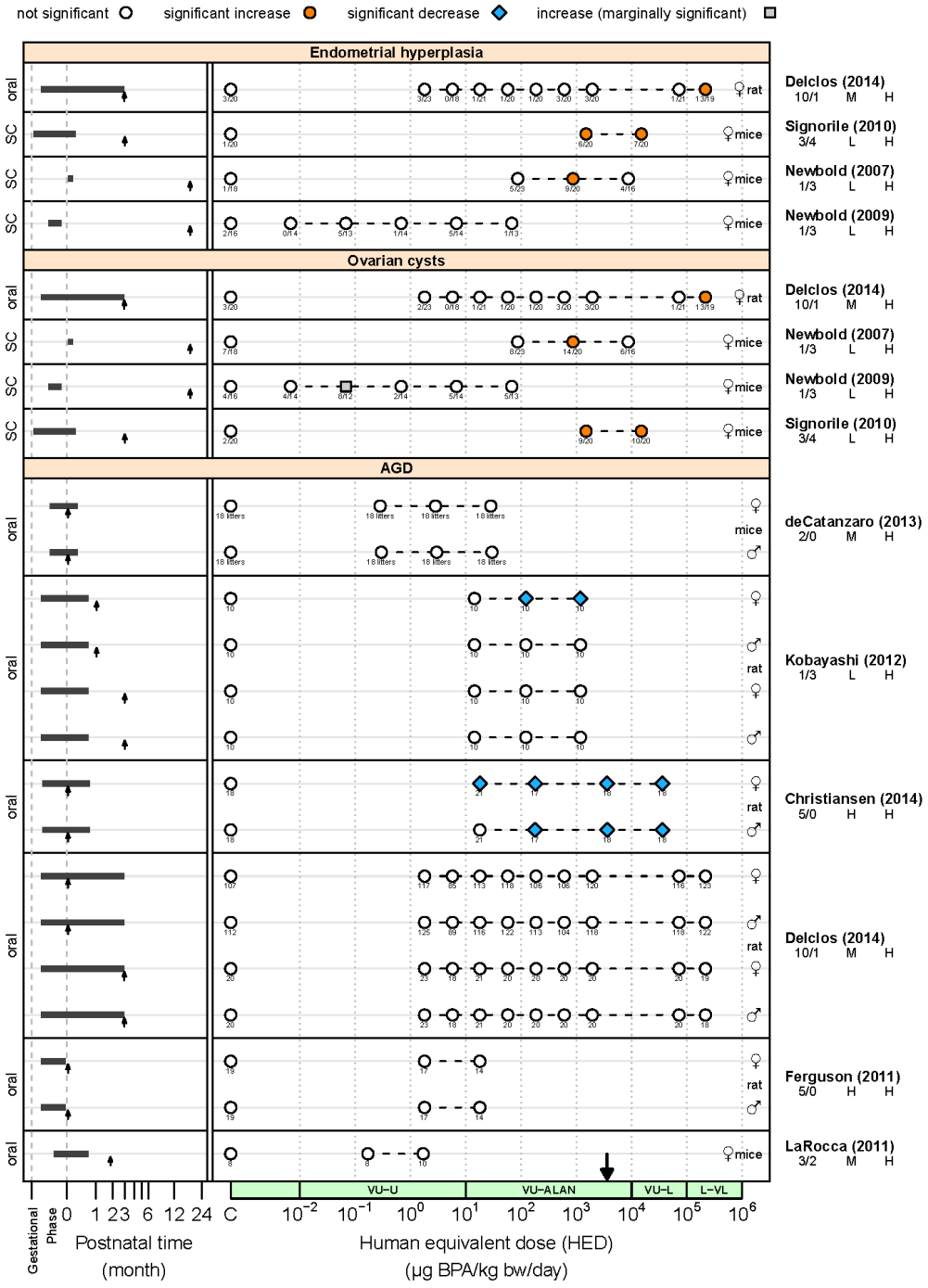


Figure 11: Summary plot for the effects of BPA exposure on the reproductive system: (a) endometrial hyperplasia and (b) ovarian cysts. The plot also shows the effect of BPA on (c) anogenital distance (AGD) as a read-out of androgen action in-utero/perinatally. The left hand text indicates the exposure route (oral or subcutaneous, SC). The left hand pane shows the experimental design with the horizontal bars showing the timing and duration of exposure (black bar: exposed as adults or during gestation and/or lactation) while the up-arrows show

when the measurements/assessments were made. The central pane shows the study results, based on the x-axis showing the human equivalent dose (HED) depending upon species, age and route of exposure. The down-arrow on the x-axis shows the HED equivalent (3,600 µg BPA/kg body weight/day) of the 5.0 mg BPA/kg body weight/day oral exposure NOAEL (Tyl et al., 2002). The lightly shaded boxes on the x-axis show the uncertainty assessment (very unlikely=VU, unlikely=U, as likely as not=ALAN, likely=L, very likely=VL and combinations thereof). The central pane also shows the sex and species of the animals under consideration (right hand-side of pane) and the nature of the symbols denotes critical information: open circles show no significant effect, red/shaded circles show significant increase and blue/shaded diamonds show significant decrease (see summaries of the studies in Appendix B for details). Numbers under the symbols either denote incidence (x/y) or samples size (n=x). The text in the right hand column shows information for each study (first author, year), the ratio of strengths/weakness of the study (taken from Appendix B), the reliability of the study (L=low, M=medium, H=high) and the relevance of the study to the biological variable and question being posed in the WoE assessment (L=low, M=medium, H=high) (see Appendix C). In the case of Newbold et al., 2009, (b) ovarian cysts, the * denotes a p value of p=0.05 rather than significance of p<0.05.

Relevance and adversity to humans for developmental and reprotoxic effects

Abnormal endometrial hyperplasia has the same fundamental physiological basis both in women and in female animals, including those that do not have a menstrual cycle. Furthermore, abnormal endometrial hyperplasia is a common gynaecological condition in women and is also a recognised precursor for endometrial carcinoma. Therefore, animal models for endometrial hyperplasia are relevant to human health and the occurrence of endometrial hyperplasia in animal models is also likely to indicate adverse consequences for women.

Ovarian cysts and endometriomas occur in various sizes (from small to large) and may be benign and/or functional (i.e. hormone producing) and are common in women. In terms of women's health, ovarian cysts/endometriomas, depending on their size and type, are associated with a range of adverse outcomes, which include pain and infertility. Therefore, the occurrence of ovarian cysts or endometriomas in animal models would be considered both relevant to and adverse for women.

Anogenital distance (AGD) needs to be considered separately for males and females and the direction of change in AGD in terms of adverse effects is opposite between the sexes. Nevertheless, AGD is a read-out of androgen action, especially during in utero development, in both animal models and in humans.

In humans and animal models, reduced AGD in males is indicative of reduced masculinisation and associated with increased adverse effects in reproductive development (hypospadias and cryptorchidism), reduced androgen levels and impaired sperm production and/or fertility. However, the magnitude of decrease in AGD that can be clearly associated with a decrease in subsequent fertility is more difficult to establish. Nevertheless, there is no doubt that reduced AGD is associated with reproductive abnormalities and reduced sperm counts/fertility (Thankamony et al., 2014; Mendiola et al., 2011). Therefore, reduced AGD in animal models is relevant to humans and indicative of an adverse effect in humans.

In humans and animal models, increased AGD in females is indicative of excess androgen action. The adverse associations in women include reduced fertility and polycystic ovary/polycystic ovarian syndrome (PCO/PCOS). Increased AGD in female humans is associated with greater follicle numbers in girls (Mendiola et al., 2012) and with prenatal stress in girl infants (Barrett et al., 2013), both suggestive of some masculinisation. Therefore, increased AGD in female animal models is relevant to humans and indicative of an adverse effect in humans.

In contrast, the implications of increased AGD in men/male animal models or reduced AGD in women/female animal models are much less clear. While these may be indicative of disturbed developmental processes, it is not known whether these effects are adverse either in animal models or in humans. Therefore it cannot be stated with any certainty that reduced female AGD or increased male AGD are definitely either relevant or adverse to women or men respectively.

The CEF Panel noted that a fundamental question to be addressed is whether developmental exposures to BPA result in reduced fertility. Many studies utilise valid measures of adverse effects of developmental exposure on the offspring. However, it has to be recognised that many of these (e.g. small but statistically significant changes in AGD or fetal/neonatal reproductive tract weights and/or cell numbers) are surrogate measures. There are recognised mismatches between animal species and human, such as the fact that sperm counts need to be reduced to a greater extent in rodents than in men before fertility is impacted. However, this can be addressed for forced/continuous breeding studies (e.g. Cabaton et al., (2011). In the final analysis, a robust approach to assess the actual fertility of the individuals exposed during development would be beneficial, especially since human fertility is considered as reflective of the combined biologies of the couple. The CEF Panel noted that few of the studies it has evaluated in this opinion have performed this analysis. A few studies have noted that the reported effects of BPA exposure are transient or reversible, i.e. loss of "adverse" phenotypes with increasing age after stopping the exposure (Nah et al., 2011, Kobayashi et al., 2012). This suggests some degree of plasticity and demonstrates the important of two generation studies. Furthermore, it should be noted that this concept of plasticity has recently also been shown to apply to a degree to AGD in male rats (Mitchell et al., 2014).

4.3.2.3. Uncertainties in neurotoxicity

Out of all studies included in this opinion, six studies were assessed to have a minor contribution to the likelihood that BPA exposure changes response in tests for various neurobehaviour in rodents.

Due to differences in results reported for female and male animals, two graphs are shown (Figure 12a and b, respectively).

The three studies investigating the possible influence of BPA exposure on anxiety-like behaviour (Jasarevic et al., 2013; Wolstenholme et al., 2011a; Xu et al., 2012), used the Elevated Plus Maze (EMP) where anxiety-like behaviours were evaluated e.g. as time spent in open and closed arms during one 5 to 10 minutes test session. The longer time spent in closed arms, the more anxiety-like behaviour, and visa versa, the more time spent in open arms, the less anxiety-like behaviour. One study for male and female rodent, respectively, reported increased anxiety-like behaviour of BPA at HED 10^0-10^3 µg/kg bw per day (males: Jasarevic et al., 2013; female: Xu et al., 2012) whereas the other two reported no significant difference compared to controls. However, Jasarevic et al., 2013 reported effects in males at a lower dose (HED 10^0 µg/kg bw per day) than the other two studies (HED 10^1 -10^2 µg/kg bw per day).

One of the three studies investigating anxiety-like behaviour, (Xu et al., 2012) used four different tests including the EPM. Additionally, one test for depression-like behaviour (forced swim task) was used. In males, prenatal BPA exposure caused anxiety-like behaviour in one test compared to controls. Additionally, males were reported to show depression-like behaviour following both exposure periods to BPA (Xu et al., 2012). In females, prenatal BPA exposure caused anxiety-like and depression-like behaviour in all tests, whereas lactational BPA exposure in three (Xu et al., 2012).

Disturbed sensory-motor function of BPA in adult male rats, not in females, was reported by Fergusson et al., 2012. The effect was evaluated by decreased acoustic startle response compared to vehicle controls, but was also seen in the naïve control. The authors presumed that the apparent effect of BPA (at HED 10^0 µg/kg bw per day) was due to an aberrant heightened acoustic startle response in the vehicle control, and did not put weight on the results. This consideration is supported by the study by Stump (2010) that, among other neurodevelopmental toxicity end points, covered the auditory

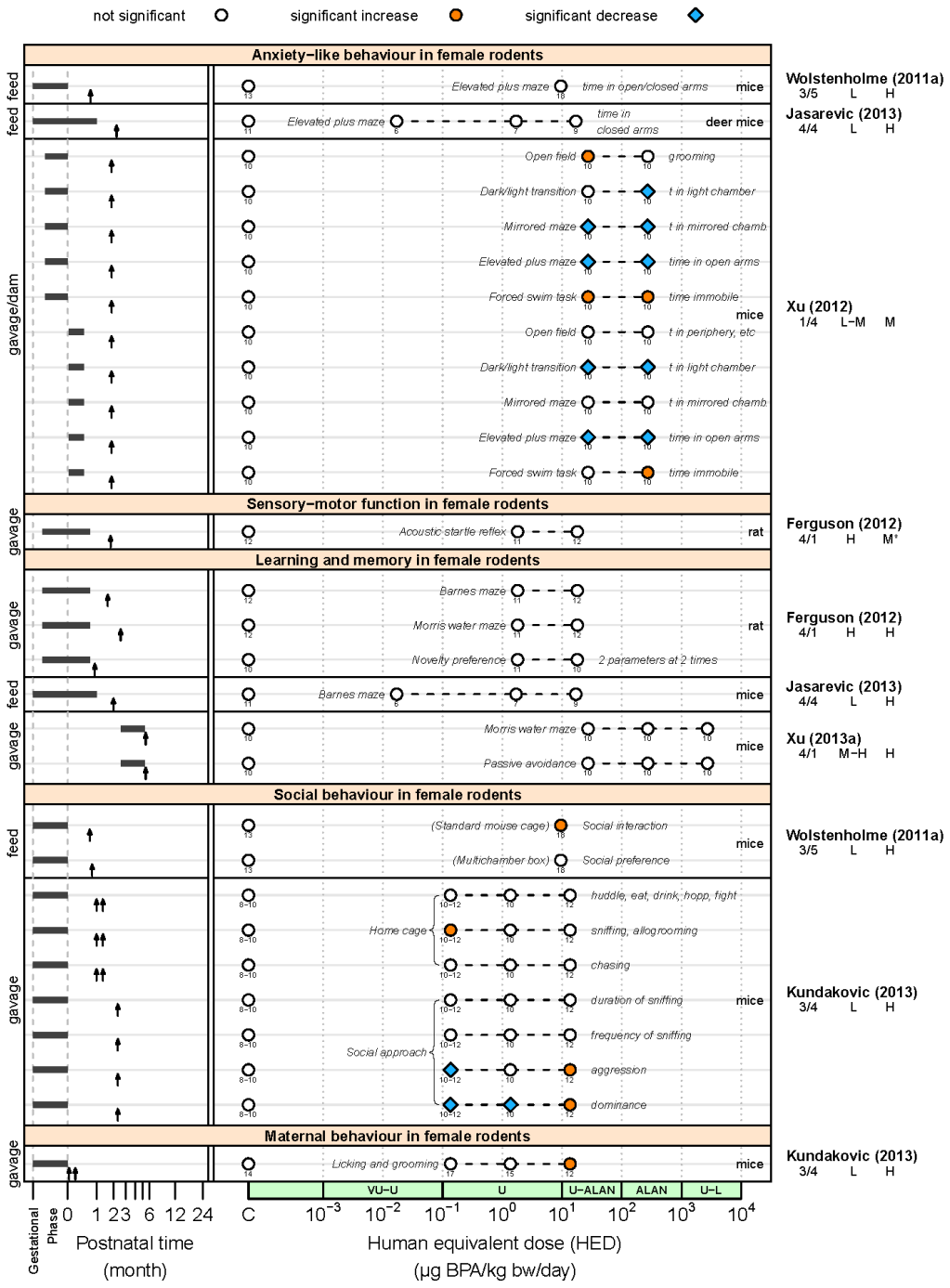
startle response. No statistically significant effects of BPA on the startle response were found, and neither the study's control data nor the historical control data showed gender related differences in auditory startle response. Also in literature auditory startle response is not known as a sexually dimorphic behaviour (Meyer, 1998). The expert group concluded disturbed sensory function of BPA as likely as not.

In females, BPA at HED 10^{-2} to 10^{-3} µg/kg bw per day produced no effects on learning and memory (Fergusson et al., 2012; Jasarevic et al., 2013; Xu et al., 2012). In males, two studies using the same test, the Barnes maze, reported no effect (Fergusson et al., 2012) and learning deficit (Jasarevic et al., 2013) following perinatal BPA exposure. However, the latter found no effect of BPA at the lowest dose (HED 10^{-2} µg/kg bw per day). At the highest dose level (HED 10^{3}-10^{4} µg/kg bw per day), another study (Xu et al., 2013a) found that BPA given to adult mice impaired learning and memory as evaluated by increased escape path length in the Morris water maze and shortened step-down latency in the Passive avoidance test.

In young male mice, no effect of prenatal BPA exposure on social interaction (standard mouse cage) and preference (multichamber box) were reported by Wolstenholme et al., 2011a, whereas Kundacovic et al. 2013 found reduced chasing in mice at the same age, and increased aggression and dominance in adult mice, within the same dose span as Wolstenholme et al., 2011a. In young female mice, Kundacovic et al. 2013 found no effect on social behaviour at the same dose level (HED 10^{1} µg/kg bw per day) as Wolstenholme et al., 2011a; did, but at a lower (HED 10^{-1} µg/kg bw per day). However, as in adult mice, increased aggression and dominance in adult females were seen at the highest dose, and decreased in at the lowest dose level (Kundacovic et al. 2013). Both studies reported some disturbances in social behaviour at lower BPA doses, but the lack of consistency made the expert group to conclude effects on social behaviour in mice as "unlikely to -as likely as not-"

Based on these results, the expert group concluded for females that at the HEDs from 10^{-3} to 10^{-1} it is "very unlikely to unlikely" that BPA exerts an effect on neurobehaviour. From HEDs 10^{-1} to 10^{1} the group judged such effects as "unlikely" (female), from HEDs 10^{1} to 10^{3} µg/kg bw per day as "unlikely to -as likely as not-", and from 10^{3} to 10^{4} µg/kg bw per day as "unlikely to likely" (see Figure 12a).

Based on these results, the expert group concluded that at the HEDs for males from 10^{-3} to 10^{-1} µg/kg bw per day it is "very unlikely to unlikely" that BPA exerts an effect on neurobehaviour. From HEDs 10^{-1} to 10^{3} µg/kg bw per day the group judged such effects as "unlikely to -as likely as not-", and from 10^{3} to 10^{4} µg/kg bw per day as "-as likely as not- to likely" (see Figure 12b).


not significant
significant increase
significant decrease
Anxiety−like behaviour in female rodents
Elevated plus maze
time in open/closed arms
mice
Wolstenholme (2011a)
3/5 L H
Elevated plus maze
time in closed arms
deer mice
Jasarevic (2013)
4/4 L H
Open field
grooming
Dark/light transition
t in light chamber
Mirrored maze
t in mirrored chamb
Elevated plus maze
time in open arms
Forced swim task
time immobile
mice
Xu (2012)
1/4 L−M M
Open field
t in periphery, etc
Dark/light transition
t in light chamber
Mirrored maze
t in mirrored chamb
Elevated plus maze
time in open arms
Forced swim task
time immobile
Sensory−motor function in female rodents
Acoustic startle reflex
rat
Ferguson (2012)
4/1 H M*
Learning and memory in female rodents
Barnes maze
Morris water maze
Novelty preference
2 parameters at 2 times
rat
Ferguson (2012)
4/1 H H
Barnes maze
mice
Jasarevic (2013)
4/4 L H
Morris water maze
Passive avoidance
mice
Xu (2013a)
4/1 M−H H
Social behaviour in female rodents
(Standard mouse cage)
Social interaction
(Multichamber box)
Social preference
mice
Wolstenholme (2011a)
3/5 L H
huddle, eat, drink, hopp, fight
Home cage
sniffing, allogrooming
chasing
duration of sniffing
frequency of sniffing
Social approach
aggression
dominance
mice
Kundakovic (2013)
3/4 L H
Maternal behaviour in female rodents
Licking and grooming
mice
Kundakovic (2013)
3/4 L H
feed
gavage/dam
gavage
VU−U
U
U−ALAN
ALAN
U−L
Gestational
Phase
0 1 23 6 12 24
Postnatal time
(month)
C
Human equivalent dose (HED)
(µg BPA/kg bw/day)

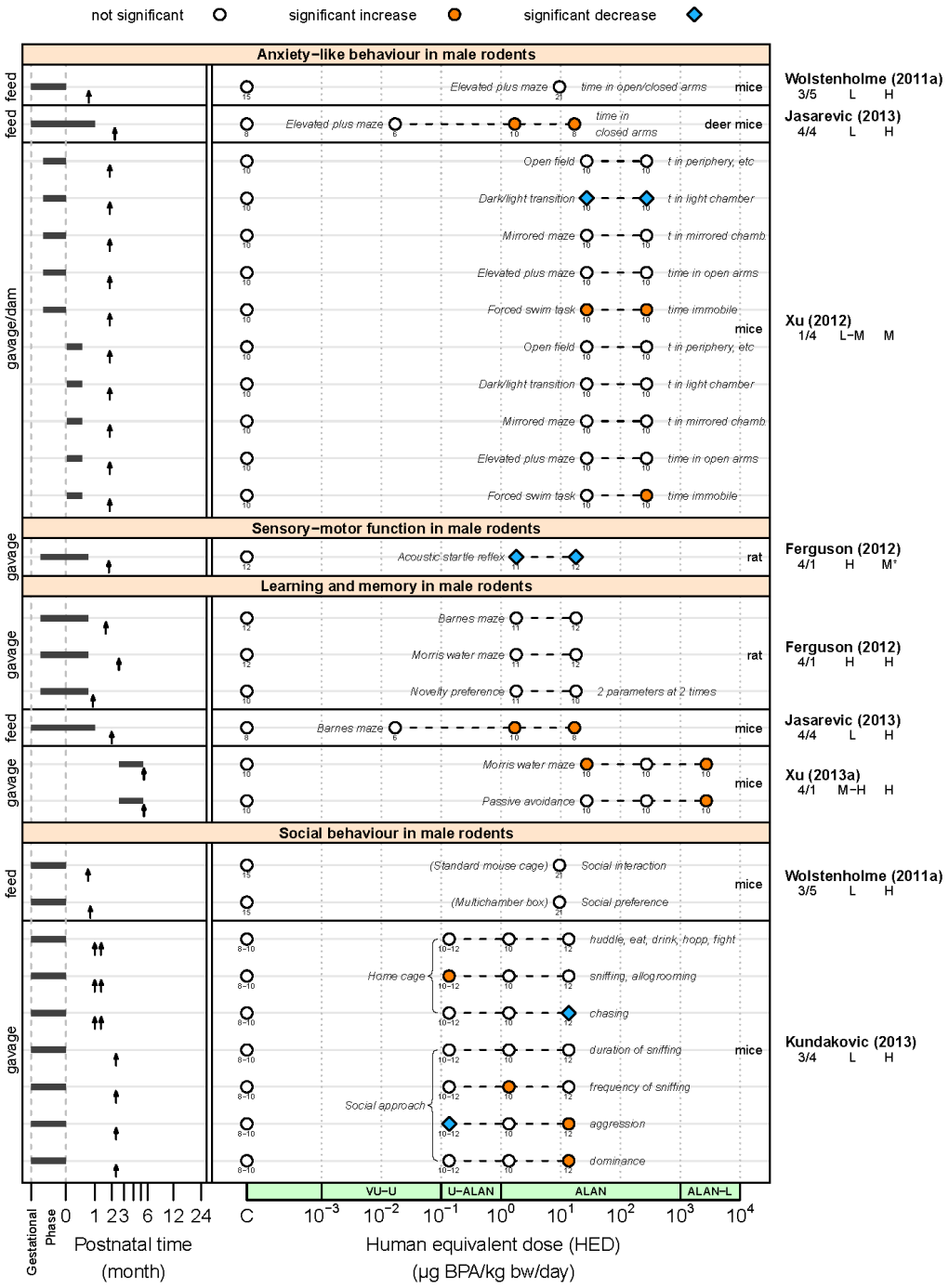


Figure 12: Summary plot for the effects of BPA exposure on the neurological system in females (a) and males (b): changes in anxiety-like behaviour, sensory-motor function, learning and memory and social behaviours. The left hand text indicates the exposure route (oral or subcutaneous, SC). The left hand pane shows the experimental design with the horizontal bars showing the timing and duration of exposure (black bar: exposed as adults or during gestation and/or lactation) while the up-arrows show when the measurements/assessments were made. The central pane shows the study results, based on the x-axis showing the human equivalent dose (HED) depending upon species, age and route of exposure The central pane shows the results for the selected studies, based on the x-axis showing the human equivalent dose (HED) depending upon species, age and route of exposure. The lightly shaded boxes on the x-axis show the uncertainty assessment (very unlikely=VU, unlikely=U, as likely as not=ALAN,

likely=L and combinations thereof). The central pane also shows the sex and species of the animals under consideration (right hand-side of pane) and the nature of the symbols denotes critical information: open circles show no significant effect, red/shaded circles show significant increase and blue/shaded diamonds show significant decrease (see summaries of the studies in Appendix B for details). Numbers under the symbols either denote incidence (x/y) or samples size (n=x). The text in the right hand column shows information for each study (first author, year), the ratio of strengths/weakness of the study (taken from Appendix B), the reliability of the study (L=low, M=medium, H=high) and the relevance of the study to the biological variable and question being posed in the WoE assessment (L=low, M=medium, H=high) (see Appendix C).

Relevance and adversity to humans for neurotoxic effects

The traditional domain of toxicology is structural damages to target tissue, i.e. pathology. However, toxicants may also cause functional changes, of which behavioural changes represent one type. Because a number of chemicals are known to produce developmental neurotoxic effects in humans and other species, behavioural changes are regarded as a relevant toxicological endpoint.

Anxiety disorders are common and relevant disorders in the general human population in the western world. Studies of anxiety-like behaviours in experimental animals are difficult to interpret. While basic neuronal pathways and modes of actions seem to be the same in mammalians (including humans), interpretation of e.g. chemical induced changes in experimental animal behaviour have to be done in the context of the animal's innate behaviour, its physiological state as well as effects such as changes of body weight or body temperature. Regarding the animal's innate behaviour, rodents have an innate aversion to open, well-lit spaces and tend to avoid them (while in humans this is not the case). Thus, if a mouse spends decreased time in the centre area of an open field task, this might be interpreted as increased anxiety-like behaviour but also as a strengthened innate behaviour - the mouse being more "cautious". There is no general agreement on the interpretation of increased anxiety-like behaviour in these tests and their relevance to humans.

Similarly, any sustained chemical-induced impairment of learning ability and/or impairment of memory in experimental animals are considered as relevant effects also for humans because the basic neuronal pathways and modes of actions are expected to be similar in mammals. However, as described for anxiety-like behaviours, caution must be taken to avoid an anthropocentric interpretation of any behavioural changes observed in tests with experimental animals. Many behavioural tests for experimental animals are linked to the reaction of the limbic system, which controls emotional behaviour (i.e. taste aversion or open field aversion in rodents) and is a system which is evolutionary well conserved. Despite the fact that emotional behaviour is organised in the same brain structures through related neurotransmitters and signalling pathways in many species, the resulting behavioural reaction to a given stressor may differ in different species. In conclusion, consistent changes in a battery of such behavioural tests are needed to predict behavioural changes in humans, although the exact nature of the effect might be different.

Sensory motor function is highly conserved and its manifestation (e.g. reflexes, startle response) is far less complex compared to e.g. emotional behaviour or learning, and is thus less sensitive to interfering factors. Therefore, chemical-induced impairment of sensory motor functions in experimental animals should be considered as relevant for humans.

On the other hand, if changes in a battery of behavioural tests are not consistent or behavioural changes are seen only in single tests, the relevance for behavioural effects in humans is questionable. Many of the behavioural tests for animals are not sufficiently standardised or validated in terms of what the test measures and how well it does so. Many of the tests use motivational factors such as rewarding incentives (or punishment) for a specific behaviour, which are also regulated in the limbic system and the hypothalamus (e.g. food/water deprivation i.e. hunger/thirst, satiety i.e. stopping

eating). As mentioned above, these neuronal centres are tightly linked to emotional behaviour and to the hormonal system. A broad variety of factors (including a variety of ingredients in food including nutrients and food additives) will influence brain chemistry (and the hormonal system) and consequently lead to behavioural changes. Equally, differing testing conditions (e.g. brightness of the light, environmental noise, arousing odours etc.) will influence the test outcome and are difficult to control. Restraining an animal (e.g. for gavage) has a very different impact in different animal species: it leads to high stress in rats but not in dogs because of the innate type of behavioural reaction to this stressor. Furthermore, laboratory animals belong to social species, and their behaviour and welfare are strongly affected by previous and present social environment. When interpreting results of any behavioural test, factors such as as animal group composition and stability, rank and previous social experience, should also be taken into account. All these factors hamper comparability and consistency of the results of the same test used in different studies when performed under different environmental conditions, unless detailed information on the different test conditions is available. It is therefore not surprising that there is disagreement among experts about the assessment and interpretation of behavioural tests such as tests for anxiety-like behavioural changes or changes in social behaviour of experimental animals.

Overall, there is substantial uncertainty in the interpretation of the results of many behavioural tests. For several of these tests (e.g. open field test, social behaviour), the relevance for humans of the effects observed in experimental animals is questionable. Only consistent changes in a battery of behavioural tests might be predictive for behavioural changes in humans, although the exact nature of the effect might be different. Furthermore, the results of functional (behavioural) testing have to be evaluated in light of other available information on the test substance such as chemical structure, results from in vitro (mechanistic) studies, other toxicity studies, toxicokinetic data, etc.

Behavioural responses are by definition adaptive reactions to environmental stimuli, accompanied by internal signals to maintain homeostasis. These environmental stimuli may be of physical (e.g. bright light, noise) or chemical nature (e.g. pheromones). Behavioural changes may become “adverse” when they are disruptive of the normal behaviour of a given species, e.g. the loss of the capability to react adequately to external stimuli/stressors, sustained (inadequate or stereotypical) behaviour in the absence (removal) of stressors. Also, an impairment of the learning ability and/or the impairment of the memory may lead to a decrease in the IQ score on a population level, which is clearly an adverse effect. Lesions in certain areas of the hypothalamus lead to irreversible loss of regulation of hunger or satiety, i.e. leading to starvation and death or gross obesity. Impaired sensory-motor performance may also indicate neuronal damage, which is clearly an adverse effect.

4.3.2.4. Uncertainties in immunotoxicity

The experiments that showed some signs of induction of pyometra (Kendziorski et al, 2012) were interpreted as not likely to be a consequence of altered resistance to infections, but rather a mechanical consequence of access of pathogens to uterus tissue during the hormonal cycle, and therefore not an indication of immunotoxic effects of BPA.

In animal studies by Lee et al. (2012) in which increased levels of total IgE and histamine are found, these effects may be interpreted as indications of increased risk of allergy, but they were found in an experiment in which only one dose of BPA was applied, and this dose was also higher than in other experiments. Studies of Bauer et al (2012) indicated a clear dose dependent facilitation of the severity of inflammation in an inhalatory ovalbumin model of respiratory allergy. No effects on eosinophils in the lavage were seen while airway hyperreactivity was not tested in this model. Nakajima et al. 2012, in an experiment with only one dose, but more or less at the higher dose level as tested by Bauer et al. (2012), noted such effects, indicating that BPA may in fact have an influence on respiratory allergy in this model.

In the model used by Bauer et al (2012) in which there was i.p. sensitization to ovalbumin, followed by inhalatory challenge, BPA diminished the number of eosinophils in the lavage. These decreased

numbers were not associated with a decrement (nor an increment) of airway hyperreactivity. For these reasons, the animal data indicating a lowering of eosinophils do not indicate an adverse effects.

Based on these results, the experts concluded that at the HEDs 10^{-2}, 10^{-1}, 10^{-0} µg/kg bw per day it is "very unlikely" to "unlikely" that BPA exerts an effect on respiratory allergy. From HEDs 10^{-0} to 10^{-1} the group judged such effects as "as likely to not", and from 10^{-6} to 10^{6} µg/kg bw per day as "unlikely to likely". It should be noted that at 10^{4} µg/kg bw per day there were no data, and some experts considered it inappropriate to give a judgment for that concentration, while others noted that this HED is between HED levels where judgments were made on the basis of available data, and felt that effects on that intermediate HED could be extrapolated, coming to a judgment of "unlikely to likely" (see Figure 13).

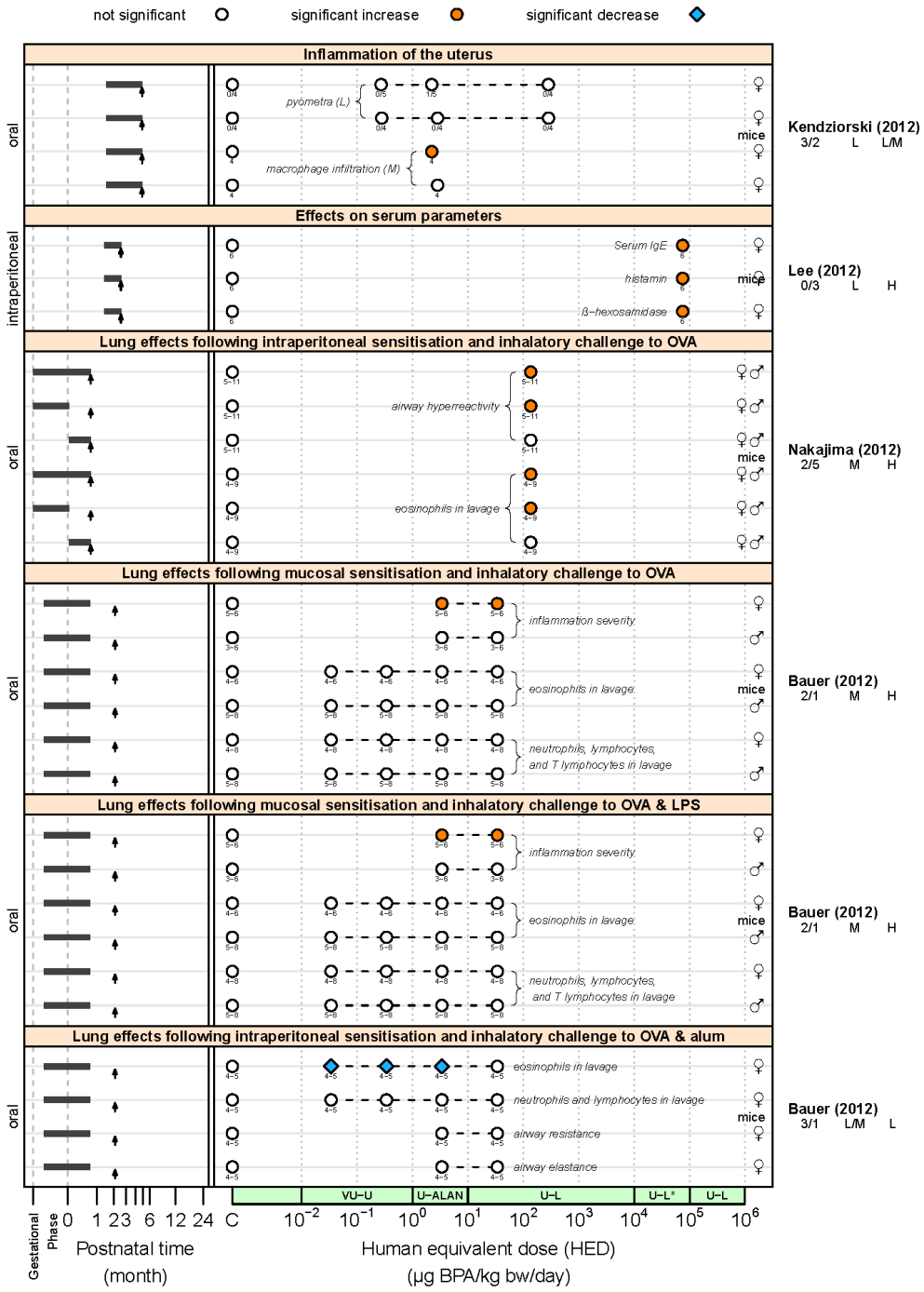


Figure 13: Summary plot for the effects of BPA exposure on the immune system: uterus inflammation, serum IgE, histamine, and β-hexosaminidase levels, and responses associated with sensitization after ovalbumin sensitization The left hand text indicates the exposure route of oral or subcutaneous (SC). The left hand text indicates the exposure route (oral or subcutaneous, SC). The left hand pane shows the experimental design with the horizontal bars showing the timing and duration of exposure (black bar: exposed as adults or during gestation and/or lactation) while the up-arrows show when the measurements/assessments were made. The central pane shows the study results , based on the x-axis showing the human equivalent dose (HED) depending upon species, age and route of exposure The central pane shows the results for the selected studies, based on the x-axis showing the human equivalent dose (HED) depending upon species, age and route of exposure. The lightly shaded boxes on the x-axis show the uncertainty assessment (very unlikely=VU, unlikely=U, as likely as not=ALAN,

likely=L and combinations thereof). The central pane also shows the sex and species of the animals under consideration (right hand-side of pane) and the nature of the symbols denotes critical information: open circles show no significant effect, red/shaded circles show significant increase and blue/shaded diamonds show significant decrease (see summaries of the studies in Appendix B for details). Numbers under the symbols either denote incidence (x/y) or samples size (n=x). The text in the right hand column shows information for each study (first author, year), the ratio of strengths/weakness of the study (taken from Appendix B), the reliability of the study (L=low, M=medium, H=high) and the relevance of the study to the biological variable and question being posed in the WoE assessment (L=low, M=medium, H=high) (see Appendix C).

Relevance and adversity to humans for immunotoxic effects

In animal studies in which increased levels of total IgE and histamine are found, these effects may be interpreted as indications of increased risk of allergy. It should be mentioned that total IgE does not necessarily indicate increased specific IgE levels, and that increased levels of IgE, be it specific of total levels, do not necessarily indicate allergy. However, if airway hyper-reactivity is found as well, this supports the notion of facilitation of respiratory allergy. Increased IgE, inflammation (especially eosinophilic inflammation) and airway hyper-reactivity are hall marks of respiratory allergy and asthma.

In case such effects would be evident in humans, in concert they would certainly indicate a risk of enhancement of respiratory allergy and asthma.

In the studies that showed a decreased number of eosinophils in lung lavage, the relevance of these effects are difficult to interpret. They do not signify a deleterious effect of respiratory allergy or asthma, while they can also not be interpreted as an improvement of such conditions, especially as in the studies presented this decrement was not associated with a decrement (nor an increment) of airway hyper-reactivity. For these reasons, the animal data indicating a lowering of eosinophils do not indicate an adverse effects, nor would such effect indicate adversity if they were to be evident in humans.

Pyometra is often seen in dogs, but also occurs in humans. It is a pathological condition, i.e. an inflammatory condition with involvement of pathogens. It is likely not a consequence of altered resistance to infections, but rather a mechanical consequence of access of pathogens to uterus tissue during the hormonal cycle. Indications of pyometra in the animal studies is most likely not an indication of immunotoxic effects of BPA.

4.3.2.5. Uncertainties in Metabolic effects

Analysis of the data in a graphical presentation (Figure 14) showed the results of the 11 studies in which parameters of glucose and insulin regulation were measured as relevant endpoints. Six of the studies had a low reliability, four studies a medium reliability and one a high reliability. Exposure was by the oral route in 10 studies and subcutaneous administration was done in one study. Exposure was prenatal (1 study), pre- and post-natal (4 studies) and post-natal (6 studies).

The doses where effects were seen ranged from 10^{-3} up to 10^{5} µg/kg bw and these studies showed contradictory results.

In addition, it can be observed that changes in one parameter (e.g. glucose) were not accompanied by changes of another parameter (e.g. insulin) in a manner which physiologically or patho-physiologically could be interpreted in a unifying hypothesis.

The assessment of the experts is concordant with this graphical presentation of the results; the probability that an effect occurs was rated very unlikely (VU) to as likely as not (ALAN) (dose range

10^{-4} – 10^{0} µg/kg bw HED) or to unlikely (UL) (dose range 10^{0} – 10^{1} µg/kg bw HED) or to as likely as not (ALAN) (dose range 10^{1} – 10^{4} µg/kg bw HED) to likely (L) (dose range 10^{4} – 10^{6} µg/kg bw HED) (see Figure 14).

The experts assessment indicates the high degree in uncertainty whether there is an effect and if so, at which level of exposure it occurs.

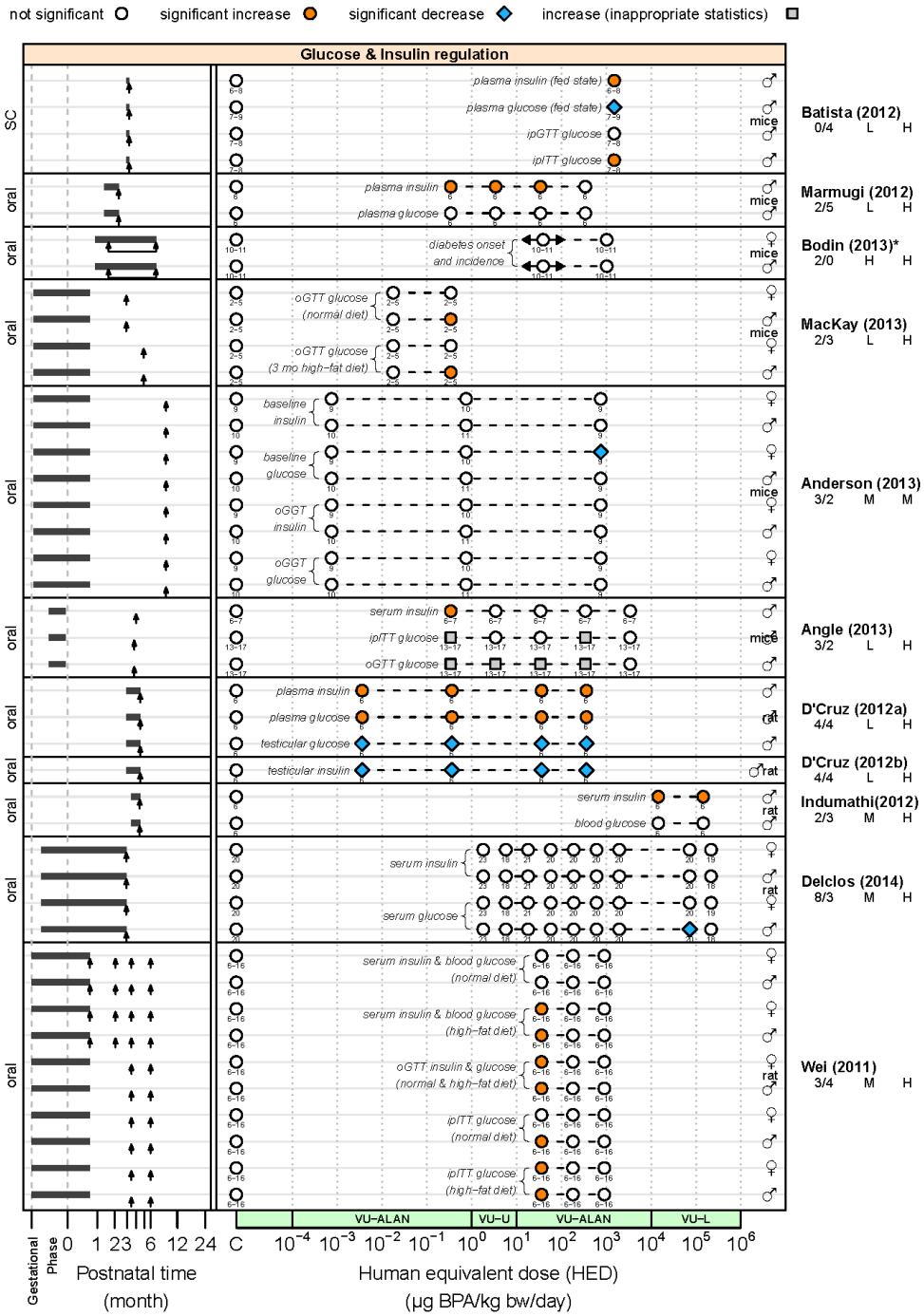


Figure 14: Summary plot for the effects of BPA exposure on the metabolic system: effects on glucose and insulin regulation. The left hand text indicates the exposure route (oral or subcutaneous, SC). The left hand pane shows the experimental design with the horizontal bars showing the timing and duration of exposure (black bar: exposed as adults or during gestation and/or lactation) while the up-arrows show when the measurements/assessments were made.

The central pane shows the study results , based on the x-axis showing the human equivalent dose (HED) depending upon species, age and route of exposure The central pane shows the results for the selected studies, based on the x-axis showing the human equivalent dose (HED) depending upon species, age and route of exposure. The lightly shaded boxes on the x-axis show the uncertainty assessment (very unlikely=VU, unlikely=U, as likely as not=ALAN, likely=L and combinations thereof). The central pane also shows the sex and species of the animals under consideration (right hand-side of pane) and the nature of the symbols denotes critical information: open circles show no significant effect, red/shaded circles show significant increase and blue/shaded diamonds show significant decrease (see summaries of the studies in Appendix B for details). Numbers under the symbols either denote incidence (x/y) or samples size (n=x). The text in the right hand column shows information for each study (first author, year), the ratio of strengths/weakness of the study (taken from Appendix B), the reliability of the study (L=low, M=medium, H=high) and the relevance of the study to the biological variable and question being posed in the WoE assessment (L=low, M=medium, H=high) (see Appendix C). oGTT and ipGTT: oral and intraperitoneal glucose tolerance test, ipITT: intraperitoneal insulin tolerance test. * This study used a non-obese diabetic mice strain. The connected arrows in Bodin (2013) indicate weekly measurements.

Relevance and adversity to humans for metabolic effects

In mammalia the regulation of carbohydrate metabolism is similar and there are no data indicating that the regulation would be species specific. The endpoints plasma/blood glucose, plasma/serum insulin and the response of blood glucose to the oral Glucose Tolerance Test (oGTT) in animals are relevant for the human situation.

In humans, low plasma/blood glucose levels are adverse if clinical symptoms are present (loss of consciousness). High plasma/blood glucose, indicating diabetes mellitus if in an oGTT levels are above 200 mg/dl at 2 hrs after glucose intake, is considered adverse. The relevance of elevated/lowered insulin plasma levels in humans is not clear in terms of metabolic situation. High insulin levels in humans indicate an insulinoma which is a benign tumour of the pancreas.

The oGTT is a widely used in humans but it is not established in animals.

Measurements of insulin after the test dose in an oGTT (in the publication refer to as oGTT response insulin) is not done in the diagnostic procedure in humans and no corresponding test in humans is known. Hence, the relevance and adversity of findings in animals for humans is not knownThe intraperitoneal insulin tolerance test (ipITT) is not done as a diagnostic procedure in humans and no corresponding test in humans is known. Hence, the relevance and adversity of findings in animals for humans is not known. Measurements of testicular glucose are not done in humans, therefore, the relevance and adversity of findings in animals for humans is not known.

4.3.2.6. Uncertainty analysis for all endpoints considering relevance and adversity to human

The experts were asked to make judgements about the overall likelihood, in each HED dose interval, that BPA has the inherent ability to cause one or more type of effect in animals and that it is relevant and adverse in humans, based on the group summaries of the judgements that had been made in the earlier stages of the assessment (table 18). The overall assessment was considered by first, considering how the likelihoods of causation, human relevance and adversity combine for each effect separately and, second, combining likelihood across effects in each dose interval. The judgements were then summarised into a range of likelihoods for each dose interval, spanning the range of those expressed by individual experts.

Although the group likelihoods are the same for $10^1 – 10^2$ and $10^0 – 10^1$ µg/kg bw per day - both are U-ALAN - there is a clear difference in the individual likelihoods between these two dose intervals,

and a clear difference again between $10^1 – 10^2$ and $10^2 – 10^3$ (in the latter, 5 of 10 experts considered the likelihood could be above 66%) (table 19)

Table 18: Summary of expert judgements of the likelihood that BPA has the inherent ability to cause effects in animals in different dose intervals and their human relevance (if they occur in animals) and adversity (if they occur in humans). (For key to likelihoods, see table 59 in Appendix D). Sexes were differentiated only for neurobehavioural effects.

Effect type	Human relevance	Adversity in humans	Likelihood that BPA causes the effect in animals in different dose intervals Human equivalent dose (HED), µg BPA/kg bw per day									
			10^{-4}-10^{-3}	10^{-3}-10^{-2}	10^{-2}-10^{-1}	10^{-1}-10^{0}	10^{0}-10^{1}	10^{1}-10^{2}	10^{2}-10^{3}	10^{3}-10^{4}	10^{4}-10^{5}	10^{5}-10^{6}
Mammary proliferation	ALAN-L	ALAN-L	VU	VU	VU	VU	U-ALAN	U-ALAN	ALAN-L	ALAN-L	ALAN-L	ALAN-L
Reproductive system	ALAN-L	ALAN-L	-	-	VU-U	VU-U	VU-U	VU-ALAN	VU-ALAN	VU-ALAN	VU-L	L-VL
Metabolic	ALAN-L	U-L	VU-ALAN	VU-ALAN	VU-ALAN	VU-ALAN	VU-U	VU-ALAN	VU-ALAN	VU-ALAN	VU-L	VU-L
Immune system	U-L	ALAN-L	-	-	VU-U	VU-U	U-ALAN	U-L	U-L	U-L	U-L	-
Neurobehaviour (males)	U-L	U-L	-	VU-U	VU-U	U-ALAN	ALAN	ALAN	ALAN	ALAN-L	-	-
Neurobehaviour (females)			-	VU-U	VU-U	U	U	U-ALAN	ALAN	U-L	-	-

-: no data available

Table 19: Expert judgements of the overall likelihood, in each HED dose interval, that BPA has the inherent ability to cause one or more type of effect (of the types shown in table 18) in animals and that it is relevant and adverse in humans. (For key to likelihoods, see table 59 in appendix D).

Expert	HED Dose interval (µg BPA/kg bw per day)									
	10^{-4}-10^{-3}	10^{-3}-10^{-2}	10^{-2}-10^{-1}	10^{-1}-10^{0}	10^{0}-10^{1}	10^{1}-10^{2}	10^{2}-10^{3}	10^{3}-10^{4}	10^{4}-10^{5}	10^{5}-10^{6}
1			U	U	U-ALAN	U-ALAN	ALAN	ALAN		
2			VU	VU-U	U-ALAN	ALAN	ALAN-L	ALAN-L		
3			U	U	U	ALAN	ALAN	ALAN-L	L	L
4	VU	VU	VU	VU	U	U-ALAN	ALAN	ALAN-L	ALAN-L	L
5	VU	U	U	U	U	ALAN	L	L	L	VL
6			VU-U	VU-U	U	U-ALAN	U-ALAN	ALAN		
7			U	U	ALAN	ALAN	L	L	L	
8	VU	VU-U	U	U	ALAN	ALAN	ALAN-L	L	L	L
9			U	U	ALAN	ALAN	ALAN-L	ALAN-L	ALAN	ALAN
10	EU	EU	VU	VU	U	U	U-ALAN	ALAN	ALAN	L
GROUP EVALUATION	**(EU - VU)***	**(EU - U)***	**VU - U**	**VU - U**	**U- ALAN**	**U- ALAN**	**U-L**	**ALAN- L**	**(ALAN- L)***	**(ALAN-VL)***

*: For these ranges of doses the experts did not provide a full evaluation because there were not data available for all the endpoints.

The overall uncertainty evaluation, including the effects on mammary gland as well as reproductive, neurobehavioural, immune and metabolic systems is presented in table 19. The CEF Panel concluded that the health-based guidance value should cover the lowest dose in the dose-range for which the likelihood approaches "likely" from the overall uncertainty evaluation, taking into account uncertainty of all the evaluated endpoints as well as their relevance and adversity to humans. The uncertainty evaluation approached "likely" in the dose (HED) range of 100-1 000 µg/kg bw per day, and therefore the CEF Panel decided that the uncertainty in the other effects at the HED of 100 µg/kg bw per day and higher should be taken into account when establishing a health-based guidance value.
The CEF Panel decided that an extra factor should be included to take into account the uncertainty in the database (mammary gland, and reproductive, neurobehavioural, immune and metabolic systems). Thus, as the reference point was 609 µg/kg bw per day based on the mean relative kidney weight and the lower end of the dose-range for which the uncertainty evaluation for other endpoints approached "likely" is 100 µg/kg bw per day, a factor of 6 was applied.

4.4. Health-based guidance value

In deriving a health-based guidance value, the CEF Panel used an uncertainty factor of 2.5 for inter-species differences (1 for toxicokinetics and 2.5 for toxicodynamics, reflecting the fact that toxicokinetic differences have been addressed by use of the HED approach) and an uncertainty factor of 10 for intra-species differences (Ref to SC opinion on the guidance on default values). In addition, the CEF Panel considered that the extra factor of 6 should be included to take into account the uncertainty in the database, i.e. mammary gland, and reproductive, neurobehavioural, immune and metabolic systems (see Section 4.3).

The CEF Panel applied, therefore, an overall uncertainty factor of 150 to the HED of 609 µg/kg bw per day and established a temporary Tolerable Daily Intake (t-TDI) for external oral exposure to BPA in humans of 4 µg/kg bw, based on the mean relative kidney weight effect in the mouse. The CEF Panel designated the TDI as temporary, pending the outcome of the long-term study in rats involving prenatal as well as postnatal exposure to BPA, currently being undertaken by NTP/FDA. This study will help resolve the uncertainties in the database.

5. Risk characterisation

In this section, the CEF Panel compares the t-TDI of 4 µg/kg bw per day with the exposure estimates for BPA.

The exposure estimates for both oral and dermal exposure (external exposures) are provided for different age groups and subpopulations in table 22 (average exposures) and 23 (high exposures) in Part I- Exposure assessment.

For the purpose of risk characterisation, the CEF Panel carried out an assessment of aggregated dietary and non-dietary exposure to unconjugated BPA using PBPK modelling. This aggregated exposure assessment included the oral-route sources diet, dust and toys (mouthing) as well as the dermal-route sources thermal paper and cosmetics. The dermal exposure estimates have been expressed as equivalent oral exposures (table 20) in order to provide an aggregated (oral plus dermal) exposure scenario for comparison with the t-TDI. This conversion is needed because the reference dose for the risk assessment (t-TDI) has been derived for external oral exposure.

This conversion of the dermal exposures to equivalent oral doses has been carried out by PBPK modelling, as described in section 3.1.7.3 of the opinion, with the outcome of the conversion being shown in table 5 and table 6 of that section (for average and high exposures). The converted exposure estimates are summarised in table 20. It should be noted that the parameters used in the PBPK model for aggregated exposure were only available for the two age groups "children (3-10 years)" and "men (18-45 years)" (see Section 3.1.7.3). The CEF Panel considered, however, that the dermal equivalent oral dose for the age group men 18-45 years is likely to be also representative for the age groups "women 18-45 years", "other adults 45-65 years" and "elderly, very elderly 65 years and over" and

adolescents, assuming that the toxicokinetics of BPA in these age groups are not significantly different to those of "men (18-45 years)". The CEF Panel noted that the highest dietary exposure resulted in toddlers and children 3-10 years, with very similar values (0.857 and 0.813 µg/kg bw per day, respectively). However the non-dietary exposure scenarios derived for "children 3-10 years" were by far the highest of any of the child populations (age below 10) identified in table 20, and for the purposes of risk characterization, the exposure estimates for this population could also be used as "worst-case" surrogates for infants and children aged below 3 years. Using the above assumptions, aggregated exposure was therefore estimated for "children 3-10 years", adolescents and adult age groups.

Table 20: Summary table on average (A) and high (H) exposure (µg/kg bw per day) from dietary and non-dietary sources to BPA in the different age groups of the general population. Dermal doses are expressed as equivalent oral doses as obtained by PBPK modelling, taken from table 22 and 23 in Part I-Exposure assessment (absolute values for dermal exposure), and table 5 and table 6 in Section 3.1.7.3.

Age group	Exposure level	Dietary	Non-dietary			
		Oral	Oral	Dermal		Sum of non-dietary
		Food & beverage	Dust & Toys	Thermal paper	Cosmetics	
Infants 1-5 days (breastfed)	A	0.225	-	-	-	-
	H	0.435				
Infants 6 days - 3 months (breastfed)	A	0.165	0.009	-	-	0.009
	H	0.6	0.015			0.015
Infants 4-6 months (breastfed)	A	0.145	0.009	-	-	0.009
	H	0.528	0.015			0.015
Infants 0-6 months (formula fed)	A	0.03	0.009	-	-	0.009
	H	0.08	0.015			0.015
Infants 6-12 months	A	0.375	0.009	-	-	0.009
	H	0.857	0.015			0.015
Toddlers 1-3 years	A	0.375	0.007	-	-	0.007
	H	0.857	0.012			0.012
Children 3-10 years	A	0.290	0.003	0.053	0.008	0.064
	H	0.813	0.005	0.424	0.016	0.445
Adolescents 10-18 years	A	0.159	0.002	0.113	0.015	0.13
	H	0.381	0.003	1.036	0.029	1.068
Women 18-45 years	A	0.132	0.0006	0.071	0.012	0.084
	H	0.388	0.001	0.650*	0.024*	0.675*
Men 18-45 years	A	0.126	0.0006	0.071	0.012	0.084
	H	0.335	0.001	0.650	0.024	0.675
Other adults 45-65 years	A	0.126	0.0006	0.071	0.012	0.084
	H	0.341	0.001	0.650*	0.024	0.675*
Elderly and very elderly 65 years and over	A	0.116	0.0006	0.071	0.012	0.084
	H	0.375	0.001	0.650*	0.024*	0.675*

* It is assumed that the dermal exposures as expressed as equivalent oral doses for the age group men 18-45 years also are representative for the age groups women 18-45 years, other adults 45-65 years, and elderly and very elderly 65 years and over, assuming that the toxicokinetics are not significantly different between these age groups.

The CEF Panel first compared the estimates for high dietary exposure for all age groups, as shown in table 20, with the t-TDI of 4 µg/kg bw per day. This comparison showed that the dietary exposure in all age groups (including all infants and toddler groups) was more than 4-fold below the t-TDI, indicating no health concern from dietary exposure alone. The additional contribution from other oral sources, like dust and toys mouthing, did not change this conclusion.

The CEF Panel then compared the aggregated exposure estimates (dietary plus non-dietary sources including dust, toys, cosmetics and thermal paper) for "children 3-10 years", adolescents and adult age groups, as presented in tables 21-23, with the t-TDI.

Table 21: Aggregated exposure from diet and non-dietary sources for the population group children 3–10 years and adolescents.

	Children 3–10 years (µg/kg bw per day)		**Adolescents (µg/kg bw per day)**	
Route of exposure	Dietary average (d)	Dietary high (D)	Dietary average (d)	Dietary high (D)
Non-dietary average (n-d)	0.064 (n-d) 0.290 (d) **0.354**	0.064 (n-d) 0.813 (D) **0.877**	0.13 (n-d) 0.159(d) **0.289**	0.13 (n-d) 0.381 (D) **0.511**
Non-dietary high (N-D)	0.445 (N-D) 0.290 (d) **0.735**	0.445 (N-D) 0.813 (D) **1.258**	1.068 (N-D) 0.159 (d) **1.226**	1.068 (N-D) 0.381 (D) **1.449**

The aggregated exposure estimates presented in table 21 show that even when the high estimates for diet and non-dietary sources are combined, the aggregated exposure for children 3–10 years (1.258 µg/kg bw per day) and adolescents (1.449 µg/kg bw per day) will be approximately 3 fold below the t-TDI. The CEF Panel noted that the exposure scenarios derived for children 3–10 years are the highest of any of the child populations (age below 10) identified in table 20 and the margin between the t-TDI and the exposures for the other child populations (i.e. younger than 3 years of age) will therefore be greater than that for children 3–10 years.

The aggregated exposure estimates for children 3–10 years presented in table 21 shows that the largest contribution to the aggregated average exposure from diet and average exposure from non-dietary sources comes from dietary exposure (82% from diet vs. 18% from non-dietary sources). For the aggregated high dietary and high non-dietary exposure, also diet contributes the most (65%). The aggregated exposure estimates for adolescents show that the largest contribution to the aggregated average dietary and average non-dietary exposure comes from diet (55% vs. 45% from non-dietary sources). For the aggregated high dietary and high non-dietary exposure from non-dietary sources, non-dietary contribute most (74%). Thermal paper contributed the most to the exposure from non-dietary sources.

Table 22: Aggregated exposure from diet and non-dietary sources for women 18-45 years and for men 18-45 years

	Women 18–45 years (µg/kg bw per day)		**Men 18–45 years (µg/kg bw per day)**	
Route of exposure	Dietary average (d)	Dietary high (D)	Dietary average (d)	Dietary high (D)
Non-dietary average (n-d)	0.084 (n-d) 0.132 (d) **0.216**	0.084 (n-d) 0.388 (D) **472**	0.084 (n-d) 0.126 (d) **0.210**	0.084 (n-d) 0.335 (D) **0.419**
Non-dietary high (N-D)	0.675 (N-D) 0.132 (d) **0.807**	0.675 (N-D) 0.388 (D) **1.063**	0.675 (N-D) 0.126 (d) **0.801**	0.675 (N-D) 0.335 (D) **1.010**

The aggregated estimates for high dietary and high non-dietary exposure for women (1.063 µg/kg bw per day) and men (1.010 µg/kg bw per day) are mostly identical, and they are lower than those for adolescents and "children 3–10 years". The CEF Panel noted that the exposure estimates for men and women (including pregnant women) are approximately 4-fold lower than the t-TDI of 4 µg/kg bw per day.

Table 23: Aggregated exposure from diet and non-dietary sources for other adults 45–65 years and for the elderly and very elderly 65 years and over

	Other adults (45–65 years) (µg/kg bw per day)		**Elderly and very elderly (65 years and over) (µg/kg bw per day)**	
Route of exposure	Dietary average (d)	Dietary high (D)	Dietary average (d)	Dietary high (D)
Non-dietary average (n-d)	0.084 (n-d) 0.126 (d) **0.210**	0.084 (n-d) 0.341 (D) **0.425**	0.084 (n-d) 0.116 (d) **0.200**	0.084 (n-d) 0.376 (D) **0.460**
Non-dietary high (N-D)	0.675 (N-D) 0.126 (d) **0.801**	0.675 (N-D) 0.341 (D) **1.016**	0.675 (N-D) 0.116 (d) **0.791**	0.675 (N-D) 0.376 (D) **1.051**

The aggregated exposure estimates for other adults and the elderly are in the same range as the those for women and men, and they are also approximately 4-fold below the t-TDI.

Overall, the CEF Panel concluded that there is no health concern from high dietary BPA exposure alone or from aggregated exposure to BPA from diet and non-dietary sources for any of the age groups: even the highest aggregated exposure of 1.449 µg/kg bw per day estimated for adolescents was approximately 3-fold lower than the t-TDI of 4 µg/kg bw per day.

5.1 Uncertainties in the risk characterisation

5.1.1. Uncertainty in the characterisation of risks from foodstuffs and non-dietary sources

The CEF Panel considered the uncertainties associated with the characterisation of risks posed by BPA in foodstuffs to different age groups. These uncertainties come from two sources, which were assessed in earlier sections of the opinion: uncertainties in the identification and characterisation of hazard (Section 4.3), and uncertainties in exposure (Section 4.7.2 in Part I- Exposure assessment). In the present section, the CEF Panel considered the implications of these uncertainties for risk characterisation. table 24 shows the main elements of the uncertainty analysis for women of childbearing age. Similar analyses were completed for males aged 18-45, adolescents (aged 10-18), and children aged 3-10. These are not shown but gave similar results to those in table 24 and the associated figures.

The CEF Panel took account of uncertainties affecting hazard identification and characterisation by applying an additional assessment factor of 6 when setting the t-TDI of 4 µg/kg bw per day (Section 4.4). The CEF Panel expressed its judgement of uncertainties affecting dietary exposure assessment using a scale of symbols representing the degree to which real dietary exposures might be higher or lower than the CEF Panel's estimates of exposure of different population groups to BPA from foodstuffs (Section 4.7.2 in Part I- Exposure assessment). In order to put this into context with exposure from non-dietary sources, including dust, thermal paper and cosmetics, the uncertainty of external exposure estimates for those non-dietary sources was also evaluated in the same way. The results of those evaluations are summarised in table 24.

The evaluations of uncertainty in dietary exposure assessment using symbols were converted to quantitative ranges, by the method described in section 4.7.2 (in Part I- Exposure assessment), in order to evaluate their impact on the comparison between exposure estimates and the t-TDI of 4 µg/kg bw per day for risk characterisation. This produced an upper and lower bound for the uncertainty of each estimate, shown in the right column at the top of table 24.

In order to put the exposures to BPA in foodstuffs into context, the CEF Panel converted its estimates of exposure from non-dietary sources, and their uncertainty ranges, into equivalent oral doses. This required the use of dermal-to-oral route equivalence factors ($EF_{D\rightarrow O}$) derived from PBPK modelling, which are themselves subject to uncertainty. An important source of uncertainty affecting the $EF_{D\rightarrow O}$

values is the choice of the PBPK model to use: there was about 7.6-fold difference between estimates produced using the Mielke/Gundert-Remy model and those that would be obtained if it would be possible to use the Fisher model (see Section 3.1.7 and Appendix D). Therefore, to provide an indication of the impact of this source of uncertainty on risk characterisation, the conversion of dermal exposure estimates to oral equivalents was done first with the Mielke/Gundert-Remy model and then with the addition of the factor of about 7.6 representing the difference between the Mielke/Gundert-Remy and Fisher models (table 24).

Another important source of uncertainty affecting the $EF_{D\rightarrow O}$ values is the absorption fractions which were used in PBPK modelling to convert the external doses from thermal paper and cosmetics into internal doses (as described at the end of section 3.1.7). It was assumed that 10% of the external dose from thermal paper is absorbed. The uncertainty of the dermal absorption factor used for thermal paper (0.1) was assessed as – –/+ (×0.2 to ×2), i.e. the true factor is considered to lie between 0.02 and 0.2. The uncertainty of dermal absorption factor used for cosmetics (0.5) was also assessed as – –/+ (×0.2 to ×2), i.e. the true factor might lie between 0.1 and 1. The effect of this uncertainty on the $EF_{D\rightarrow O}$ factors can be calculated by applying the same uncertainty ratings (e.g. ×0.2 to ×2 for thermal paper exposure) to them.

table 24: Evaluation of uncertainties affecting exposure assessment for females aged 18–45 years

Source of uncertainty	Estimates for each component	Uncertainty of the estimates	Approximate maximum plausible range*
EXTERNAL EXPOSURE BY EACH ROUTE:			
Average Oral exposure			
Average dietary exposure to BPA (µg/kg bw per day)	0.13	–/●	0.066 - 0.16
Average exposure from dust (µg/kg bw per day)	0.0006	– –/●	0.00012 - 0.001
High Oral exposure			
High dietary exposure to BPA (µg/kg bw per day)	0.39	–/●	0.19 - 0.47
High exposure from dust (µg/kg bw per day)	0.001	– – –/+	0.0001 - 0.002
Average Dermal exposure			
Average exposure from dermal contact with thermal paper (µg/kg bw per day)	0.059	– – –/++	0.006 - 0.29
Average exposure from cosmetics (µg/kg bw per day)	0.002	– –/++	0.0004 - 0.010
High Dermal exposure			
High exposure from dermal contact with thermal paper (µg/kg bw per day)	0.54	– –/++	0.11 - 2.7
High exposure from cosmetics (µg/kg bw per day)	0.004	– –/++	0.0008 - 0.020
Dermal absorption fractions			
Dermal absorption fraction for thermal paper	0.1	– –/+	0.02 - 0.2
Dermal absorption fraction for cosmetics	0.5	– –/+	0.1 - 1
Conversion of dermal exposure to oral equivalent using PBPK modelling			
Conversion for thermal paper exposure (ratio)	1.2	not considered	
Conversion of cosmetics exposure (ratio)	6.0	not considered	
Conversion of dermal exposure including model uncertainty factor of 7.6**			
Conversion for thermal paper exposure (ratio)	9.1	not considered	
Conversion of cosmetics exposure (ratio)	45.6	not considered	
Dermal exposures as oral equivalents converted with & without uncertainty factor			
Average exposure from dermal contact with thermal paper (µg/kg bw per day, no factor)	0.071		0.001 - 0.71
Average exposure from dermal contact with thermal paper (µg/kg bw per day, with factor)	0.54		0.011 - 5.4
Average exposure from cosmetics (µg/kg bw per day, without factor)	0.012		0.000 - 0.12
Average exposure from cosmetics (µg/kg bw per day, with factor)	0.091		0.004 - 0.91
High exposure from dermal contact with thermal paper (µg/kg bw per day, without factor)	0.65		0.026 - 6.5
High exposure from dermal contact with thermal paper (µg/kg bw per day, with factor)	4.9		0.20 - 49
High exposure from cosmetics (µg/kg bw per day, without factor)	0.024		0.001 - 0.24
High exposure from cosmetics (µg/kg bw per day, with factor)	0.18		0.007 - 1.8

**Using full width of range implied by the uncertainty symbols (-/+/●) in the third column*

*** Model uncertainty factor of 7.6 representing difference between Mielke & Fisher models*

The results of these evaluations are presented together with the t-TDI in Figure 15. This shows the average and high estimates for dietary exposure for women of childbearing age, together with the ranges expressing their uncertainty, and corresponding estimates for the two largest non-dietary sources of exposure (thermal paper and cosmetics), expressed as oral equivalents. It can be seen from Figure 15 and Table 24 that:

- The estimates and uncertainty ranges for both average and high dietary exposure are well below the t-TDI for all 4 age groups considered (upper bound of high estimate: 0.47 µg/kg bw per day for women aged 18–45 years; maximum: 0.98 µg/kg bw per day for children aged 3–10 years).
- The estimated equivalent oral exposures for non-oral routes (thermal paper and cosmetics) are very much more uncertain than the dietary estimates for the oral route, as indicated by the width of the dashed lines in Figure 15.
- The difference in the predictions of the Mielke and Fisher PBPK models (see Section 3.1.7 and Appendix D) is also a substantial source of uncertainty when the doses for non-oral routes are

converted to oral equivalents, as can be seen from the distance between the triangles and circles in Figure 15.

- The estimate of high equivalent oral exposure from thermal paper is 4.9 µg/kg bw per day and its upper bound (including model uncertainty) is 10-fold higher, due to the combined effects of the factor of about7.6 representing PBPK model uncertainty together with the uncertainty of estimated external exposure and of the dermal absorption fraction. However, the lower bound is 0.2 µg/kg bw per day, a factor of 20 below the estimate, so it is clear that the contribution from thermal paper is highly uncertain (factor of 200 overall).
- The upper bound (including model uncertainty) for high equivalent oral exposure from cosmetics reaches 1.8 µg/kg bw per day. This results from the upper bound for the dermal absorption fraction (1.0, the maximum possible), the upper bound of the estimated external exposure to cosmetics (a factor of 5 above the estimate), and the factor of 7.6 that takes account of the PBPK model uncertainty.
- Similar analyses for men aged 18–45 years, adolescents and children aged 3–10 years gave very similar patterns of results.

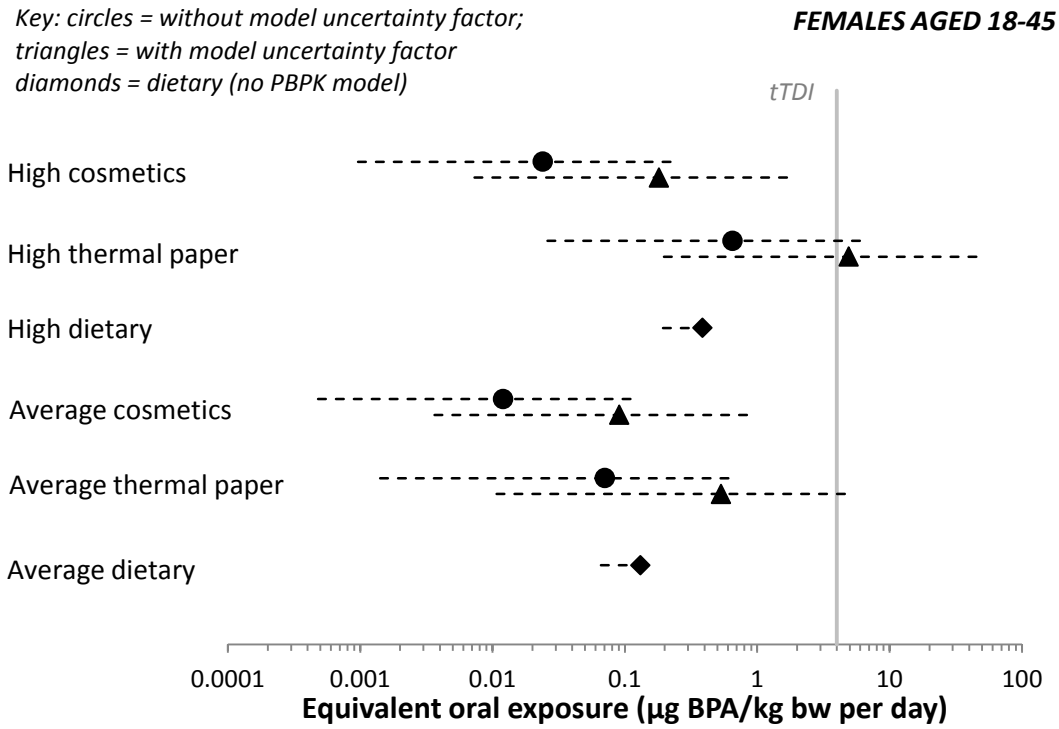


Figure 15: Analysis of uncertainty affecting the comparison of the proposed t-TDI of 4 µg/kg bw per day with the estimated exposure to BPA in foodstuffs (oral route) for women of childbearing age (18–45 years). Also shown are estimates for the largest non-oral sources of exposure (thermal paper and cosmetics), converted to oral equivalents by PBPK modelling with and without a model uncertainty factor. Note the exposures and t-TDI are plotted on a $\log_{10}$ scale, with labels in the natural scale. Dashed lines show approximate ranges for the uncertainty of the estimates.

5.1.2. Uncertainty of aggregate exposure assessment

Estimates of aggregate equivalent oral exposure were calculated using the following following equation:

$$Aggregate\ exposure = Dietary + Dust + Paper \times Paper\ EF_{D\rightarrow O} + Cosmetic \times Cosmetic\ EF_{D\rightarrow O}$$

Dermal exposure estimates for thermal paper and cosmetics are converted into equivalent oral exposures by source-specific dermal-to-oral route equivalence factors (EF_{D_O}). Estimates of (oral equivalent) exposures for each of the sources identified in the equation are shown in Table 24, for women aged 18–45 years, together with their uncertainty ranges.

As explained in section 4.7.2 (in Part I- Exposure assessment), the exposure assessment is intended to estimate, for each population group, the 95th percentile exposure in the EU country where this is the highest. For each individual source of exposure, 'average' and 'high' estimates have been made as approximate estimates for the mean and 95th percentile. For estimating aggregate exposure we explore different combinations of average and high estimates. Air is a very minor contribution to the aggregate exposure and is excluded from all aggregate exposure estimates but would have a negligible influence on the total if it were included. Tables 21-23 and Figure 16 show four aggregations of estimates for each population group:

1. Averages for both dietary exposure and non-dietary exposure (A1)
2. High estimates for dietary exposure and averages for non-dietary exposure (A2)
3. Averages for dietary exposure and high estimates for non-dietary exposure (A3)
4. High estimates for both dietary and non-dietary exposure (A4).

Aggregate 1 is an approximate estimate of the average aggregate exposure. It is expected that the 95th percentile aggregate exposure will be in the region of the higher of aggregates 2 and 3, and below Aggregate 4, which is expected to approximate a 99th percentile or higher. Together aggregates 1-4 provide an indication of where the 95th percentile exposure is likely to be. To obtain firmer estimates of 95th percentile aggregate exposure would require probabilistic exposure modelling, as it will depend on the detailed shape and variance of the distributions for each route of exposure and any dependencies between them.

In order to evaluate the impact of the uncertainties in the different parts of the exposure assessment on the estimates of aggregate exposure, the calculation shown in the equation above was repeated three times: once with the CEF Panel's estimate of each parameter in the equation, once with the upper end of the uncertainty range for each parameter, and once with the lower bound of the uncertainty range for each parameter. These three calculations were conducted for each of the four aggregates (A1-A4, above), providing an estimated aggregate exposure and an uncertainty range for each aggregate.

The results of these calculations are presented together with the t-TDI in Figure 16 (the numerical estimates are given in Table 57 in Appendix D). These show four aggregations of average and high exposure estimates (aggregates 1-4, as explained above). Each aggregate is estimated twice, once with the dermal exposures being converted into equivalent oral exposures without a PBPK model uncertainty factor (circles) and the other including a PBPK model uncertainty factor of about 7.6 (triangles), representing the expected difference in the predictions of Mielke/Gundert-Remy model and the Fisher model.
It can be seen from Figure 16 that:

1) All of the aggregated equivalent oral exposure estimates have wide uncertainty ranges. It can be seen from Figure 16 that this is very largely due to the high uncertainty of the contributions from thermal paper, which are much more uncertain than the dietary exposure.

2) The difference in the predictions of the Mielke/Gundert-Remy and Fisher PBPK models is a substantial source of uncertainty (see Section 3.1.7 and Appendix D).
3) All the estimates of aggregate equivalent oral exposure are below the t-TDI, except for those that result from Aggregates 3 and 4 and include the factor of about7.6 for PBPK model uncertainty. These estimates are highly uncertain, with two orders of magnitude between the lower and upper bounds.
4) The assessment is dominated by the high uncertainty of the exposure from thermal paper. If further investigation confirms that this lies in the lower half of its current uncertainty range, then the largest source of exposure would be dietary and Aggregate 2 would be a better guide to the true 95th percentile than Aggregate 3. If, on the other hand, further investigation shows that thermal paper lies in the upper half of its current uncertainty range, this would be the dominant source and Aggregate 3 would be a better guide.
5) Similar analyses for men aged 18–45 gave a very similar pattern of results to those in Figure 16. The pattern for adolescents and children aged 3–10 was similar, but shifted a little to the right.

Additional uncertainties that were not quantified in the uncertainty analysis are considered in table 58 (Appendix D), and do not alter the above conclusions.

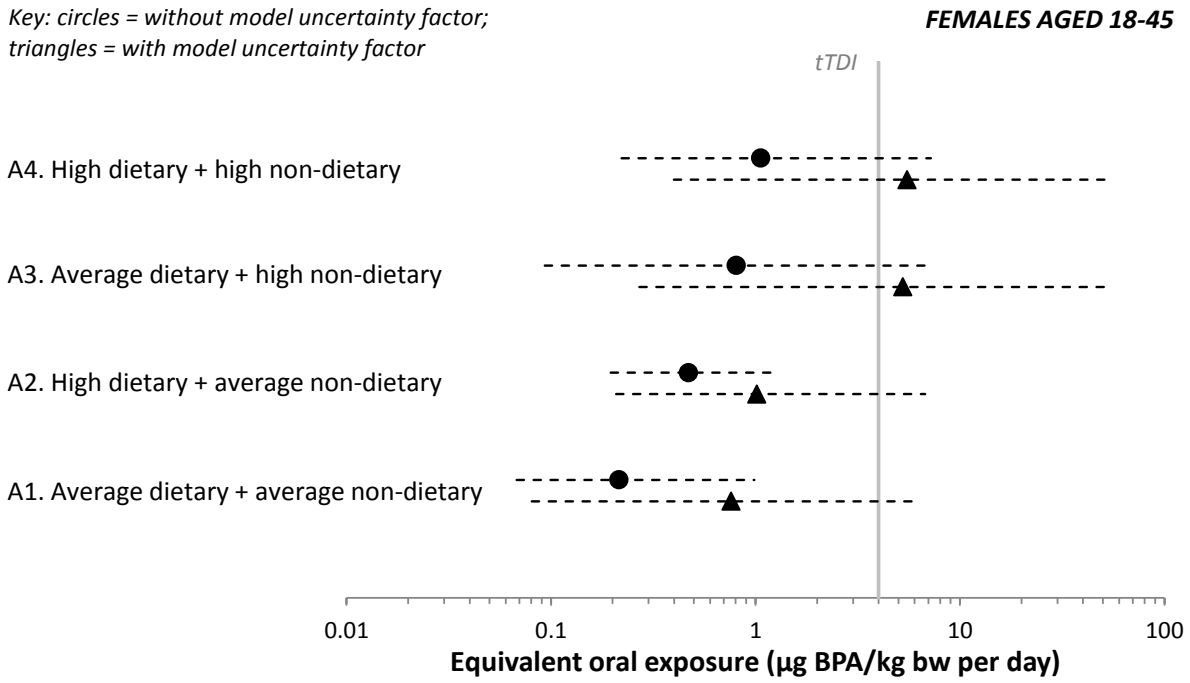


Figure 16: Comparison of alternative estimates of aggregate equivalent oral exposure for females aged 18-45 and their uncertainty. Dashed lines show approximate uncertainty ranges for each estimate. The proposed t-TDI of 4 µg/kg bw per day is also shown. A1-A4 denote aggregate estimates 1-4, see text for explanation and Appendix D for numerical estimates.

5.1.3. Conclusion of the uncertainty analysis

Overall, having evaluated the overall uncertainty of this assessment, the upper bound of the uncertainty interval for dietary BPA exposure alone did not exceed the t-TDI for any age group. Upper bound for the uncertainty of high but not average aggregate exposure estimates to BPA exceed the t-TDI but in all cases the lower bounds are considerably lower than the t-TDI. The wide uncertainty intervals are caused by uncertainty about the magnitude of external exposure to BPA from thermal

paper, about the fraction of BPA which is absorbed through the skin, and about the choice of PBPK model for converting dermal exposures to oral equivalents.

6. Conclusions

The CEF Panel was asked to address the risk to public health related to the presence of BPA in foodstuffs as well as to estimate exposure from all sources (dietary and non-dietary). The CEF Panel felt that from a public health point of view the exposure from food needs to be seen in the context of all sources of exposure for the general population. Therefore, the risk characterisation was carried out for food-related exposure to BPA and for aggregated exposure, which covers dietary as well as non-dietary sources.

6.1. Exposure assessment

The exposure assessment involved the estimation of consumer exposure in terms of the following three different metrics:

- External exposure to BPA (in Part I - Exposure assessment)
  The assessment of external exposure considered the different routes (ingestion, inhalation, dermal uptake) and sources (e.g. diet, drinking water, air, thermal paper). It estimated the source-specific doses reaching the physical barriers in the gastrointestinal and respiratory tracts and of the skin. Route-specific external exposures to BPA were calculated via the so-called forward modelling approach by multiplying source concentrations with the corresponding use frequencies (e.g., food intake, handling of thermal paper).

- Internal exposure to total BPA (in Part I - Exposure assessment)
  The assessment of internal exposure to total BPA considered the route- and source (thermal paper/cosmetics)-specific absorption fractions to estimate the doses that passed the physical barriers to enter the body. For this purpose the estimates for average and high internal exposure to total BPA from all sources and over all routes were combined (Combinations C1-C4).
  These internal doses are expressed as doses of total BPA (as no differentiation is made between the unconjugated and conjugated BPA). As BPA is completely eliminated via urine, the sum of these internal doses is directly comparable to exposure estimates obtained by the so-called backward modelling approach from urinary biomonitoring data.

- Aggregated exposure to unconjugated BPA (in Part II - Toxicological assessment and risk characterisation)
  The assessment of aggregated exposure used PBPK modelling to translate the contributions from the relevant sources (diet and house dust for the oral route, thermal paper and cosmetics for the dermal route) into a toxicologically relevant dose metric, the area under the curve (AUC) for the serum concentration of unconjugated BPA. A translation into AUC is necessary because, in contrast to dermally absorbed BPA, BPA absorbed from the gastrointestinal tract is subjected to the first-pass metabolism before entering the systemic circulation. Based on the AUC information, external dermal exposures (thermal paper, cosmetics) were converted to equivalent oral exposures which could then be summed up with the external oral exposures (diet, house dust) to arrive at an aggregated exposure estimate.

The assessment of exposure to BPA from all sources showed that diet is the main source in all population groups for external exposure only Specifically, canned food and non-canned meat and meat products are the two main dietary contributors to external BPA exposure in the large majority of countries and age classes. The pattern was different for aggregate exposure (see Section 5).

While canned food as a main source of external dietary exposure to BPA is confirmed by the data presented in this opinion, exposure from non-canned meat and meat products and fish had not been anticipated until the 2013 report of ANSES on concentrations of BPA in French food. Investigation of

these findings is currently under way, but, until further results are available, there is no substantiated explanation for the presence of unconjugated BPA in foods of animal origin.

Among the population older than six months, infants (6-12 months) and toddlers had the highest estimated external average (0.375 µg/kg bw per day) and high (0.857 µg/kg bw per day) dietary exposure. This was mainly due to their higher consumption of foods and beverages per kilogram body weight. The modelled dietary exposure for adolescents, adults (including women of childbearing age) and elderly/very elderly ranged from 0.116 to 0.159 µg/kg bw per day for the average external exposure and from 0.335 to 0.388 µg/kg bw per day for the high exposure, respectively. Dietary exposure to BPA estimated by EFSA in 2006 in the population older than six months was far higher (up to 5.3 µg/kg bw per day in toddlers) compared with the current assessment (up to 0.857 µg/kg bw per day for the high exposure of toddlers), owing to the lack of data at that time which led to the use of very conservative assumptions in relation to both the level of consumption of canned food and the estimated BPA concentration in these foods.

The availability of more data now, as compared to 2006, has allowed the CEF Panel to carry out a more refined dietary exposure assessment for infants. According to the current estimate, BPA exposure for infants up to six months (0.03 to 0.225 µg/kg bw per day for average external exposure) is much lower than that estimated by EFSA in 2006 for infants within six months of age (≤ 11 µg/kg bw per day)bw per daybw per day. This was due to the use at that time of very conservative assumptions in relation to BPA concentration in infant formula and to BPA migration from PC bottles to account for the lack of data.

Dietary external exposure in women of childbearing age (0.132 and 0.388 µg/kg bw per day for average and high exposure, respectively) was similar to that in men of the same age (0.126 and 0.335 µg/kg bw per day for average and high external exposure, respectively). The minimal differences may be related to women consuming different food items, as reported in the individual surveys.

The uncertainty around the estimates of dietary exposure was judged as relatively low compared to other sources of exposure such as thermal paper.

Thermal paper was the second largest source of external exposure in all population groups above three years of age. The modelled estimates for 3–10 years old children, adolescents, adults (including women of childbearing age) and elderly/very elderly ranged from 0.059 to 0.094 µg/kg bw per day for the average exposure and from 0.542 to 0.863 µg/kg bw per day for the high external exposure, respectively. The CEF Panel considers that more data would be needed for BPA absorption through the skin, on skin metabolism and for patterns of thermal paper handling by the general population in order to provide a refined estimate of exposure through this source to reduce the uncertainty in the estimate.

In children under the age of three years (except for infants in the first few days of life) dust was the second largest source of external exposure to BPA and ranged from 0.009 to 0.015 µg/kg bw per day for average and high external exposure, respectively.

Average external exposure to BPA from other sources such as toys and cosmetics was estimated to be less than 0.001 µg/kg bw per day and 0.005 µg/kg bw per day, respectively, in all population groups.

For the four age classes covering infants from 1 day up to 6 months, the average internal exposure to total BPA, as estimated by forward modelling, ranged from 0.042 µg/kg bw per day to 0.226 µg/kg bw per day. The average internal exposure for the population older than six months ranged from 0.301 to 0.387 µg/kg bw per day in children aged 3 to 10 years and infants aged 6 to12 months and from 0.124 to 0.172 µg/kg bw per day in the elderly/very elderly and adolescents.

For the four age classes covering infants from 1 day up to 6 months the high internal exposure to total BPA ranged from 0.101 µg/kg bw per day to 0.621 µg/kg bw per day. The high internal exposure for populations older than six months ranged from 0.873 to 0.878 µg/kg bw per day in toddlers and infants aged 6 to 12 months, and from 0.393 to 0.473 µg/kg bw per day in men and adolescents.

Internal exposures to total BPA, as estimated by forward modelling, are in good agreement with the backward-modelling estimates obtained from urinary biomonitoring, suggesting that it is likely that no major exposure sources have been missed for the forward-modelled exposure assessment. It is, however, important to highlight the fact that the internal exposure estimation of total BPA includes conservative assumptions resulting in likely overestimation of exposure that could in theory have hidden other possible sources of exposure. The CEF Panel noted also that there are considerable uncertainties in the forward and backward estimates of internal exposure.

The CEF Panel has carried out an assessment of aggregated dietary and non-dietary exposure tounconjugated BPA using PBPK modelling. This aggregated exposure assessment included diet and house dust (the main oral-route sources) as well as thermal paper and cosmetics (the main dermal-route sources).

6.2. Hazard identification

The overall conclusions of the CEF Panel on the hazard identification step for BPA in relation to each endpoint considered are summarised in the following sections.

6.2.1. Toxicokinetics

- Species- and life stage-dependent differences in the toxicokinetic profile of BPA must be considered when comparing toxicokinetic data from different species.

- Conjugation to BPA-glucuronide is the major metabolic pathway of BPA in humans, non-human primates and rodents. Glucuronidated BPA is a biologically inactive form of BPA at the oestrogen receptors (ERs), however it cannot be excluded that the glucuronidated form may have effects at ER independent sites. BPA can also be conjugated via sulfation to a lower extent.

- The oral systemic bioavailability of unconjugated BPA in adults is as follows: 2.8 % in rats, 0.45 % in mice and 0.9 % in monkeys, based on oral versus intravenous toxicokinetic dataUnconjugated BPA and BPA-conjugates are observable at low concentrations in the amniotic fluid of rats and monkeys in comparison with serum levels. In early pregnancy exposure of the fetus might be greater compared with later pregnancy after i.v. exposure to BPA.

- BPA is present in rat milk from BPA-treated dams in both unconjugated and conjugated forms. In rat milk, BPA-glucuronide comprises about 80% of the total BPA concentration. Pup exposure via lactation is low, i.e. about 1/300 of the maternal dose. Unconjugated BPA has also been reported in human milk.

- BPA-conjugating enzymes (UDP-glucuronyl-transferases (UGT) and sulfotransferases (SULT)) are polymorphic in humans. The default intraspecies uncertainty factors used to derive a health-based guidance value are considered sufficient to account for possible differences in rates of metabolism of BPA between human individuals.

- A solid base of toxicokinetic studies in various laboratory animal species provides internal dose metrics for neonatal-to-adult stages and for different routes of exposure. Moreover, PBPK models have been developed to predict the internal exposures in laboratory animals and humans in a route-specific manner.

- Overall, this body of information permits extrapolation to humans and the application of the human equivalent dose (HED) concept for providing HEDs for points of departure derived from animal data. This was achieved by estimating human equivalent dose factors (HEDF) from the ratio of the Area Under the Curve (AUC) for the test species and AUCs for humans. Available experimental evidence indicates a 24-h percutaneous penetration of BPA across human skin of 2.3–8.6%. For exposure scenarios with dermal contact to thermal paper, the CEF Panel used a conservative value of 10% dermal absorptionThe CEF Panel did not consider skin metabolism (conservative decision). For scenarios with aggregated oral and dermal exposures, PBPK modelling was used to estimate the internal dose metrics (AUCs) for unconjugated BPA, with which equivalent oral exposures were subsequently calculated.

6.2.2. General toxicity

- BPA was found to affect kidney and liver weight in parental animals and in all the generations of rats and mice examined in multi-generation studies. These effects were considered by EFSA (2006) and EFSA CEF Panel (2010) as relevant systemic effects for the identification of a NOAEL. In mice the increased kidney weight was associated with nephropathy at the highest BPA dose. Liver weight was increased in rats (relative weight) and mice (both absolute and relative weight). The latter species also showed hepatocellular hypertrophy.

- The CEF Panel re-confirmed that the changes in the kidney and liver are critical endpoints in BPA toxicity and therefore took them forward for hazard characterisation.

6.2.3. Reproductive and developmental effects

- Only limited conclusions can be drawn from human studies on the likelihood of associations between BPA exposure during pregnancy and disturbed fetal growth, or maternal and infant decreased thyroid function. The evidence is not sufficient to infer a causal link between BPA exposure and reproductive effects in humans.

- Data considered in previous EFSA opinions show that BPA is a reproductive toxicant at high dose levels. On balance, the evidence from new lower dose (below 3.6 mg/kg bw per day, that corresponds to the NOAEL for general toxicity transformed in HED) animal studies for changes in reproductive function arising from in utero exposure to BPA remains contradictory and highly variable between studies. Furthermore, the biological relevance to humans of some of the effects of BPA exposure observed in some animal studies (e.g. reduced ano-genital distance in females) is not well understood. The CEF Panel noted that there is some evidence for effects of BPA exposure on several parameters indicative for changes in the reproductive system in adult male animals at dose levels below 3.6 mg/kg bw per day, although these effects were modest. It is not possible to conclude that these changes are reflective of changes in reproductive performance, since the studies rarely included a forced/continuous breeding phase in adulthood to establish reduced fertility. However, in several multigeneration studies no effects were observed at dose levels as low as 3 µg/kg bw per day up to at least 50 mg/kg bw per day.

- The CEF Panel re-confirmed that BPA is a reproductive toxicant at high dose levels.

- Using a WoE approach, the CEF Panel assigned a likelihood level of “as likely as not” to reproductive and developmental effects of BPA at low doses (below the HED of 3.6 mg/kg bw per day). Since the likelihood level for this endpoint is less than "likely" (see Appendix A), this endpoint was not taken forward for assessing the toxicological reference point, but was taken into account in the evaluation of uncertainty for hazard characterisation and risk characterisation.

6.2.4. Neurological, neurodevelopmental and neuroendocrine effects

- There are indications from prospective studies in humans that prenatal BPA exposure (BPA exposure during pregnancy) may be associated with altered child behaviour in a sex-dependent

manner. However, the associations were not consistent across the studies and it cannot be ruled out that the results are confounded by diet or concurrent exposure factors. The associations reported do not provide sufficient evidence to infer a causal link between BPA exposure during pregnancy or childhood and neurodevelopmental effects in humans.

- A number of new studies report changes that may indicate effects of BPA on brain development (effect on neurogenesis and on gene expression, neuroendocrine effects, effects on the morphology of certain brain regions, etc.). Whether such changes are mechanistically related to the neurobehavioral responses reported following exposure is attempted addressed by some studies but with inconsistent results.

- Several new animal studies investigated anxiety-like behaviour, learning and memory, social behaviour and sensory-motor function. Some studies report changes in anxiety-like behaviour after BPA exposure. Some, but not all, studies reported significant impairment of either learning and/or memory capacities. A few studies also report effects on social behaviour and sensory-motor function. However, the studies present methodological shortcomings, such as small sample size, lack of consideration of the litter effect, not properly controlled variability of exposure through diet and inadequate statistics. Moreover the results from different studies are inconsistent.

- Using a WoE approach, the CEF Panel assigned a likelihood level of "as likely as not" to neurological, neurodevelopmental and neuroendocrine effects of BPA. Since the likelihood level for this endpoint is less than "likely" (see Box 1), this endpoint was not taken forward for assessing the toxicological reference point, but was taken into account in the evaluation of uncertainty for hazard characterisation and risk characterisation.

6.2.5. Immune effects

- Based on recent studies, there are indications that BPA exposure may be linked to immunological outcomes in humans, although these studies had limitations and confounding factors may have been present. A causal link between BPA exposure during pregnancy or in childhood and immune effects in humans cannot be established.

- Studies in animals lend support to the possibility of immunological effects of BPA. Most of these studies suffered from shortcomings in experimental design and reporting. Although dose-responses could not be confidently established in most studies, a dose-related effect was observed in allergic lung inflammation.

- Using a WoE approach, the CEF Panel assigned a likelihood level of "-as likely as not- to likely" to immunotoxic effects of BPA. Since the likelihood level for this endpoint is less than "likely" (see Appendix A), this endpoint was not taken forward for assessing the toxicological reference point, but was taken into account in the evaluation of uncertainty for hazard characterisation and risk characterisation.

6.2.6. Cardiovascular effects

- All but one study, among the newly considered human studies in relation to cardiovascular effects since the 2010 EFSA opinion, are cross-sectional and thus unsuitable to study BPA exposure-disease associations on their own. There are indications from one prospective study that BPA may be associated with such effects but overall a causal link between BPA exposure and cardiovascular effects in humans cannot be established.

- There are insufficient animal data to suggest that BPA has an effect on cardiac function or causes cardiotoxicity.

- Using a WoE approach, the CEF Panel assigned a likelihood level of "as likely as not" to cardiovascular effects of BPA. Since this likelihood level is less than "likely", this endpoint was not taken forward for risk characterisation. It cannot be ruled out that the observed association between exposure to BPA and cardiovascular effect in the epidemiological studies is confounded by diet or other concurrent exposure factors. This association does not provide sufficient evidence to infer a causal link between BPA exposure and cardiovascular effects in humans. Since there is also a lack of information on the dose-relationship for this association, and since there were only very limited number of animal studies on this endpoint, this endpoint was not included in the uncertainty analysis.

6.2.7. Metabolic effects

- Of the reviewed human studies on metabolic effects only two were prospective while 22 were cross-sectional and thus not suitable on their own to study exposure-disease associations. Inconsistent with the results of cross-sectional studies one prospective study found that a higher BPA concentration in maternal urine during pregnancy was associated with a lower level of obesity in daughters. A causal link between BPA exposure and metabolic effects in humans cannot be established.

- A number of studies in pre- and postnatally exposed rats and mice indicate that BPA exposure could have an effect on metabolic function as evidenced by effects on glucose or insulin regulation or lipogenesis, and body weight gain (short-term studies). Based on the results from other studies with a longer duration (e.g. 90 days) there is no convincing evidence that BPA is obesogenic after intrauterine exposure or in longer-term studies.

- Using a WoE approach, the CEF Panel assigned a likelihood level of "as likely as not" to metabolic effects of BPA. Since the likelihood level for this endpoint is less than "likely" (see Appendix A), this endpoint was not taken forward for assessing the toxicological reference point, but was taken into account in the evaluation of uncertainty for hazard characterisation and risk characterisation.

6.2.8. Genotoxicity

- The available data support that BPA is not mutagenic (in bacteria or mammalian cells), or clastogenic (micronuclei and chromosomal aberrations). The potential of BPA to produce aneuploidy in vitro was not expressed in vivo. The positive finding in the postlabelling assays in vitro and in vivo is unlikely to be of concern, given the lack of mutagenicity and clastogenicity of BPA in vitro and in vivo.

- Using a WoE approach, the CEF Panel assigned a likelihood level of "unlikely" to BPA genotoxicity.

6.2.9. Carcinogenicity

- Very few epidemiological studies published to date have investigated a possible association between exposure to BPA and incidence of certain cancers, specifically breast cancer and meningioma. These studies do not allow any conclusion to be drawn regarding the carcinogenicity of BPA in humans.

- BPA was not carcinogenic in two standard oral carcinogenicity studies in rats and mice. In a more recent study, female but not male mice, exposed to approximately 10 mg/kg bw per day BPA from *in utero* up to postnatal day (PND) 21, developed significantly more hepatocellular tumours (adenomas and carcinomas together) with or without preneoplastic lesions after a stop-exposure period of 10 months. Additional rodent studies on perinatal exposure to BPA investigated the potential carcinogenic effect in mammary gland. Due to weaknesses in these studies the results do

not provide convincing evidence that BPA is carcinogenic to the liver during adult life or in mammary gland following perinatal exposure.

- Using a WoE approach, the CEF Panel assigned a likelihood level of "unlikely to - as likely as not -" to carcinogenic effects of BPA. Since the likelihood level for this endpoint is less than "as likely as not" (see Appendix A), this endpoint was not taken into account in the evaluation of uncertainty for hazard characterisation and risk characterisation.

6.2.10. Proliferative and morphological changes potentially related to carcinogenesis

- Earlier evidence for BPA effects on cell proliferation and differentiation in the mammary gland and other tissues (e.g. prostate or testis) has been supported by recent studies. The proliferative changes in the mammary gland reported in these new studies, including a non-human primate study, are insufficient to conclude that there is a link to cancer development in later life. However, there might be a possible role of BPA in increasing the susceptibility to mammary gland carcinogenesis later in life.

- The proliferative responses and possibly enhanced sensitivity to mammary gland carcinogens seen in animal studies might be of relevance for human health and are therefore included in the risk assessment.

- Using a WoE approach, the CEF Panel assigned a likelihood level of "likely" to BPA induced proliferative changes in the mammary gland. Therefore, this endpoint was brought forward for hazard characterisation and for uncertainty analysis.

- The CEF Panel considered that the evidence for proliferative changes induced by BPA in other organs (e.g. prostate or testis) is currently too limited to reach any conclusion.

6.2.11. Mechanistic studies with BPA including epigenetic effects

- Mechanistic studies published since 2010 continue to support the conclusion that BPA affects a number of receptor-dependent and independent signalling pathways, resulting in effects on hormone homeostasis and gene expression as well as cytogenetic and in epigenetic effects.

- The CEF Panel confirmed its conclusion in its opinion of 2010 that no single clearly defined mode of action of BPA can be identified that can contribute substantially to the understanding of the potential effects of BPA in humans.

6.3. Hazard characterisation

- The CEF Panel developed criteria for nonmonotonic dose-responses (NMDRs) and reviewed studies reporting a NMDR for BPA. None of the studies fulfil these criteria. Overall the CEF Panel concluded that the available data do not provide evidence that BPA exhibits a NMDR for the endpoints considered (reproductive/developmental toxicity, neurotoxicity/behavioural effects, metabolic effects, proliferative changes in mammary gland).

- Hazard characterisation was carried out only for those endpoints for which the overall likelihood for a specific effect of BPA was considered "likely" in the WoE approach. Dose-response relationships were examined for the most reliable studies supporting "likely effects", in order to provide a reference point for establishing a health-based guidance value.

- The CEF Panel has carried out dose-response (Benchmark Dose [BMD]) modelling on the data for general toxicity and for the proliferative changes in the mammary gland. The analysis on the mammary gland data revealed large differences in the BMD estimates obtained with the various models and the wide BMD confidence intervals obtained with some of the models. The CEF Panel

concluded that the dose-response data were not suitable to derive with any confidence a reference point for this endpoint.

- The CEF Panel therefore used only the endpoint "general toxicity" for risk characterisation, and specifically the mean relative kidney weight in a two generation study in mice, and calculated a $BMDL_{10}$ of 8 960 µg/kg bw per day.

- Subsequent to the 2010 opinion, new toxicokinetic data have become available which allow a more accurate substance-specific extrapolation of data from animals to humans, using the human equivalent dose (HED) approach. The human equivalent dose factor (HEDF) of 0.068 for oral exposure of adult mice was applied to the $BMDL_{10}$ of 8 960 µg/kg bw per day, which results in a HED of 609 µg/kg bw per day. The CEF Panel decided to use the HED value of 609 µg/kg bw per day as the reference point for establishing a health-based guidance value for BPA.

6.3.1. Uncertainty

- The overall uncertainty evaluation included the effects on mammary gland as well as reproductive, metabolic, neurobehavioural and immune systems. The CEF Panel concluded that the health-based guidance value should cover the lowest dose in the dose range for which the likelihood approaches "likely" from the overall uncertainty evaluation, taking into account uncertainty of all the evaluated endpoints as well as their relevance and adversity to humans. The uncertainty evaluation approached "likely" in the (HED) dose range of 100-1000 µg/kg bw per day. The CEF Panel therefore decided that the uncertainty regarding the above mentioned effects at the HED of 100 µg/kg bw per day and higher should be taken into account when establishing a health-based guidance value by including an extra factor in establishing the TDI. Thus, as the reference point was 609 µg/kg bw per day based on the mean relative kidney weight and the lower end of the dose-range for which the uncertainty evaluation for other endpoints approached "likely" is 100 µg/kg bw per day, a factor of 6 was applied.

6.3.2. Health-based guidance value

- In deriving a health-based guidance value, the CEF Panel used an uncertainty factor of 2.5 for inter-species differences (1 for toxicokinetics and 2.5 for toxicodynamics, reflecting the fact that toxicokinetic differences have been addressed by use of the HED approach) and an uncertainty factor of 10 for intra-species differences. In addition, the CEF Panel considered that the extra factor of 6 should be included to take into account the uncertainty in the database, i.e. mammary gland and reproductive, neurobehavioural, immune, and metabolic systems.

- The CEF Panel applied, therefore, an overall uncertainty factor of 150 to the HED of 609 µg/kg bw per day and established a temporary Tolerable Daily Intake (t-TDI) for external oral exposure to BPA in humans of 4 µg/kg bw, based on the mean relative kidney weight effect in mice. The CEF Panel designated the TDI as temporary, pending the outcome of the long-term study in rats involving prenatal as well as postnatal exposure to BPA, currently being undertaken by NTP/FDA.

6.4. Risk characterisation

- Comparison of the estimates for high dietary exposure for all age groups with the t-TDI of 4 µg/kg bw per day showed that the dietary exposure in all age groups (including the most exposed groups, i.e. 6-12 months infants and toddlers with levels of 0.857 µg/kg bw per day) was more than 4-fold below the t-TDI, indicating no health concern from dietary exposure alone. The additional contribution from other oral sources, like dust and toys mouthing (high exposure up to 0.015 µg/kg bw per day), did not change this conclusion.

- Comparison of the aggregated estimates for exposure to dietary and non-dietary sources of "children 3-10 years" and adolescents with the t-TDI showed that even when the high exposure estimates for dietary and non-dietary sources are combined, the aggregated exposure for children

3-10 years (1.258 µg/kg bw per day) and adolescents (1.449 µg/kg bw per day) will be approximately 3-fold below the t-TDI.

- The aggregated high dietary and non-dietary exposures (including oral and dermal sources) for women (1.063 µg/kg bw per day) and men (1.010 µg/kg bw per day) are mostly identical and they are lower than those for adolescents and children 3-10 years. The CEF Panel considered that these exposure estimates (up to approximately 1 µg/kg bw per day) for men and for women including pregnant women and prenatally exposed children, will be approximately 4-fold below the t-TDI of 4 µg/kg bw per day.

- Having evaluated the overall uncertainty of this assessment, the upper bound of the uncertainty interval for dietary BPA exposure alone did not exceed the t-TDI for any age group. Upper bounds for the uncertainty of high but not average aggregate exposure estimates to BPA exceed the t-TDI but the lower bounds are considerably lower than the t-TDI. The wide uncertainty intervals are caused by uncertainty about the magnitude of external exposure to BPA from thermal paper, about the proportion of the amount of BPA which is absorbed through the skin, and about the choice of PBPK model for converting dermal exposures to oral equivalents.

7. Overall conclusions

The CEF Panel concludes that the dietary exposure to BPA for the highest exposed groups, which include infants children and adolescents, is below the t-TDI of 4 µg/kg bw per day, indicating that there is no health concern for BPA at the estimated levels of exposure. These conclusions also apply to prenatally exposed children and to the elderly.

In addition, the CEF Panel concludes that the central estimates for aggregated exposure to BPA via the dietary and non-dietary sources (dust, toys, cosmetics and thermal paper) for the highest exposed groups, which includes infants, children, and adolescents, is also below the t-TDI of 4 µg/kg bw per day, indicating that the health concern for BPA is low at the estimated levels of exposure. However, the CEF Panel noted that there is considerable uncertainty in the exposure estimate for the non-dietary sources.

8. Recommendations

Reflecting the uncertainties surrounding this risk assessment of BPA as outlined in the previous section, the CEF Panel considers that further research in the following areas would be useful:

- Further work to refine the Human Equivalent Dose approach used in this opinion to extrapolate from experimental results in animals to humans, including further refinement of the toxicokinetics of unconjugated BPA in mice

- Further refinement of the human PBPK modelling applied in the opinion

- Further studies on the frequency and extent of dermal contact with BPA containing materials

- Further studies on the extent of dermal absorption following exposure to BPA by the dermal route in humans and the toxicokinetics of BPA following dermal absorption in humans and experimental animals

- Mechanistic studies in the kidney to determine the mode of action of BPA in this organ

- Further research on the significance of proliferative and morphological changes in mammary gland following exposure to BPA and the possible relevance for the development of breast cancer

- Further research on the potential adverse health effects of BPA for which there are uncertainties and that were therefore not definitevely considered as “likely” in this opinion, in particular reproductive, neurobehavioural, immunological and metabolic endpoints, using validated, robust methodology.

- Further investigations designed to explore the occurrence of non-monotonic dose-responses following in vivo exposure to BPA.

- Investigations to clarify the extent and the sources of unconjugated BPA in meat and fish.

GLOSSARY

External exposure: the amount of BPA with which the body comes into contact *before* it is absorbed through skin, lungs or GI-tract.

Internal exposure: the total amount of BPA which is present in the body irrespective of its form (i.e. the sum of conjugated plus unconjugatd BPA in the body)

Aggregated exposure: the sum of unconjugated BPA in the body, irrespective of the route through which it entered ther body. The aggregated exposure is the ultimate exposure estimate that will be at the basis of the risk assessment. Since the reference dose of BPA (i.e. the t-TDI) is expressed as an external oral exposure, aggregated exposure is equal to oral exposure to which contributions to the exposure via other routes are added, after conversion of these contributions to their equivalent oral exposures, with use of PBPK modelling.

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APPENDICES

Appendix A. Detailed methodology applied to perform hazard identification and characterisation and risk characterisation of BPA

Appendix A describes in detail the methodology applied in this opinion to perform hazard identification and characterisation and risk characterisation of BPA. A brief overview of this methodology is also presented in section 2 of the scientific opinion.

Graphical figures showing toxicological data were generated in the statistical computing environment R (R Core Team, 2014) in combination with R using the R lattice package (Sarkar et al., 2008).

Identification and selection of evidence relevant to hazard identification and characterisation

For identifying relevant studies for hazard identification and characterisation, different sources of evidence were considered as illustrated below. All studies considered for hazard assessment and risk characterization are reported in Appendix B. In the next sections details are given on the process for identifying and selecting relevant studies.

The sources of studies considered for hazard identification and characterisation

Study sources
Studies that EFSA (EFSA, 2006; EFSA CEF Panel, 2010) or other risk assessment bodies had previously identified as crucial for BPA toxicological assessment
In vitro and *in vivo* studies on genotoxicity published after the 2006 EFSA opinion
Studies that were present in the list of the retrieved articles for the preparation of the EFSA opinion of 2010 (EFSA CEF Panel, 2010), but were not then evaluated because they did not match the inclusion criteria established at the time, e.g. non oral studies, exposure during adult age, single dose
Studies retrieved via a literature search for the period August 2010-December 2012
Studies included in the report of Réseau Environnement Santé (RES, 2012) on BPA-related risks
Additional studies becoming available after 2012
Studies cited in the public consultation (ended on 13 March 2014) comments and original raw data submitted in this context (if relevant to the questions addressed by the opinion)

Evidence available until July[14] 2010

The background information for this risk assessment was provided by the earlier evaluations of BPA by a number of expert bodies (SCF, 2002; EU RAR, 2003; 2008; EFSA, 2006; 2008; 2010; Health Canada, 2008; NTP, 2008, US FDA, 2010a, FAO/WHO, 2011; ANSES, 2011; 2013 - see summary in Section 1.1).

For the WoE approach, the conclusions from the EFSA opinions on BPA of 2006 and/or 2010 were taken as starting point by the members of the working group on BPA toxicology.

The working group also revisited some individual studies that EFSA and/or other risk assessment bodies had previously identified as crucial for BPA toxicological assessment. These studies were included, along with new studies published from August 2010 onwards (see below), in the WoE approach, as starting point.

In vitro and in vivo studies on genotoxicity published after the EFSA opinion from 2006 were also considered for this risk assessment, since genotoxicity was not specifically dealt with in the 2010

[14] Time of adoption of the 2010 EFSA Opinion on BPA dealing with hazard identification and characterisation (EFSA CEF Panel, 2010).

EFSA opinion. These studies were identified by performing a thorough literature search and selected for relevance in line with the EFSA Scientific opinion on genotoxicity testing strategies applicable to food and feed safety assessment (http://www.efsa.europa.eu/en/efsajournal/pub/2379.htm). The studies on genotoxicity that were reviewed are reported in Appendix B.

In addition, studies that were present in the list of the retrieved articles for the preparation of the EFSA opinion of 2010, but were not then evaluated because they did not match the inclusion criteria established at the time, e.g. non-oral studies, exposure during adult age, single dose studies, were identified by the EFSA secretariat and provided to the experts for review. When considered relevant, they were included in the WoE approach as "lines of evidence".

Evidence available between August 2010 and December 2012

In addition to the above, EFSA outsourced a thorough literature search[15] aiming at identifying as many relevant studies as possible published between August 2010 and December 2012.

The approach to searching was sensitive, i.e. the search terms contained only the term "Bisphenol[16]" or "BPA" without any additional search terms, in order to retrieve as many studies as possible relevant to hazard identification and characterisation of BPA and minimize the risk of publication bias. The details and results of the searches are reported in table 24 of this Appendix.

The studies retrieved were screened against pre-defined selection criteria, by the external contractor, EFSA secretariat and the members of the EFSA WG on BPA toxicology. The criteria for study selection are illustrated below. All studies included in this risk assessment are listed in Appendix B.

Criteria for study selection

- Relevant studies were defined as primary research studies[17] published in peer-reviewed journals in the period August 2010 - December 2012 and dealing with human/animal/in vitro toxicity of Bisphenol A (studies dealing with other forms of BPA/metabolites were excluded);
- Reviews were considered as sources of studies (and not considered in the risk assessment as such);
- Only studies in English were included;
- Studies not in the field of animal and human health were excluded (e.g. ecotoxicity studies);
- Studies in the fields of chemistry or physics, where BPA was used or involved in the synthesis of other compounds were excluded;
- Human studies:
    - Biomonitoring studies and epidemiological studies addressing associations between BPA exposure and an adverse health outcome in a particular population, all designs (e.g. cross-sectional studies were also included);
    - All routes of exposure (also non-oral routes);
    - Including ex vivo studies.

- Animal toxicity studies, including non-oral routes of exposure:
    - single dose studies were included as potential supporting evidence for hazard identification;
    - For reproductive and developmental toxicity studies in animals, studies were excluded if all the doses used exceeded the oral HED of 3.6 mg BPA/kg bw per day (see list in Appendix B).

[15] Contract CT/EFSA/CEF/2011/01 – Screening of literature on BPA
[16] It was decided to use "Bisphenol" instead of "Bisphenol A" to avoid excluding studies where in text it wass reported "*the form A of Bisphenol*".
[17] i.e. studies generating new data, as opposite to "secondary research" studies (i.e. reviews).

This was justified on the basis that the study of Tyl et al. (2002) offered a well-established oral NOAEL of 5 mg BPA/kg bw per day in the rat, with a higher NOAEL of 50 mg BPA/kg bw per day for reproductive effects. Depending on the animal species and route of administration, a correspondent oral HED (see Section 3.1.5 and Appendix D) was calculated for each dose level used in a particular in vivo study, using the HEDFs for adults and infants. Any study employing BPA doses exceeding the HEDs of 3.6 mg BPA/kg bw per day (equivalent to the NOAEL of 5 mg BPA/kg bw per day in the rat) was excluded from the assessment, unless it also included a dose level or dose levels (HEDs) < 3.6 mg BPA/kg bw per day. The data for sheep were not available to calculate the HED and therefore a ratio of 1:1 was assumed, which led to the inclusion of 1 study and exclusion of 1 study. Also studies addressing the toxicity of BPA in mixtures were condifered relevant to the purpose of this review.

In the case of the in vitro studies not addressing genotoxicity, an additional exclusion criterion was applied, i.e. studies using concentrations equal or above 100 nM were excluded. In vitro studies performed at high concentrations of BPA were not considered relevant due to either the induction of cytotoxic effects or the use of concentrations not reachable in human serum after BPA intakes at or below the current TDI of 50 µg/kg bw per day. Several publications indicate that BPA at or below 10-100 µM reduces cell viability in vitro, e.g. in human breast cancer cells (Zhang et al., 2011), a murine Sertoli cell line (Wang et al., 2012d), stem cell lines (Biemann et al., 2012) or human oocytes (Brienõ-Enriquezet al., 2012). In addition, based on results from toxicokinetic studies (Doerge et al., 2010a,b, and 2011b; Taylor et al., 2011) the CEF Panel is of the view that human BPA intake at the current TDI of 50 µg/kg bw per day would lead to unconjugated BPA serum concentrations in the order of 0.1-2 nM. Human kinetic studies suggested that consumption of food resulted in BPA intakes which were at least two orders of magnitude below the TDI (see EFSA CEF Panel (2013) for draft exposure part of the BPA opinion). Therefore, based on the above considerations and also taking into account potentially different effects of low and high BPA concentrations for some endpoints in in vitro studies (non-monotonic dose-response curves, see Section 1.2 on NMDR) and assuming a maximum factor of 50 applied to the nominal concentration to account for unspecific binding of BPA to cell culture devices, it was decided to exclude in vitro studies with nominal concentrations at or higher than 100 nM BPA for the purpose of this evaluation.

The results of the literature search were also compared with the compilation of published scientific studies on BPA submitted by Réseau Environnement Santé to the European Commission and received by EFSA on 19 February 2013. The few publications identified as missing were screened by the EFSA secretariat against the relevance criteria illustrated above.The excluded studies and the underlying motivations are presented in Appendix B.

Table 24: Details and results of the literature search process (August 2010-December 2012)

Pubmed (http://www.ncbi.nlm.nih.gov/pubmed/)	**Sciencedirect** (http://www.sciencedirect.com/)	**Scopus** (http://www.scopus.com)	**Web of Knowledge/Science** (http://apps.webofknowledge.com)	**DOAJ**[18] (http://www.doaj.org/)
Date of the search: August 2010-December 2012 (searches performed almost every day)	**Date of the search**: August 2010- December 2012 (searches performed almost every day)	**Date of the search**: January 2010- December 2012 (searches performed at least once a month)	**Date of the search**: January 2010 - December 2012 (searches performed almost every day)	**Date of the search**: January 2010 - December 2012 (searches performed at least once a month)
Search terms (a) *Bisphenol* (Total records retrieved: 1485) *BPA* (Total records retrieved: 808)	**Search terms** (a) *Bisphenol* (Total records retrieved: 1061) *BPA* (Total records retrieved: 646)	**Search terms** (a), (b) *Bisphenol* (Total records retrieved: 1551) *BPA* (Total records retrieved: 813)	**Search terms** (c) *Bisphenol* (Total records retrieved: 3542; toxicology: 988) *BPA* (Total records retrieved: 1088; toxicology: 545)	**Search terms** (c) *Bisphenol* (Total records retrieved: 85) *BPA* (Total records retrieved: 59)
Total number of records retrieved: 2293	**Total number of records retrieved**: 1707	**Total number of records retrieved**: 2364	**Total number of records retrieved**: 4630 (toxicology: 1533)	**Total number of records retrieved**: 144
Total number of records retrieved after removing duplicates: 1612	**Total number of records retrieved after removing duplicates**: 1192	**Total number of records retrieved after removing duplicates**: 1696	**Total number of records retrieved after removing duplicates**: 3731 (toxicology: 1003)	**Total number of records retrieved after removing duplicates**: 115

(a): Two searches run separately using "Bisphenol" and "BPA" in all fields (Text, Abstracts, Keywords, etc).
(b): The filters "Life Sciences" and "Health Sciences" were applied. [c] Two searches run separately using "Bisphenol" and "BPA" only in the field "Topic". The filters with fields dealing with "Life Sciences" and "Health Sciences" were applied.
(c): Two searches run separately using "Bisphenol" and "BPA" only in the field "Abstract".

[18] The Directory of Open Access Journals (DOAJ) is website that lists open access journals and is maintained by Infrastructure Services for Open Access. The project defines open access journals as scientific and scholarly journals that meet high quality standards by exercising peer review or editorial quality control and "use a funding model that does not charge readers or their institutions for access DOAJ was searched as few journals are not indexed by other databases (e.g. ISRN Pulmonology).

Evidence available after 2012

Additional studies made available in 2013 were considered in this review on a case by case basis (see Appendix B). Although the CEF Panel acknowledges that these studies may not represent the entire body of evidence that has become available after 2012 , these studies were considered based on expert judgement because of their relevance to the review questions and/or their methodological soundness.

Other evidence in the context of the public consultation

The papers that were mentioned in the comments submitted during the public consultation on the draft assessment of BPA health risks (and the full comments themselves) are reported in the EFSA Technical report "Outcome of the public consultations on the Draft Scientific Opinion of the EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids on the Risks to Public Health related to the presence of Bisphenol A (BPA) in foodstuffs" (EFSA, 2015).

Among these papers and irrespective of their publication date, those relevant to BPA hazard assessment were screened against the reference list in the pre-public consultation draft opinion. The missing studies that the Panel considered relevant to address the review questions were included in the revised evaluation and treated as all the other evidence as further explained below.

Upon discretion of the CEF Panel and on the basis of their relevance and methodological soundness, original raw data submitted as a result of the public consultation were also considered for the assessment.

1. Assessment of individual studies for BPA toxicological evaluation

The studies relevant to hazard identification were then grouped according to macro-areas of interest, e.g. toxicokinetics and metabolism, general toxicity, reproductive and developmental effects, and relative study type, i.e. human, animal or *in vitro* study (see table 25). The studies selected for inclusion in the Opinion were considered by the working group as described in the right column.

Table 25: Macro-areas by which the relevant studies for BPA hazard identification were grouped and consideration of the studies used for the toxicological evaluation

Study content	How the study was considered
1. Toxicokinetics and metabolism (human and animal studies) 2. General toxicity (animal studies)	Appraisal of strengths and weaknesses (see Appendix B)
3. Reproductive and developmental effects (human and animal studies) 4. Neurological, neurodevelopmental and neuroendocrine effects (human and animal studies) 5. Immune effects (human and animal studies) 6. Cardiovascular effects (human and animal studies) 7. Metabolic effects (human and animal studies) 8. Genotoxicity (in vitro and in vivo studies) 9. Carcinogenicity (human and animal studies)	Appraisal of strengths and weaknesses (see Appendix B) and inclusion in the WoE approach used for hazard identification (see Appendices B and C)
10. Mechanisms of action of BPA (including epigenetics and gene expression studies) 11. In vitro studies	Examination and use as supplementary information for the toxicological evaluation (see Appendix B and Section 3.10)

In particular, the appraisal of the strengths and weaknesses of each study was performed by two reviewers from the working group on BPA Toxicology (a rapporteur and a co-rapporteur) and their evaluation was presented to and further discussed by the entire working group. During this evaluation, studies not relevant to the review questions on the association between BPA and toxicological effects were excluded from the WoE approach (as specifically stated in Appendix B).

The criteria and principles applied for assessing study strengths and weaknesses are illustrated in the following sections. All studies assessed and the overall conclusions on each study are reported in Appendix B.

Criteria and principles for assessing the strengths and weaknesses of human studies

The CEF Panel took into consideration the following aspects for evaluating epidemiological studies.

Cross-sectional studies assess exposures and health outcomes at the same time point and are inappropriate for making causal inference because reverse causation cannot be ruled out. For example, a reported association between BPA exposure and obesity is not sufficient evidence that BPA is a cause of obesity; it is a plausible assumption that obese people ingest more food and hence, that they ingest more BPA.

Case-control studies examine multiple exposures in relation to a disease; subjects are defined as cases and controls, and exposure histories are compared. Case-control studies generally depend on the collection of retrospective data, thus introducing the possibility of recall bias.

Cohort studies can be either retrospective or prospective. In a prospective cohort study, participants are followed over time and exposures are assessed prior to the incidence of the health outcome. In a retrospective cohort study, both the exposures and outcomes have already occurred when the study begins. Well designed and conducted cohort studies have more weight than case-control and cross-sectional studies, but for all types of studies it is questionable to relate single BPA measurements to a health outcome developing over many years because of BPA's short half-life.

Limitations in study design, power, statistical methods, and study population may lead to inconsistent results. A study that has a valid exposure assessment, an appropriate sample size, a prospective design, a valid and reliable outcome and sound statistical handling provides more robust findings than a small study with obvious limitations independent of the statistical outcome.

The health outcome reported should clearly be identifiable as an adverse event in humans, and it is important to evaluate the reliability and validity of the outcome measures used. Often, epidemiological studies rely on self-reported information, and this information can be biased. Doctors' reports and health registries are less prone to reporting error, but it is important that the methods include concise and transparent information on how outcomes were assessed. The importance of using scientifically and clinically supportable exclusion criteria and outcome definitions has been highlighted by Lakind et al. (2012). For example, when reanalysing NHANES data, exposure-disease associations between BPA exposure and chronic disease outcomes were no longer statistically significant when using a-priori selected methods to address the research question (Lakind et al., 2012).

Valid and precise exposure assessment is a major challenge in studies of chemicals and human health outcomes. For substances like BPA that are rapidly eliminated, a single measurement, such as a single spot urine sample, does not provide a reliable estimate of long term exposure. The time interval between the onset of patho-physiological changes that precede the health outcome considered in a study and sample collection, on which the exposure assessment is based, is for such substances a major factor of concern. If in a study the biological samples were taken at a time that only reflects the exposure to the substance in question for a very limited time period before the taking of the samples, it is inappropriate to relate the exposure to health outcomes that may take years or decades to develop.

The special considerations which apply for BPA because of analytical challenges and its toxicokinetics which were made for assessing study quality are covered in the Draft Scientific Opinion on the risks to public health related to the presence of bisphenol A (BPA) in foodstuffs – Part: exposure assessment (EFSA CEF Panel, 2013). The criteria and principles for assessing the strengths and weaknesses related to the analytical challenges are illustrated in table 24 of this Appendix.

In epidemiological studies of exposures and adverse health effects there is the need for sufficient confidence that the reported effects are indeed related to the exposure of interest and not confounded by correlated factors. Most epidemiological studies use statistical models to adjust for potential confounding by demographic, socioeconomic, lifestyle (including diet) and other factors. The appropriateness of such models was evaluated when assessing the individual studies. When an exposure is associated with an adverse health outcome, the association may in fact be explained by another factor, not measured in the study, which actually causes the disease. It is of particular importance to adjust the analyses for key confounding factors, of which the two most important in the case of BPA are socioeconomic position and dietary intake. The main source of BPA ingestion is from food packaging (polycarbonate drink bottles, tinned items etc), and consumption of energy-dense, processed (packaged) and tinned food tend to be higher in disadvantaged groups. NHANES data has revealed that individuals with lower incomes, who may also be more likely to suffer from other disparities in health and exposures, have a greater burden of exposure to BPA (Nelson et al., 2012). Even when relevant confounding variables are taken into account, the possibility of unmeasured or residual confounding cannot be excluded. Detailed discussion on the points to be considered in epidemiological studies is given in Geens et al. (2012) and such points were considered when assessing the individual studies.

Epidemiological studies can demonstrate statistically significant associations between BPA and health outcomes, but it should be noted that statistical significance does not imply a causal relationship. Criteria for objectively evaluating the level of causality of associations observed in epidemiology have been formulated by Bradford Hill (1965) and include consistency, strength of association, dose-response, time order, specificity, consistency on replication, predictive performance, biological plausibility and coherence.

The strengths and weaknesses considered for appraising epidemiological studies are summarised in table 26.

Table 26: Criteria applied to assess the strengths and weaknesses of epidemiological studies

Criteria	Interpretation / Assessment		Comments
	Strengths:	***Weaknesses:***	
Study design			
Type of study	Prospective design Longitudinal follow up	Cross-sectional design Short time frame	Well designed and conducted prospective cohort studies have more weight than case-control and cross-sectional studies. All cross-sectional studies were considered "weak by default" but included in the assessment for comparison of BPA concentrations across different populations and because cross-sectional studies can be considered as hypothesis- generating studies. However, they do not provide any meaningful information on exposure-disease associations.
Selection of the population	------	Selection bias (give details)	For cohort studies selection bias was considered to arise when the comparison groups (exposed and unexposed) were not truly comparable. For case-control studies selection bias was considered to arise when cases were not representative of all cases within the defined population or controls were not representative of the population which produced the cases

Criteria	Interpretation / Assessment		Comments
	Strengths:	***Weaknesses:***	
Sample size	Large sample size	Small sample size	For a non-persistent compound like BPA, the large variability in the exposure may to some degree be compensated by a sufficiently large sample size and by including repeated measures of exposure. Although the exposure estimate may be inaccurate at the individual level, ranking of subjects within a study population can give a fairly accurate indication of exposure at the group level. This was considered for evaluating study quality.
Recall period	Reporting by two different sources (e.g. teachers and parents)	Long recall period (retrospective collection of data)	
BPA exposure assessment			
Matrix and containers	Urine, container specified	Serum BPA measurement (invalid exposure measurement) Plasma BPA measurement (invalid exposure measurement) Blood BPA measurement (invalid exposure measurement) Urinary BPA measurement not adjusted (for creatinine or specific gravity)	At current levels of oral and dermal exposure the concentrations of unconjugated BPA in blood/plasma/serum are typically below the LOD of specific analytical methods (< 0.1 ng/ml) and cannot be measured unless they result from a contamination
Sampling time(s)	Repeated measurements (>1) Standardized samples e.g. morning spot or 24-h urine collections	Single measurements Single spot urine BPA measurement	Single measurements are interpreted as a weakness due to the short BPA half-life (< 6 hours) Repeated measurements are interpreted as such when >1
Analytical method, accuracy and precision, handling of values below LOQ)	Analytical method (SPE LC-MS-MS or GC-MS-MS or RIA) Quality control, including blanks quality assurance procedures	Analytical method (ELISA) No quality control (e.g. blanks) or quality assurance procedures No distinction between conjugated and unconjugated	Unspecific and cross-reactivity with other phenols and conjugates (to avoid sample contamination during collection, handling, and analysis)

Criteria	Interpretation / Assessment		Comments
	Strengths:	***Weaknesses:***	
		BPA Handling of values below LOQ) not reported	
Confounding factors	------	Confounding by diet, or by concurring exposure factors (other chemicals, drugs) not considered or not reported	
Study results documentation / study reporting			
Study reporting	------	Insufficient study reporting	
Statistical modelling	------	Inappropriate statistics (give details),	e.g. incomplete model description, too many categories, etc)
Plausibility of the study design and results			
Clinical relevance	------	Unclear clinical relevance	e.g. unclear adversity of the effect, small effect size, etc)
Outcome assessment	Multiple outcome assessment	Unclear/invalid/imprecise/unreliable outcome	e.g. outcome based on self-reported information
Generalisability to the total population	------	Generalisability to the overall population (give details)	e.g. study performed only in couples undergoing in vitro fertilisation, etc.
Consistency of results	Consistent results amongst different studies or tests	Inconsistent results amongst different studies or tests	
Occupational exposure	------	Occupational exposure	Professional exposure may occur by a route different from and not relevant to the general population. If studies were accompanied by urinary BPA measures, they were rated less weak than those without such measurements

2. Criteria and principles applied for assessing the strengths and weaknesses of animal studies

Table 27: Criteria applied to assess the strengths and weaknesses of animal studies

Criteria	Interpretation/assessment		Comments
	Strengths:	***Weaknesses:***	
Test substance identification			
Vehicle	------	Vehicle not reported	
Test organism characterisation			
Species and strain of the animal	------	Animal species and/or strain not reported	
Is the age and body weight of the test organisms given?	------	Animal age and/or body weight not reported	
Is the sex of the test	------	Sex of the animals tested	

Criteria	Interpretation/assessment		Comments
	Strengths:	***Weaknesses:***	
organism given?		not reported	
Study design description			
Use of a priori study protocol/study plan	------	Lack of a priori study protocol or study plan	
Sample size – power of the study (number of animals)	Large sample size	Small sample size	This is based on expert judgement
Control procedures (Were negative and/or positive controls included (where required)?	Both naïve controls and vehicle controls available Adequate positive controls included (if appropriate)	No vehicle controls were tested	
Number of BPA doses	≥ 3 dose levels tested	Single dose level study	Not mentioned as a strength or weakness if 2 dose levels were tested
BPA dose levels		Too wide dose spacing *Too high dose levels tested	Wide dose spacing makes the study inadequate to study a dose-response relationship Testing of BPA at very high dose levels is not informative of effects occurring at current human exposure levels
BPA exposure assessment	------	Feed consumption (BPA given by the diet) not measured BPA concentration and homogeneity in the feed mixture not guaranteed analytically (BPA given by the diet) Drinking water consumption (containing BPA) not measured	The exact BPA doses received by the animals cannot be established
Route and type of administration / administration scheme	Oral administration via gavage (except for neurobehavioural studies)	Maternal administration via ip injection during pregnancy	Not mentioned if: BPA was given via diet or drinking water and food/water consumption was measured; BPA was given via sc injection; Maternal dosing via ip injection during pregnancy was considered as a weakness due the uncertain fetal dosing

Criteria	Interpretation/assessment		Comments
	Strengths:	***Weaknesses:***	
			Oral administration via gavage was considered as a strength due to exact dosing: only exceptions were neurobehavioural studies addressing anxiety-like behaviours due to animal handling
Frequency and duration of exposure: Are frequency and duration of exposure as well as time-points of observations explained?	------	Single acute dose administration	Acute exposure is not representative of human exposure which is prolonged in time
BPA exposure assessment	BPA measurement in biological samples		The quality of the analysis is also checked
Test performance		Test performed in one sex only Low number of animals tested (in a test)	
Blind treatment	Blind treatment or Blind evaluation of samples….	------	Blind treatment was considered as a strength if reported, and was not mentioned if not reported
Study results documentation/ Study reporting			
Study reporting	------	Insufficient study reporting (give details)	Details, e.g. number of animals tested for each test unclear or not reported, time points unclear, dose levels etc
Statistical analysis	------	Inappropriate statistics (give details)	Details, e.g. litter effect not considered, inappropriate analysis
Plausibility of the study design and results			
Is the study design chosen appropriate for obtaining the substance-specific data aimed at?	------	Study design not appropriate to the scope	
Diet	Phytoestrogen-free diet (e.g. soy free diet) or feed content of oestrogens negligible at E-screen	Animal diet and phytoestrogen content not reported (or poorly described)	Confounding by diet
Housing conditions/ Environmental contamination	Use of non-polycarbonate (non-PC cages), and of non plastic (e.g. glass) or BPA-free water bottles	Use of polycarbonate cages (PC) and plastic water bottles OR Type of cages and drinking bottles not reported	Confounding by environmental contamination

Criteria	Interpretation/assessment		Comments
	Strengths:	***Weaknesses:***	
Quality assurance principles			
GLP/other quality assurance system	Study/analysis performed under GLP or XX quality assurance system	------	
Protocol according to existing guidelines, e.g. OECD guidelines or EU guidelines (or other e.g. national guidance)	Study/test performed according to XX guidelines	------	
Others	On a case by case basis	On a case by case basis	This is based on expert judgement

*"Too high dose levels" is an exclusion criterion for the studies on reproductive and developmental toxicity and therefore this weakness is reported only for non reproductive studies.

3. WoE approach to hazard identification

The studies appraised against their strengths and weaknesses (see table 26 and 27) were included in the WoE approach to perform hazard identification.

First of all for each toxicological endpoint different questions (Qn) were defined addressing the association between BPA and the endpoint (e.g., "*does BPA cause ... (type of effect)*?") (See all toxicological endpoints and related questions in Appendix C).

The conclusions from the EFSA opinions on BPA of 2006 and/or 2010 were taken as starting point for answering each question. Then the relevant studies were organised into a number of "lines of evidence", addressing different findings that relate to the question concerned. Some lines of evidence referred to a single study, whereas others referred to a group of studies addressing the same issue.

To draw its conclusion for each association question, the CEF Panel first summarised the strengths and weaknesses of each line of evidence and pre-2010 assessments in an overall reliability assessment (expressed qualitatively on a scale of low, medium or high) and expressed it in terms of *weight* or *influence* on the likelihood of a positive answer to each question, when considered independently of the other lines of evidence.

Then the CEF Panel evaluated the overall likelihood of a positive answer, taking into account the individual influences of all the lines of evidence and considering how they combine.

The CEF Panel expressed its conclusions in terms of the *likelihood* that the answer to the question was positive in order to take into account uncertainties affecting the balance of evidence. The CEF Panel's conclusion lied on the continuum between a definite negative answer and a definite positive answer.

The approach described above is generically summarized in table 28. All WoE tables filled in are reported in Appendix C.

Table 28: Example of table used in the WoE approach

Q1: Is BPA……..?	**Answer to the question as reported by the study authors**	**Reliability of evidence**	**Influence on Likelihood**
Starting point based on previous assessments (EFSA, 2006; 2010): (summarise conclusions of previous assessments relating to this question) *Strengths:* *Weaknesses:* *(of the evidence considered in the previous assessments)*	**Positive, Negative or Uncertain**	**Low, Medium or High**	**See table 29 for key to symbols**
Line of Evidence 1: new evidence on ……. *Strengths:* *Weaknesses:*			
Line of Evidence 2: increased effect on…… *Strengths:* *Weaknesses:*			
Overall conclusion on Likelihood:			**Chosen likelihood level (see Box below)**

The CEF Panel found it helpful to include separate columns in table 28 summarising steps in the evaluation of each line of evidence.

The second column indicates the answer to the question as reported *by the study authors* (e.g. a positive, negative or uncertain answer to the question), i.e. before the CEF Panel assessed strengths and weaknesses.

The third column gives the CEF Panel's assessment of the *reliability* based on the evaluation of the strengths and weaknesses of each line of evidence, expressed qualitatively on a scale of low, medium or high. Note that a low score for reliability does not necessarily imply a poor quality study: e.g. it may relate to a well-conducted study with results not reaching statistical significance, but the treatment groups are not large enough to be statistically confident there is no effect (see Section on 'absence of evidence' in EFSA 2011). The CEF Panel did not use a fixed formula to assess reliability of a study from its number of strengths and weaknesses, because this would not take appropriate account of the varying weights of different strengths and weaknesses. Instead, the reliability of the evidence as well as the influence on likelihood were agreed based on collective expert judgement (starting from a proposal made by the study's rapporteur and co-rapporteur, that was discussed at least at one but more commonly at many WG meetings and that then was reviewed by the CEF Panel during Plenary meetings In reaching a collective view varying opinions within the WG and the specialist expertise of individual members were taken into account.

The evaluation of the weight or influence of each line of evidence was then recorded in the right hand column using a defined set of symbols as described in table 29.

Table 29: Definition of symbols used for expressing the influence on likelihood of each line of evidence in the WoE tables (see Appendix C).

Symbols	Interpretation
↑	minor contribution to increasing likelihood
↑↑	moderate contribution to increasing likelihood
↑↑↑	major contribution to increasing likelihood
↓	minor contribution to decreasing likelihood
↓↓	moderate contribution to decreasing likelihood
↓↓↓	major contribution to decreasing likelihood
●	negligible influence on likelihood
Pairs of symbols indicate uncertainty about the influence, e.g., ●/↑ = between negligible and minor positive influence on likelihood.	

The number (from one to three) of upward and downward arrows indicates the degree (small, medium, high) of the impact of the new evidence to increase or decrease, respectively, the likelihood of a positive answer to the question. In developing its judgment on the influence or weight of each line of evidence, the CEF Panel took into account all the strengths and weaknesses it identified in the left hand column of the WoE table.

The "dot" is used when the reliability of the new line of evidence is considered as insufficient as to have an impact on the likelihood of a positive answer to a question. When the evidence base was too weak to make a firm judgement about the influence, a range of symbols was given to reflect that additional uncertainty. For example,●/↑ indicates between negligible and minor positive influence on likelihood.

A conclusion on the overall likelihood that BPA exposure was associated with a particular effect in humans and/or animals was expressed in the bottom row both as a narrative statement and using a standard set of likelihood terms (Box below), which was adapted from a similar set used by the Intergovernmental Panel on Climate Change (Mastrandrea et al., 2010) ranging from "very unlikely" to "very likely". Such conclusion was drawn after considering the individual influences of all the lines of evidence and the starting point. As already explained above for the assessment of reliability, the CEF Panel drew its conclusions on the overall likelihood by expert judgement after a thorough discussion process at a Working Group and /or CEF Panel meeting level and not by any standardised combination of scores for reliability and influence, which would be simplistic and preclude the consideration of other factors. Each likelihood was accompanied by a narrative text briefly summarising the rationale for the conclusion, in the bottom row of the WoE table (table 28).

It is also important to emphasise that the likelihood assessed by the WoE approach refers specifically to hazard identification, i.e. it refers to the likelihood of an association between BPA and the effect under consideration. It does *not* refer to the likelihood or frequency of the effect actually occurring in humans, which depend on additional factors including the dose-response relationship for the effect (considered in hazard characterisation) and the levels of human exposure to BPA (considered in exposure assessment).

Set of standard terms used for expressing the overall likelihood in the WoE tables from Appendix C (adapted from Mastrandrea et al., 2010)

Likelihood
Very likely
Likely
From -as likely as not- to likely
As likely as not
From unlikely to -as likely as not-
Unlikely
Very unlikely

4. Approach to hazard characterization

The WoE approach to hazard identification has been used to identify the critical toxicological effects ("likely effects") for BPA, in relation to specific time windows of exposure. Endpoints were only considered for setting a reference point in hazard characterisation, if they were judged “likely” or “very likely” in the hazard identification. The possibility of the other, less likely effects was taken into account together with the other uncertainties affecting hazard characterisation.

For effects that were considered "likely” or “very likely", the CEF Panel considered the adversity of the effects and their relevance to humans. Uncertainty affecting these considerations was dealt with in a conservative way, by retaining effects for hazard characterisation unless there was strong evidence that they were not adverse or relevant.

The most reliable study(ies) supporting “likely” or “very likely" effects were used to study dose-response relationships and to identify the critical reference point (NOAEL or LOAEL or BMDLs, depending on the suitability of the data set) for setting a health-based guidance value.

To set a TDI (see Section 4 of the opinion), the CEF Panel converted the lowest reference point identified from this/these animal studies into a correspondent oral HED (see Section 3 and Appendix D), by multiplying it by a factor that takes account of quantitive differences in toxicokinetics between the animal species used in the study and humans. This so-called HEDF is calculated from the ratio of the AUCs for the test species and AUCs for humans. HEDF, which is based on real data, replaces the default uncertainty factor of 4 generally attributed to interspecies kinetic differences. To derive a TDI an additional uncertainty factor of 25 is then applied to the HEDF. This default factor should cover for (i) the remaining dynamic component of inter-species-related differences, i.e. 2.5, and for (ii) intra-species-related kinetic and dynamic differences, i.e. 10.

5. Approach to risk characterization

To assess the risks (see Sections 5) for consumers from current levels of BPA exposure the TDI is compared to estimates for oral exposure for different age groups and subpopulations (both average and high dietary exposure scenarios) and with dermal exposure estimates based on PBPK modelling (see Part I – Exposure assessment).

Appendix B. All Studies Evaluated

Toxicokinetics and Metabolism

Human and animal studies on toxicokinetics and metabolism were appraised against strengths and weaknesses but did not undergo WoE analysis.

(i) Human studies

Cao X-L, Zhang J, Goodyer CG, Hayward S, Cooke GM and Curran IHA, 2012a. Bisphenol A in human placental and fetal liver tissues collected from Greater Montreal area (Quebec) during 1998–2008. Chemosphere, 89, 505-511.

This ex-vivo human study was aimed at measuring unconjugated and conjugated BPA levels in human placental and fetal liver tissues. In the study, human placental samples (n = 128) and fetal liver samples (n = 28) were obtained from induced abortions between 1998 and 2008. The determination of BPA and its glucuronide (by gas chromatography coupled to a mass selective detector (GC-MSD)) was not the primary aim of the tissue collection. Unconjugated BPA was detected in a total of 113 placental samples. The LOQ was estimated for each placental and liver sample and was on average 0.99 and 1.4 ng/g, respectively. The unconjugated BPA concentrations ranged from 0.55 ng/g up to 165 ng/g. The authors also determined BPA-glucuronide by subtracting the concentration of BPA (parent compound) from the concentration of total BPA (sum of parent compound and BPA-glucuronide), obtained after treatment with ß-glucuronidase. The concentration of BPA-glucuronide detected in 50 samples varied between 0.1 and 178 ng/g tissue. The ratio of the mean unconjugated BPA/BPA-G was 0.73. In fetal liver samples the unconjugated BPA was 1.02–37.7 ng/g (detected in 20 samples) and that of BPA-glucuronide 1.41–93.9 ng/g (detected in 13 samples out of 16 samples analysed). The ratio of the mean unconjugated BPA/BPA-G was 0.47.

Comments from the CEF Panel:
The CEF Panel identified the following strengths and/or weaknesses in the study:
Strengths:

- Container specified (PP vials)
- Analytical method (SPE GC-MS)
- Quality control, including blanks

Weaknesses:

- Single measurement
- Quality assurance, precaution to avoid contamination not described
- Conjugated BPA analysed in a subset of samples

Overall, the CEF Panel considers that concerning the methodology, there is no indication in the study description that special precautions were taken to avoid contamination of the samples during the collection process. Also, tissue samples should be handled deep frozen to avoid that the ß-glucuronidase present in the tissue can release unconjugated BPA from conjugated BPA. It cannot be excluded that high levels of unconjugated BPA result from BPA deconjugation during sample processing and storage.

Carwile JL, Ye X, Zhou X, Calafat AM and Michels KB, 2011. Canned soup consumption and urinary bisphenol A: a randomized crossover trial. JAMA, 306, 2218-2220.

The authors carried out a randomized, single-blinded, 2x2 crossover study in 84 volunteers with the aim to measure urinary BPA concentration after a week of canned vs. fresh soup consumption. Each phase consisted of 5 consecutive days. For the first 5 days, one group consumed a 12-ounce serving of a soup that was made of fresh ingredients, whereas the other group consumed a 12-ounce serving of canned soup. After a 2 day wash out, treatment assignments were reversed. The ingestion was between

12:15 and 2:00 pm; spot urine was collected at day 4 and/or 5 between 3:00 and 6:00 pm. Measurements were done by a specific and sensitive method based on solid phase extraction (SPE) coupled to isotope dilution high-performance liquid chromatography-tandem mass-spectrometry (ID LC-MS-MS). The LOD of the method is 0.4 ng/ml. The concentration measurements were corrected for different urine volume by a factor which uses specific gravity measurement of the individual urine. Seventy-five volunteers completed the study. The corrected geometric mean concentration of total BPA was 1.1 µg/l after intake of fresh soup, and 20.8 µg/l after canned soup consumption. The authors commented that the increase in urinary BPA concentrations following canned soup consumption is likely a transient peak of yet uncertain duration.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:
- Study design
- Sample size
- Container specified (collected in PE vials, stored in PP cryogenic vials)
- Analytical method (SPE ID LC-MS-MS)
- Quality control, including blanks
- Standardized BPA concentration (specific gravity)
- Consistency in results among different studies

Weaknesses:
- Single measurement
- Quality assurance, precaution to avoid contamination not described
- Confounding by diet (other than canned soup) and other exposures not considered
- Generalisability to the overall population

Overall, the CEF Panel considers that the study design was appropriate, as appropriate was the analytical method, and the urine sample collection and storage. The CEF Panel noted that the urinary concentrations which were measured in urine are in the same order of magnitude as the urinary concentrations reported by the study by Teeguarden et al. (2011; mean: 5.9 µg/l with a broad wide range the highest concentration was 125 µg/l).

Christensen KLM, Lorber M, Koslitz S, Brüning T and Koch HM, 2012. The contribution of diet to total bisphenol A body burden in humans Results of a 48 hour fasting study. Environment International, 50, 7–14.

The study evaluated the excretion of conjugated BPA in five volunteers during a course of a two day-fasting (0–48 hrs). Total BPA concentration in urine was determined by method based on solid phase extraction (SPE) coupled to isotope dilution high-performance liquid chromatography-tandem mass-spectrometry (ID LC-MS-MS). The LOD and the LOQ of the method are 0.05 and 0.1 µg/L, respectively. Free BPA was measured in a subset of samples. In four of the five volunteers the amount of conjugated BPA excreted in the urine declined during the fasting period to 5 % on the second day of the amount on day 1, in one of them urinary excretion increased between 32 and 42 hours without a defined exposure. The study shows that even after intake of BPA by meal ceases BPA is still excreted from the body indicating (a) non-food exposure towards BPA or (b) excretion of BPA from store tissue such as lipid tissues.

Comments from the CEF Panel:

The CEF Panel identified the following strengths and/or weaknesses in the study:
Strengths:
- Study design
- Multiple measurements (sampling)

- Container specified (PP)
- Analytical method (SPE ID LC-MS-MS)
- Quality control, including blanks and quality assurance (precaution to avoid contamination described)
- Standardized samples (BPA concentration in urine by creatinine)
- Confounding by diet and other exposures considered (water, hygiene products, medications)

Weaknesses:

- Small sample size (one is an outlier)
- Selection bias (healthy adult volunteers)
- Single measurement
- Generalisability to the overall population (particularly to children)

Overall, the CEF Panel considers that the study design was appropriate, as appropriate was the analytical method, and the urine sample collection and storage.

Edlow AG, Chen M, Smith NA, Lu C and McElrath TF, 2012. Fetal bisphenol A exposure: concentration of conjugated and unconjugated bisphenol A in amniotic fuid in the second and third trimesters. Reproductive Toxicology, 34, 1-7.

This study was aimed at determining the levels of free and conjugated BPA in second and third trimester amniotic fluid. Amniotic fluid was collected for medical reasons in 20 pregnant women between week 15 and 23.9 (second trimester) and in 20 pregnant women in the third trimester. Liquid chromatography coupled with mass spectrometry (LC-MS) was used to measure BPA concentrations (LOD = 0.1 ng/ml; LOQ: 3LOD) after solid phase extraction (SPE). The method was validated and specific investigations were performed to ensure that no cross-contamination took place. In the second trimester samples, total BPA levels ranged from non-detectable to 0.75 ng/ml (median 0.47 ng/ml) and in 4 out of 20 samples no total BPA was detected. Unconjugated BPA was detected in 9/20 second trimester samples, with levels ranged from 0.31 to 0.43 ng/ml (median 0.38 ng/ml). In the third trimester samples, total BPA was detected only in 2/20 samples and unconjugated BPA (0.42 ng/ml) only in 1/20. When detected, unconjugated BPA comprised 83 % and 91 % of total BPA in second and third trimester amniotic fluid, respectively. The authors concluded that the results indicate that placental ß-glucuronidase may deconjugate BPA. Deconjugation of BPA by the placenta, and limited capacity of the fetal liver to conjugate BPA, may increase fetal exposure to unconjugated BPA.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- Repeated measurements (sampling, second and third trimester)
- Container specified (PETF tube, HDPE caps)
- Analytical method (SPE LC-MS)
- Quality control, including blanks and quality assurance (precaution to avoid contamination described)

Weaknesses:

- Small sample size
- Single measurement
- Inability to detect BPA in the majority of third trimester samples
- Maternal samples (serum or urine) were not collected (maternal exposure not characterized)
- Unclear clinical relevance

Overall the CEF Panel notes that the analytical part of the study is done according to the current scientific standard, including the check of accuracy, precision (coefficient of variation), BPA stability

and the use of blanks. The observed results might be explained by deconjugation of BPA-conjugates in the amniotic fluid.

Geens T, Neels H and Covaci A, 2012. Distribution of bisphenol-A, triclosan and n-nonylphenol in human adipose tissue, liver and brain. Chemosphere, 87, 796-802.

The study of Geens et al. (2012) used human material obtained by autopsies in 11 deceased patients, aged 9–62 years, and measured BPA, triclosan and n-nonylphenol in brain, liver and fat. BPA measurement was done by gas chromatography coupled with mass spectrometry operated in electron negative ionization mode (GC-ECNI/MS) after liquid liquid extraction and derivation to pentafluorobenzoyl derivatives. Whereas in brain tissue and fat only unconjugated BPA was measured, in liver tissue BPA content was estimated both without (unconjugated BPA) and with previous pretreatment with glucuronidase (total BPA, as the sum of unconjugated and its conjugated BPA). The resulting median concentrations of unconjugated BPA in tissues were: 2.1 ng/g in fat, 0.6 ng/g in brain and 1.0 ng/g in liver. In liver, the median concentration of total BPA was 1.0 ng/g.

Comments from the CEF Panel:
The CEF Panel identified the following strengths and/or weaknesses in the study:
Strengths:

- Container specified (PP, BPA free, evaluated for contamination)
- Analytical method (GC-ECNI/MS)
- Quality control, including blanks and quality assurance (precaution to avoid contamination described)

Weaknesses:

- Small sample size
- Selection bias (deceased patients)
- Single measurement
- Collection of sample in hospital settings (less contamination control)
- Confounding factors (no information on medical treatments of patients before death)
- Generalisability to the overall population
- Unclear clinical relevance
- Inconsistency in results compared to other studies

Overall the CEF Panel notes that the ratio of the concentrations in different tissues is in fair accordance with measurements done by Csanady et al. (2002) in rat tissue incubated with blood containing BPA. It should be considered that in experimental studies in humans nearly 100 % of an oral dose was excreted in the urine, in the form of metabolites, and only traces of parent compound were found. The authors acknowledge that the results of the study partly disagree with the fast elimination of BPA and postulate that the reason that unconjugated BPA was detected in the tissues was due to deconjugation of the glucuronyl metabolite in the cells, releasing unconjugated BPA. The CEF Panel considered that this could not take place in the living organism as the calculated tissue/blood partition coefficient (calculated after Schmitt, 2008) is 0.1. This means that only 10 % of the blood concentration will go into the tissues, which is due to the high polarity and water solubility of BPA-glucuronide. The findings that the authors did not find metabolites, even in the liver tissue, might be due to post mortem changes. When the amount of BPA in the human body is calculated, based on the data of Geens et al. (2012), the amount is about 82.6 µg or 1.235 µg/kg bw which might be in line with high exposure by medical devices used before the death. The authors did not report whether the deceased were treated before because of diseases in a hospital. In this case, the amount of BPA in the body could be caused because of leaching from medical devices. There were no indications of how many persons/age were evaluated in the study.

Genuis SJ, Beesoon S, Birkholz D and Lobo RA, 2012. Human Excretion of Bisphenol A: Blood, Urine, and Sweat (BUS) Study. Journal of Environmental and Public Health, ID 185731, 10 pages, doi:10.1155/2012/185731

This study was designed to assess the relative concentration of BPA in three body fluids—blood, urine, and sweat—and to determine whether induced sweating may be a therapeutic intervention with potential to facilitate elimination of this compound. Blood, urine, and sweat were collected from 20 individuals (10 healthy participants and 10 participants with assorted health problems) and analyzed for total BPA (after hydrolysis of conjugated BPA). BPA in blood urine and sweat was measured by liquid chromatography coupled to tandem mass spectrometer (LC-MS-MS) after hydrolysis and solid phase extraction. The LOD of the method was 0.2 ng/ml. BPA was detected at different levels in blood, urine, and sweat. In 16 of 20 participants, BPA was identified in sweat, even in some individuals with no BPA detected in their serum or urine samples. The authors conclude that biomonitoring of BPA through blood and/or urine testing may underestimate the total body burden of this potential toxicant. They further conclude that sweat analysis should be considered as an additional method for monitoring bioaccumulation of BPA in humans and furthermore that induced sweating appears to be a potential method for elimination of BPA.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- Container specified for all matrices (glass)
- Analytical method (SPE LC-MS-MS)
- Quality control, including blanks and quality assurance (precaution to avoid contamination described)

Weaknesses:

- Small sample size
- Single measurement
- Collection of samples at different times
- No distinction between unconjugated and conjugated BPA
- Concentration in urine not standardized
- Confounding by diet and other exposures not considered
- Generalisability to the overall population
- Unclear clinical relevance
- Inconsistency in results compared to other studies
- No possibility of comparison across studies for sweat

The CEF Panel noted that concentrations in the three body fluids were taken at different times. Thus, it is difficult to make a comparison between the concentration in urine (which has been taken as a spontaneous sample in the morning), sweat (mainly taken in an infrared sauna) and blood (taken in a laboratory). It is not explained why in this study the concentration in the urine was higher than reported in other studies and why the two subjects with a measurable serum concentration showed levels 10-fold higher that measured in other studies (Teeguarden et al., 2011).

Gerona RR, Woodruff TJ, Dickenson CA, Pan J, Schwartz JM, Sen S, Friesen M, Fujimoto VY and Hunt PA, 2013. BPA, BPA glucuronide, and BPA sulfate in mid-gestation umbilical cord serum in a northern California cohort. Environmental Science & Technology, 47, 12477-12485.

Gerona et al. (2013) used isotope-dilution LC/MS/MS to directly measure concentrations of unconjugated BPA, glucuronidated BPA, and sulfated BPA in umbilical cord serum collected from 85 study participants during elective second trimester pregnancy terminations. The LODs were 0.05 ng/ml (unconjugated and glucuronidated BPA) and 0.025 ng/ml (sulfated BPA), the LOQ was 0.1 ng/ml for all three analytes. d16-BPA was used as (surrogate) internal standard for all analytes.

Procedural blanks and quality control (QC) materials at low and high concentrations (0.5 and 20 ng/ml) were included. To reduce the likelihood of contamination, the authors used glass/polypropylene containers for sample collection, processing, and storage containers. Umbilical cord blood was drained directly into collection tubes during the procedure to avoid contact with medical devices and the environment. The authors additionally performed a field blank testing by simulating the sample collection, extraction, and analytical run using double-charcoal stripped serum and synthetic human serum. No BPA was detected ≥ LOD in any of the sample collection and extraction materials or in any of the instruments used in the analysis. The possible contamination from the IV equipment (IV bag, needles, and tubing) and medical equipment (gloves, laminaria) used during the termination procedure was also evaluated. The analysis of the umbilical cord serum samples revealed detection levels of 47%, 76%, and 96% for for unconjugated, glucuronidated, and sulfated BPA. The GM concentrations were 0.16 ng/ml for unconjugated BPA (range: <LOD–52.26 ng/mL), 0.14 ng/ml for glucuronidated BPA (range: <LOD–5.41 ng/mL), and 0.32 ng/ml for sulfated BPA (range: <LOD–12.65 ng/mL). The concentration ratio of unconjugated BPA/conjugated BPA ranged from 1:100 to >400:1. The CEF Panel noted that extreme concentration ratios such as 400:1 between the unconjugated and conjugated BPA forms are not in line with the evidence from toxicokinetic studies in pregnant monkeys (vom Saal et al., 2014) and questions the attempts of the authors to have the sample contamination and the exposure by medical devices fully under control. The CEF Panel also noted the specific exposure situation of pregnant women and fetuses during the termination procedure, which makes an extrapolation of the results to pregnant women in general (i.e. outside a hospital setting) questionable.

Comments from the CEF Panel:
Comment: They authors describe that they did not found BPA eluting from iv drips. Calafat et al. (2009) described that in every of 41 neonatal patients in two hospitals excreted conjugated BPA in the urine indicting exposure towards BPA.
Comment: Umbilical cord blood is a mixture of maternal and fetal blood. The concentrations measured in this matrix are henceforth not clearly attributable to the mother or the child

The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- Analytical method (LC-MS/MS)

Weaknesses:

- .
- Insufficient study reporting (the authors state that they tested the medical equipment for possible BPA content. They did not describe how they took the samples (at random?) and how many replicates they performed).

Kosarac I, Kubwabo C, Lalonde K and Foster W, 2012. A novel method for the quantitative determination of free and conjugated bisphenol A in human maternal and umbilical cord blood serum using a two-step solid phase extraction and gas chromatography/tandem mass spectrometry. Journal of Chromatography B, 898, 90-94.

The aim was to develop a new analytical method for the analysis of both unconjugated and conjugated BPA in human material. Unconjugated BPA was determined after liquid-liquid extraction (LLE) followed by a two-step solid-phase extraction (SPE), derivatization by N-methyl-N-(trimethylsilyl)trifluoro-acetamide (MSTFA) and gas chromatography/tandem mass spectrometry (GC/EI-MS/MS). Conjugated BPA was determined after enzymatic deconjugation and bisphenol A-d6 β-glucuronide served as internal standard. The LOD and LOQ for BPA were 0.026 ng/ml and 0.087 ng/ml, respectively. Blank levels ranged between <0.026 (LOD) and 0.083 ng/ml, and results were corrected for their respective blank samples. Matched human maternal at mid-pregnancy, at delivery and umbilical cord blood serum samples were obtained from 12 pregnant women. Total BPA concentrations in human maternal serum at mid-pregnancy and at delivery ranged from <0.026 ng/ml to 10.425 ng/ml (median 0.548 ng/ml, n=12) and <0.026 ng/ml to 3.048 ng/ml (median 1.461 ng/ml),

respectively. Matching umbilical cord blood serum BPA concentrations were in the range of <0.026-2.569 ng/ml (median 1.823 ng/ml).

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- Repeated measurement (mid-term pregnancy and at delivery)
- Analytical method (LLE SPE GC-MS-MS)
- Quality control, including blanks and quality assurance (precaution to avoid contamination described)
- Consistency in results among different studies

Weaknesses:

- Small sample size
- Confounding by diet and other exposures not considered
- Unclear clinical relevance

The CEF Panel considered that the study has some shortcomings and that the biomonitoring data reported have low credibility due to limited reporting in particular with respect to sample coollection and handling, and discrepancies with other studies.

Krotz SP, Carson SA, Tomey C and Buster JE, 2012. Phthalates and bisphenol do not accumulate in human follicular fluid. Journal of Assisted Reproduction and Genetics, 29, 773-777.

The study examined whether phthalates and BPA could be detected in human follicular fluid after exposure to medical plastics during an in vitro fertilisation (IVF) cycle. Ovarian follicular fluid was prospectively collected from five women, although it is not clear how many follicles were aspirated from each woman. Within each woman a single pooled follicular fluid sample was used for BPA and phthalate measurement by liquid chromatography tandem mass spectrometry (LC-MS-MS, LOD not given). The authors report that no BPA was detectable in any of the five, pooled, follicular fluid samples (although phthalates were quantifiable).

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- Container specified (glass)
- Analytical method (SPE LC-MS-MS)

Weaknesses:

- Small sample size
- Single measurement
- No quality control (including blanks) and quality assurance (precaution to avoid contamination not described)
- Limit of detection not reported
- No distinction between unconjugated and conjugated BPA
- Confounding by diet and other exposures not considered
- Generalisability to the overall population

Overall, the CEF Panel considers that the number of participants is very low (n=5). Moreover, the authors did not specify whether unconjugated, conjugated or total BPA was measured. BPA was below the LOD in all samples, but the LOD was not given and thus the study cannot be compared with other studies. However, as a pilot study this shows that BPA is unlikely to accumulate in the microenvironment around the oocyte in humans.

Mose T , Mathiesen L, Karttunen V, Nielsen JKS, Sieppi E, Kummu M, Mørck TA, Myöhänen K, Partanen H, Vähäkangas K, Knudsen LE and Myllynen P, 2012. Meta-analysis of data from human ex vivo placental perfusion studies on genotoxic and immunotoxic agents within the integrated European project NewGeneris. Placenta, 33, 433-439.

In the EU integrated project NewGeneris, the placental transport of thirteen immunotoxic and genotoxic agents was studies in three *ex vivo* placental perfusion laboratories. In the present publication, all placental perfusion data have been re-analyzed and normalized to make them directly comparable and rankable. Antipyrine transfer data differed significantly between the studies and laboratories, and therefore normalization of data was necessary. An antipyrine normalization factor was introduced making the variance significantly smaller within and between the studies using the same compound but performed in different laboratories. Non-normalized (regular) and normalized data showed a good correlation. The compounds were ranked according to their transplacental transfer rate using either antipyrine normalized AUC120 or transfer index (TI120(%)). Based on their results the authors concluded that BPA has a high transplacental transfer rate (concentration at the fetal site/concentration of the maternal site =1) which is explained by passive diffusion.

Comments from the CEF Panel:

The result of the study is comparable to that of a study published earlier (Balakrishnan B, Henare K, Thorstensen EB, Ponnampalam AP, Mitchell MD (2010). Transfer of bisphenol A across the human placenta. Am J Obstet Gynecol 202:393.e1–e7.) which also reported a factor of 1 for transplacental transfer rate.

Nachman RM, Fox SD, Golden WC, Sibinga E, Veenstra TD, Groopman JD and Lees PSJ, 2013. Urinary Free Bisphenol A and Bisphenol A-Glucuronide Concentrations in Newborns. The Journal of Pediatrics, 162, 870-872.

The study population consisted of 11 healthy neonates plus 1 young infant (median age 17 days) born from healthy non smoking mothers. Urine samples were collected using BPA-free pediatric urine collection bags (U-Bag; Hollister, Inc, Libertyville, Illinois) during the neonates' regular well-child care visits. After voiding the urine was transferred on ice to the laboratory, transferred to a pre-cleaned glass vial which was stored at -80 °C until analysis. Free and conjugated BPA were determined directly in urine by liquid chromatography tandem mass spectrometry (LC-MS-MS) without hydrolysis and extraction. The LOD and the LOQ were 0.02 and 0.1 ng/ml, respectively. The average concentration of BPA glucuronide, as measured in all of the duplicate urine samples, was 0.87 ± 0.51 ng/ml (median: 0.66 ng/ml. Unconjugated BPA was not found in any of the urine samples with the exception of 1 sample (subject 6) whose replicate sample was a non-detect. With the exception of one fully breast feed baby al babies received infant formula. The study demonstrates that neonates and infants are capable of conjugating BPA to the BPA-glucuronide.

The CEF Panel identified the following strengths/weaknesses in the study:

Strengths:

- Container specified (BPA-free pediatric urine collection bags)
- Analytical method (LC-MS-MS)
- Quality control, including blanks and quality assurance (precaution to avoid contamination described)
- Repeated measurements (n=2)

Weaknesses:

- Single measurement
- BPA concentration in urine not standardized
- Generalisability to the overall population

Overall the CEF Panel noted that the study was well performed and the results are in accordance with what is known from the expression of UTGs in the fetus and neonates. The study showed that BPA-

glucuronide is the only detectable BPA compound in the urine of the newborn. This finding is not in conflict with the results of another study showing that levels of unconjugated BPA might be found.in the urine of premature infants in intensive care (Calafat et al., Environ Health Perspect 2009;117:639-44). Infants in intensive care are exposed to BPA by others than the oral route at doses exceeding the doses on the oral route by breast feeding or bottle feeding. As non-oral routes of exposure are characterized by lacking presystemic elimination of BPA, the concentration of unconjugated BPA in the systemic circulation is higher compared with the oral administration. Hence, levels of unconjugated BPA might be found in the urine. The study has a limitation as it did not measure the sulphate conjugate of BPA.

Nahar MS, Liao C, Kannan K and Dolinoy DC, 2012. Fetal Liver Bisphenol A Concentrations and Biotransformation Gene Expression Reveal Variable Exposure and Altered Capacity for Metabolism in Humans. Journal of Biochemical and Molecular Toxicology, 27, 116-123.

In this study, the internal dose of unconjugated BPA and conjugated BPA was measured and gene expression of biotransformation enzymes specific for BPA metabolism was evaluated in 50 first- and second-trimester human fetal liver samples. The concentration of unconjugated BPA and conjugated BPA concentrations in the fetal livers (measured by liquid chromatography tandem mass spectrometry (LC-MS-MS)) varied widely, with unconjugated BPA (geometric mean 2.26 ng/g tissue) exhibiting three times higher concentrations than conjugated BPA (geometric mean 0.65 ng/g tissue). As compared to gender-matched adult liver controls, UDP-glucuronyltransferase, sulfotransferase, and steroid sulfatase genes exhibited reduced expression whereas β-glucuronidase mRNA expression remained unchanged in the fetal tissues. According to the authors, the study provides evidence that there is considerable exposure to BPA during human pregnancy and that the capacity for BPA metabolism is altered in the human fetal liver.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- Container specified (PC-free PP tubes)
- Analytical method (LC-MS-MS)
- Quality control, including blanks and quality assurance (precaution to avoid contamination described, but only for analysis)

Weaknesses:

- Single measurement
- Quality assurance (precaution to avoid contamination during sampling not described)
- Confounding by diet and other exposures not considered
- Generalisability to the overall population
- Inconsistency in results compared with other studies

Overall the CEF Panel notes that the study results are in marked contrast to what has been observed in experimental studies where lower plasma concentrations of BPA was detected in the fetus compared to the dams in rats and rhesus monkeys (Doerge et al., 201, and Patterson et al., 2012). The fetal liver samples were obtained from the University of Washington Birth Defects Laboratory fetfetal tissue bank. The procedures of surgery, the sort of surgical instruments used and the liver sample isolation from fetal tissues are not described. Hence, it is open to discuss that the results were mainly due to contamination by in hospital processing of the samples. The ratio of 3 of unconjugated/conjugated BPA is also a strong indication for sample contamination given the fact that this ratio is 0.1 to 0.01 in the experimental study in fetuses of rhesus monkeys.

Teeguarden JG, Calafat AM , Ye X, Doerge DR, Churchwell MI, Gunawan R and Graham MK, 2011. Twenty-four hour human urine and serum profiles of bisphenol A during high-dietary exposure. Toxicological Sciences, 123, 48-57.

Teeguarden et al (2011) have reported the results of a clinical exposure study conducted to better understand the internal exposure of adult humans to BPA and the relationship between the serum and urinary pharmacokinetics of BPA. Blood and urine samples were collected approximately hourly over a 24-hour period from 20 adult volunteers who per sitting (breakfast, lunch, and dinner) ingested 100 % of one of three specified meals comprising standard grocery store food items (including canned food). In between the meals the volunteers were only allowed drinking of BPA-free water. Both conjugated and total BPA (after enzymatic hydrolysis) were determined in serum samples by solid phase extraction coupled to liquid chromatography tandem mass spectrometry (SPE LC-MS-MS). In urine samples total BPA only was determined by SPE LC-MS-MS. The LOD of the method was 0.3 ng/ml in serum and 0.4 ng/ml in urine. The volunteers' average consumption of BPA, estimated from the urinary excretion of total BPA (as the sum of conjugated and unconjugated BPA) was 21 μg (range 3.3 to 73 μg). Assuming 100 % absorption and urinary excretion and using individual body weights this is equivalent to an oral exposure of 0.27 μg/kg body weight (bw) (range, 0.03–0.86), 21 % greater than the 95th percentile of aggregate (all routes) daily exposure in the adult US population (0.22 μg/kg bw; equivalent to approximately ~15 μg/person). A serum time course of total BPA was observable only in individuals with exposures 1.3–3.9 times higher than the 95th percentile of aggregate US exposure. Total BPA urine concentration Tmax was 2.75 hours (range, 0.75–5.75 hours) post meal, lagging the serum concentration Tmax by ~1 hour. During these high dietary exposures, total BPA concentrations in serum were below the LOD for 86 % of the 320 samples collected. Unconjugated BPA concentrations were always below the LOD (1.3 nM; 0.3 ng/ml). In six individuals, serum total BPA concentrations could be measured (concentrations up to 5.7 nM; 1.3 ng/ml) and the serum levels found were, on average, 42 times lower than urine concentrations. For these individuals, serum total BPA area under the curve per unit BPA exposure (i.e. normalised to urinary BPA excretion, expressed as μg/kg bw) was between 21.5 and 79.0 nM•hr•kg/μg.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- Large sample size
- Repeated measurements (sampling)
- Urine, container specified
- Analytical method (SPE LC-MS-MS)
- Quality control, including blanks and quality assurance (precaution to avoid contamination described)
- Distinction between unconjugated and conjugated BPA in serum samples
- Repeated measurement (analysis of serum samples by an independent laboratory)
- Confounding by diet and other exposures considered

Weaknesses:

- Serum BPA
- No distinction between unconjugated and conjugated BPA in urine
- Single measurement (urine samples)
-

Overall the CEF Panel notes that this study endorses the view that no unconjugated BPA in serum and only very low levels of conjugated BPA can be found in humans at levels of dietary exposure. The study report indicates that exposures to BPA from retrospectively determined dietary exposure (e.g. from canned foods) is at the most 73 μg person per day (three meals). The exposures were at the high end of the NHANES population-based exposure estimates (spot samples) from the 2005-2006 NHANES biomonitoring report. The study encompasses quite a large group of human volunteers and

is very rigorously controlled with respect to possible sample contamination (e.g. plasma samples were analysed in a contra-expertise set-up to identify problems with reproducibility and were further analysed if inexplicable results were obtained to identify possible contamination). A limitation of this study is the lack of data concerning the content of BPA in the canned food.

(ii) Animal studies

Churchwell MI, Camacho L, Vanlandingham MM, Twaddle NC, Sepehr E, Delclos KB, Fisher JW, and Doerge DR, 2014. Comparison of life-stage-dependent internal dosimetry for bisphenol A, ethinyl estradiol, a reference estrogen, and endogenous estradiol to test an estrogenic mode of action in Sprague Dawley rats. Toxicological Sciences, 139, 4-20.

A subchronic BPA exposure study with daily oral administration throughout perinatal and adult life was performed in SD rats. As a companion paper to the published evaluation of BPA toxicity (Delclos et al., 2014), the present paper evaluated the internal dosimetry in newborn (PND 4), weanling (PND 21), and adult (PND 80) animals. Native BPA in 0.3% aqueous carboxymethyl-cellulose (CMC) was administered by gavage (2.5–300,000 µg/kg bw per day) to pregnant dams and their offspring. Blood samples were collected at ~0.5 h postdosing (time window: 0.25–0.75 h). Unconjugated and total BPA concentrations in serum were measured by isotope-dilution LC-MS/MS (LOD: 0.1–1.1 nM, see below). Similarly, the internal dosimetries for the reference estrogen ethinyl oestradiol (EE2, dose: 0.5–5 µg/kg bw per day) and for the endogenous hormones 17β-oestradiol (E2) and testosterone were evaluated. Finally, the toxicokinetics following the gavage administration of 100 µg/kg bw of deuterated d6-BPA (LOD: 0.2 nM) was analysed in PND 4 rats.

Several quality control measures were applied which included, among others, the use of (i) uncontaminated rat serum for negative control samples and spiked samples, (ii) incurred rat serum containing both unconjugated and conjugated BPA to check for the completeness of enzymatic hydrolysis for the analysis of total BPA, and (iii) procedural blanks to establish background BPA levels from sample preparation. A limit of blank (LOB) was determined daily from procedural blanks as mean + 2 SD and subtracted from each serum sample concentration. LOB values ranged from 0.3–1.1 nM (without enzymatic hydrolysis) to 0.5–1.8 nM (with enzymatic hydrolysis). The LOD was set to a signal-to-noise ratio of 3 above the LOB and ranged from 0.1–0.4 nM (without enzymatic hydrolysis) to 0.2–1.1 nM (with enzymatic hydrolysis).

Serum samples from naïve (NC) and vehicle control (VC) rats of all age groups consistently contained measurable levels of unconjugated BPA (mean values: 0.1–2 nM) accounting for 17–58% of total BPA, which the authors found to be best explained as resulting from contamination by native BPA during sample collection and storage. The levels of total BPA in NC and VC rats were consistently observed in the 1–10 nM range. Verification measurements were conducted by direct determination of glucuronidated BPA (BPA-G) since BPA-G is the predominant BPA conjugate. The presence of BPA-G in serum indicated an unintentional exposure of the control animals. The concentration of BPA-G in NC and VC rats were similar to the levels of conjugated BPA observed in the 2.5 µg/kg bw per day dose groups at all ages tested. According to the authors, these findings are best interpreted as resulting from similar levels of aggregated exposures. Although several potential BPA inputs (diet, drinking water, vehicle, cages, bedding, and dust) were evaluated, the relevant source(s) of BPA to which the control animals were supposedly be exposed could not be identified.

In the BPA-treated rats, increases in serum levels of unconjugated and total BPA above the levels measured in control animals were observed at doses above ~80 µg/kg bw per day. In PND 4 rats, the fraction of total BPA accounted for by unconjugated BPA plateaued at ~4% in the intermediate dose range but increased to >10% at the two highest doses. A lower unconjugated fraction of 1–2% was seen in the PND 21 and PND 80 rats. These findings reflected the immaturity, and the saturation at high dose levels, of phase II metabolism in PND 4 rats.

The analysis of serum toxicokinetics (TK) in PND 4 animals yielded a C_{max} for unconjugated d6-BPA of 14 nM (observed at the first sampling time point at 0.25 h after dosing), an AUC (i.e. the $AUC_{0-\infty}$) of 26.3 nM×h, and a terminal elimination half-life ($t_{1/2}$) of 4.1 h. Unconjugated BPA represented 4.9% of total BPA when determined from the concentrations measured at 0.25 h, and 0.97% when judged on the derived AUC values. The TK parameters (C_{max}, AUC, and $t_{1/2}$) were intermediate between those reported for PND 3 and PND 10 animals in a previous TK study (Doerge et al., 2010a), in which the same rat strain and dose but a different vehicle (10% ethanol in water instead of 0.3% CMC) were used. Churchwell et al. (2014) concluded that the results of both TK studies are "consistent with the age-dependent development of metabolic and excretory function in neonatal rats that combine to decrease internal exposures as maturation proceeds".

Serum levels of the reference estrogen EE2 were determined in controls, 0.5 and 5 µg/kg bw-treated rats at PND 4, PND 21, and PND 80. As seen with BPA, the internal exposures to unconjugated EE2 were highest in PND 4 rats. When maximal serum concentrations from the two highest BPA doses (100,000 and 300,000 µg/kg bw) and both EE2 doses were compared with concurrent levels of endogenous hormone E2, the estrogen receptor α (ERα) binding equivalents were similar to or above those of endogenous E2 in male and female rats at all ages tested, suggesting the possibility of ERα-mediated signaling for the administered EE2, especially at the high EE2 dose, for BPA at the two highest doses.

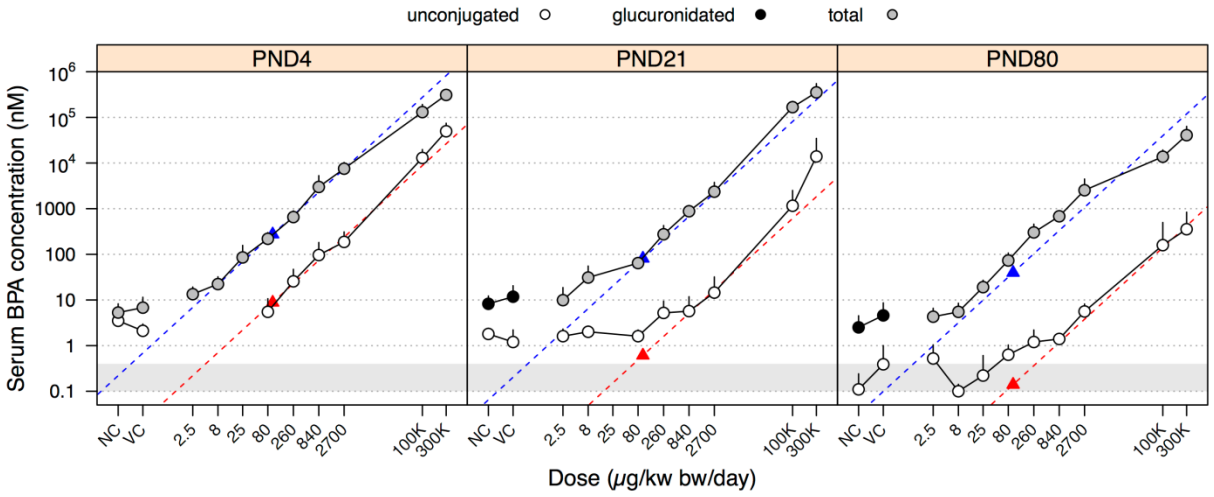

Figure 17: Serum concentrations of unconjugated and total/glucuronidated BPA at ~0.5 h postdosing across all control and dose groups for PND 4, PND 21, and PND 80 rats. The gray-shaded area indicates the LOD range (0.1–0.4 nM) for unconjugated BPA. The red and blue triangle symbols represent the serum concentrations of unconjugated and total BPA as measured in the toxicokinetic experiments with PND 4 rats (this study) and PND 21 and PND 80 rats (Doerge et al., 2010a) at 0.5 h after gavage administration of 100 µg/kg bw of d6-BPA. The associated red and blue dashed lines show dose extrapolations under the assumption of linear kinetics. NC: naïve control, VC: vehicle control.

Comments from the CEF Panel:
The CEF Panel identified the following strengths and weaknesses in this study:
Strengths:
- Quality control measures (BPA contamination, completeness of enzymatic deconjugation)
- Life-stage-dependent internal dosimetry for unconjugated and total BPA in all study groups
- Verification experiments with direct determination of glucuronidated BPA
- Toxicokinetic analysis in newborns with administration of deuterated BPA
- Results consistent with linear kinetics and age-dependent maturation of toxicokinetics
- Internal dosimetry for the reference estrogen EE2 and for endogeneous hormones (E2, testosterone)

Weaknesses:

- Unintended exposure of controls at levels similar to the intended exposure of lowest dose group (2.5 µg/kg bw/d)
- Relevant source(s) of unintended BPA exposure could not be identified

The CEF Panel noted that the presence of unintended BPA exposure source(s) resulted in similar internal levels of unconjugated and conjugated BPA in the control animals and lowest dose group. BPA-related toxicity effects, if occurring specifically in the lower dose range, would not be discernible in the absence of unexposed controls. However, as reported by Delclos et al. (2014), the unintended exposure did not affect the interpretation of the toxicology study since there were no biologically significant effects of BPA observed in the "low dose" range (2.5-2700 µg/kg bw/d). Similarly, the unintended exposure to BPA did not affect identification of significant adverse effects in either of the two EE2 dosing groups or the two highest BPA dosing groups.

Doerge DR, Twaddle NC, Vanlandingham M and Fisher JW, 2010a. Pharmacokinetics of bisphenol A in neonatal and adult Sprague-Dawley rats. Toxicology and Applied Pharmacology, 247, 158-165.

Doerge DR, Twaddle NC, Woodling KA and Fisher JW, 2010b. Pharmacokinetics of bisphenol A in neonatal and adult rhesus monkeys. Toxicology and Applied Pharmacology, 248, 1-11.

These two pharmacokinetic studies in rats and monkeys (Doerge et al. 2010a, 2010b) had already been revied by EFSA in 2010 as stated below.

"A very recent pharmacokinetic study by Doerge et al. (2010a) measured by LC/MS/MS serum levels of free and conjugated deuterated BPA in neonatal, immature (PND 3, 10 and 21) and adult Sprague-Dawley rats dosed via oral (gavage) and injection routes (i.v and s.c.). Animals were given a single dose of 100 µg/kg b.w. BPA, which was demonstrated to be within the linear range of pharmacokinetics, so that extrapolation to lower doses is feasible. The use of labelled BPA (methyl-d6-BPA) avoided confounding background contamination (from laboratory materials or other sources), which was reported by the authors to be as high as 2 ng/ml in buffer blanks. In adult rats, direct comparisons of i.v. and oral routes of administration indicate: i) extensive absorption from the gastrointestinal tract; ii) rapid elimination of free BPA from the circulation (> 50% of circulating BPA was conjugated 5 min after i.v. injection); iii) importance of first-pass conjugation in liver and gut after ingestion: indeed, at maximum serum concentration (Cmax), the fraction present as conjugated BPA was substantially higher following oral compared to i.v. administration (99.5% vs. 55%). Moreover, the higher volume of distribution following i.v. vs. oral administration was due to higher distribution of free BPA to tissues. In addition, the occurrence of enterohepatic recirculation of BPA in adult rats is reflected by a secondary peak in the concentration of total BPA later in the rat serum concentration-time profiles.

The Cmax of free BPA observed in individual rats ranged from 0.1-0.6 nM (average value of 0.39 ± 0.19 nM, corresponding to about 89 ng/L) so that internal exposures of adult rats to free BPA are below 1% of the total (Cmax = 73 ± 29 nM, corresponding to 16.6 µg total BPA/L).

A comparison of BPA pharmacokinetics in adult vs. neonatal rats was also performed.

Oral administration of BPA (100 µg/kg b.w.) to PND 3 pups produced higher Cmax in serum of total (6-fold) and free (74-fold) BPA when compared to adults: the fraction present as conjugates was 93.4% (PND 3) and rapidly increased with age up to 96.9 and 98.9% (PND 10 and 21, respectively), indicating a regular development of metabolic and excretory functions toward the adulthood situation (99.5% BPA in conjugated form). The percentage of total BPA present in neonatal blood (PND 3) as free BPA was very limited (1.4% of AUC, Cmax: 6.6%).

Administration of an identical dose (100 µg/kg b.w.) by s.c. injection to PND 3 rats produced 34-fold higher Cmax and 17-fold higher AUCs for free BPA compared to oral. The age-related changes (described above) after oral administration were not observed after s.c. injection, indicating that even in early postnatal pups, which possess lower conjugation activity, the first pass effect is extremely relevant. This confirms that the effect due to the route of administration is very relevant, and highlights the limitations in interpreting toxicity data from studies in which BPA is administered via routes other than the oral one, without any internal dosimetry.

With a similar study design, free deuterated BPA (100 µg/kg b.w.) was administered to adult and neonatal rhesus monkeys orally (PND 5, 35 and 70) and i.v. (PND 77). Free and conjugated BPA were measured in the blood (Doerge et al., 2010b).

In adult rhesus monkeys, results for the first sampling points show that the percentage of free BPA was higher following parenteral (i.v) administration (29 ± 19% of total BPA at 5 min post-injection) than after oral administration of the same dose (0.21 ± 0.14% of total BPA at 30 min post-gavage). This confirms that in monkeys the systemic availability of free BPA is much lower after oral administration. When BPA was administered to neonatal non-human primates orally (PND 5, 35 and 70) or i.v. (PND 77), the toxicokinetic parameters were not statistically significantly different from those in adults.

The CEF Panel noted that following the same oral dose of 100 µg/kg b.w. BPA to adult rat and monkeys, free BPA concentrations in both species was similarly low (<1%), the only notable difference being the longer elimination half-time in rats, due to the enterohepatic re-circulation in the rat. On the contrary, comparing newborn animals, PND3 rats have longer elimination half-time and approximately 10 times higher plasma levels of free BPA than PND 5 monkeys, when treated with the same oral BPA dose (Doerge, 2010b). These data provide evidence for a different developmental profile of hepatic and intestinal conjugation of BPA in rats and monkeys, consistent with literature data describing a higher degree of immaturity of rats at birth as compared to primates, in relation to UGT activity (Coughtrie et al, 1988; Matsumoto et al, 2002).”

Doerge DR, Vanlandingham M, Twaddle NC and Delclos KB, 2010c. Lactational transfer of bisphenol A in Sprague–Dawley rats. Toxicological Letters, 199, 372–376.

Female pregnant Sprague Dawley rats were given 100 µg/kg bw deuterated BPA using either oral administration or IV injection and starting at day of delivery. Conjugated and unconjugated forms of BPA were then measured by using liquid chromatography tandem mass spectrometry (LC-MS-MS) in milk from lactating dams on PND 7 and in serum from dams and their pups on PND 10. The limit of detection (LOD) for deuterated BPA from analysis of 100 µL of either serum or milk was 0.2 nM. All samples were collected 1 h after dosing, a time selected to produce nearly maximal levels. In milk and serum samples of dams, conjugated material represented most of the BPA present (87 % in milk and > 99 % in serum). While unconjugated BPA was detected in all dam serum (0.55 nM) and milk (0.87 nM) samples, none was detected in pup serum (<0.2 nM). Total serum BPA in pups amounted to 0.41 nM. Doses delivered to pups lactationally, estimated from milk concentrations and body weights, were 300–fold lower than the dose administered to the dams. Similarly, serum concentrations of total BPA in pups were 300–fold lower than those in their dams. Plasma concentrations of total BPA in PND 10 rat pups were 500–fold lower than peak levels achieved following direct oral delivery of the same dose to the same age pups (by comparison with data from another study (Doerge et al., 2010a). According to the study authors the significant dose attenuation for the active unconjugated form of BPA, relative to that of the dam, suggest that if effects are observed in offspring exclusively due to lactational transfer, this would mean that BPA would have high potency for toxicological effects. Alternatively, studies that include lactational exposure and report minimal effects from BPA should consider the possibility that inadequate internal exposures were achieved during the critical postnatal period (i.e. relatively high exposures resulting from e.g. baby bottles is not covered by normal multi-generation studies, since during lactation the exposure of neonatal rats is far too low to be comparable).

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:
- Oral administration by gavage
- Phytoestrogen-free diet (e.g. soy-free diet)
- Analytical method (LC-MS-MS)

Weaknesses:
- Single dose levels study
- Single acute dose administration
- Type of cages and drinking bottles not reported

The CEF Panel noted that in the study report some mean values can be found which cannot be explained from the reported results for individual animals. However, recalculation of these means from the individual data would not change the study outcome. Low lactational exposure has already been inferred from the available data in the EFSA opinion of 2010. In fact in this opinion it was estimated that rat pups would receive 300–fold lower dose of BPA than the mother animals, which is confirmed by the study by Doerge et al (2011b). The use of stable isotope-labeled BPA reassures that no contamination by ubiquitous BPA had occurred.

Doerge DR, Twaddle NC, Vanlandingham M and Fisher JW, 2011b. Pharmacokinetics of bisphenol A in neonatal and adult CD-1 mice: Inter-species comparisons with Sprague-Dawley rats and rhesus monkeys. Toxicology Letters, 207, 298-305.

Adult and neonatal CD-1 mice were administered deuterated BPA (100 µg/kg bw) by oral (gavage) or subcutaneous (sc) routes and the concentration of unconjugated and conjugated (inactive) BPA in serum were measured at the time points 0.25, 0.5, 1, 2, 4, 8 and 24 hours after dosing by isotopic dilution ($^{13}C_{12}$-BPA) liquid chromatography tandem mass spectrometry (LC-MS-MS). The limit of detection (LOD) for deuterated BPA in serum was 0.2 nM (0.05 ng/ml). Pharmacokinetics of BPA was measured in neonatal mice at postnatal day (PND) 3, 10 and 21. Neonatal mices were delivered and culled to 6 males and 6 females per litter (i.e. 2 litters for each of the 3 neonatal ages providing one male and female pup from each litter for each of the post-dose time points). Administration of BPA (100 µg/kg bw) by gavage to adult CD-1 mice (n = 12) produced levels of unconjugated BPA that were below the LOD in the preponderance of samples at all time points. Levels of unconjugated BPA that were above the LOD were observed only at the earliest three time points, and only in one or two samples out of the twelve determinations at each time. Oral administration in adults gave a rapid absorption phase (max at the first time point) with similar distribution kinetics for unconjugated and total BPA. Unlike adult mice, serum levels of unconjugated BPA were consistently detected in pups of all ages at early post-dosing time points for both oral and sc administration. Elimination half time after oral exposure decreased with postnatal age and became similar to that of adults at PND 21. The percentage of Cmax values as the unconjugated BPA form following oral exposure showed statistically significant effect of age. On the contrary SC administration showed an almost constant Cmax from PND 3 to PND 21. The developmental profile on pharmacokinetics observed in mice (and rats) was quite different from neonatal rhesus monkeys in which small insignificant age-related differences were observed.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:
- Oral administration by gavage
- Phytoestrogen-free diet (e.g. soy-free diet)
- Analytical method (LC-MS-MS)

The CEF Panel noted that there is a large difference in Cmax of unconjugated BPA following the oral and subcutaneous routes, especially after PND 10. For adult mice (and rats) the unconjugated BPA levels were mostly below the detection limit in serum after oral exposure. The use of stable isotope-labeled BPA reassures that no contamination by ubiquitous BPA had occurred.

Doerge DR, Twaddle NC, Vanlandingham M, Brown RP and Fisher JW, 2011a. Distribution of bisphenol A into tissues of adult, neonatal, and fetal Sprague-Dawley rats. Toxicology and Applied Pharmacology, 255, 261-270.

Sprague-Dawley rats were administered deuterated BPA (100 µg/kg bw) by oral (gavage) or iv routes. The concentration of both unconjugated and conjugated (inactive) BPA in tissues (mammary, liver, ovary, uterus, adipose, brain and muscle in adults, liver and brain in fetus) and placental transfer were measured by isotopic dilution ($^{13}C_{12}$-BPA) liquid chromatography tandem mass spectrometry (LC-MS-MS).The limits of detection (LODs) for deuterated BPA were 0.2 nM in serum and 0.4 pmol/g in tissues. Following iv and oral dosing at gestation day (GD) to 12 pregnant rats (n = 6–7), serial blood samples were collected at designated time points (5–1440 min for iv, and 30–1440 for oral). For the fetfetal collections, whole concepti (n = 4 from each dam) were collected at GD 12 (0.5 hour after iv dosing and 24 hours after oral dosing), whole fetus (n = 4 from each dam) were collected at GD 16 (0.5 hours after iv dosing and 8 hours after oral dosing), and at GD 20 (n = 4 from each dam) fetal serum was obtained by cardiac puncture, and liver and brains were removed and flash frozen (0.5 hours after iv dosing and 8 hours after oral dosing). Amniotic fluid was collected from different ages of fetuses. Aliquots of each sample were analysed for both unconjugated BPA and total BPA, the latter following incubation with glucuronidase/sulfatase mixture. Thawing and/or cutting of liver samples released glucuronidase that converted conjugated BPA to unconjugated. All tissue aliquots were therefore processed in the frozen state.

Administration of BPA by iv to pregnant rats led to rapid distribution ($t_{1/2}$ = 0.29 ± 0.04 h) and elimination ($t_{1/2}$ = 0.78 ± 0.11 h) of the parent compound from serum. Conjugated BPA were eliminated more slowly (t1/2 = 13 ± 7.4 h). Conjugated BPA were eliminated more quickly after oral exposure ($t_{1/2}$ = 7.5 ± 1.9 h) than iv exposure, and the mean pharmacokinetic parameters were similar to those previously reported for oral dosing of non-pregnant rats of comparable age. Orally administered BPA to pregnant rats resulted in the presence of predominantly BPA-glucuronide (BPA-G) in fetfetal tissues, while no measurable levels of unconjugated BPA was found in any GD tested. The maternal serum levels of unconjugated BPA after oral exposure were close to the LOD. However, after iv dosing of BPA to pregnant rats placental transfer were observed for unconjugated BPA into fetus after several GD. At GD 20 the ration of unconjugated BPA in fetfetal brain versus maternal serum was 4.5 ± 0.9, showing that the fetal brain contained more unconjugated BPA than maternal serum at this age. For the fetal tissues/fluids, amniotic fluid, serum and liver, the levels in the fetus were lower than in the maternal serum after iv injection of BPA. Oral exposure of BPA to neonatal rats postnatal day (PND) 3, 10 and 21 showed measurable concentrations of unconjugated BPA in liver, muscle, brain and serum, with highest concentration in the liver of PND 3 (14 nM unconjugated BPA). There was a significant age-dependent decrease in the levels of unconjugated BPA in both serum and tissues, with 2 nM unconjugated BPA in the liver at PND21. Concentrations of the conjugated BPA were considerable higher than the unconjugated in the neonatal tissues after oral exposure.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- Oral administration by gavage
- Analytical method (LC-MS-MS)
- Phytoestrogen-free diet (e.g. soy-free diet)

The CEF Panel noted that no measurable levels of unconjugated BPA were found in the fetuses after oral exposure, while unconjugated BPA was transferred to the fetus after iv injection. Also important to notice is the sensitive window of the neonatal exposure, with age-decreasing levels of unconjugated BPA in the neonates. This has not been observed to the same extent in monkeys, and therefore it can be questionable whether the most sensitive time-window for rats is relevant for humans. The use of stable isotope-labeled BPA ensures that no contamination by ubiquitous BPA had occurred.

Doerge DR, Twaddle NC, Vanlandingham M, Fisher JW, 2012. Pharmacokinetics of Bisphenol A in serum and adipose tissue following intravenous administration to adult female CD-1 mice. Toxicology Letters, 211, 114-119.

In the study liquid chromatography tandem mass spectrometry (LC-MS-MS) was used to measure serum concentrations of unconjugated and conjugated BPA in adult female CD-1 mice following intravenous (iv) injection of deuterated BPA (100 µg/kg bw). The limits of detection (LODs) for deuterated BPA were 0.2 nM (0.05 ng/ml) in serum and 0.4 pmol/g (0.1 ng/g) in tissues. Additionally, the unconjugated BPA was measured in adipose tissue. After iv injection, unconjugated BPA had a distribution half-life of 0.2 h and a terminal elimination of 0.8 h. Consistent with the degree of aqueous solubility, lipid/water solubility ratio, and partitioning from blood into adipose tissue in vivo, the levels of unconjugated BPA in mouse adipose tissue rapidly reached a maximal level (0.25 h) that did not exceed the serum maximum at the initial sampling time (0.08 h). Terminal elimination of unconjugated BPA from adipose tissue ($t_{1/2}$ = 7.0 h) was similar to that for conjugated BPA in serum ($t_{1/2}$ = 6.6 h) and <0.01 % of the administered dose remained in adipose tissue after 24 h. These plasma and tissue kinetics are consistent with rapid equilibria and underscore the non-persistent nature of BPA. By comparing the AUC in serum found in this study with the AUC in serum from an earlier oral study (Doerge, 2011a) a systemic availability of 2 % resulted after oral administration.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- Phytoestrogen-free diet (e.g. soy-free diet)
- Analytical method (LC-MS-MS)
- Distinction between unconjugated and conjugated BPA
- Type of cages and drinking bottles not reported

The CEF Panel considered that this well performed study adds to the toxicokinetic knowledge on BPA. It demonstrates that BPA is not retained in the adipose tissue. The results are plausible and the use of stable isotope-labeled BPA reassures that no contamination by ubiquitous BPA had occurred.

Kohda N, Inoue S, Noda T, Saito T, 2012. Effects of Chitosan Intake on Fecal Excretion of Bisphenol A and Di(2-ethyl)phthalate in Rats. Bioscience, Biotechnology, and Biochemistry, 76, 732-736.

The This study investigated the effect of chitosan oral intake on faecal excretion of bisphenol A (BPA) in rats. The rats were fed a chitosan diet or a control diet (control group) for 10 days and orally administered BPA (100 mg/kg bw) on day 4. Faecal excretion rates of BPA was significantly higher in the CHI group than in the control group. Furthermore, accelerated BPA excretion into the faeces was observed in the CHI group.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- Use of non-PC cages (metal cages)
- Analytical method (GC-MS)

- Distinction between unconjugated and conjugated BPA

Weaknesses:

- Single dose level study
- Single acute dose administration
- Test performed in one sex only

Overall the CEF Panel notes that this study in rats is of no biological significance for humans as in man BPA is not excreted in the bile and eliminated in the faeces via this mechanism.

Marquet F, Payan JP, Beydon D, Wathier L, Grandclaude MC and Ferrari E, 2011. In vivo and ex vivo percutaneous absorption of [14C]-bisphenol A in rats: a possible extrapolation to human absorption? Archives of Toxicology, 85, 1035-1043.

This study is also described and discussed in Appendix D.

This is the only *in vivo* study investigating BPA dermal absorption. The "absorbed dose" of total ^{14}C-BPA after application of a concentrated acetone solution (4 mg/ml, 500 µL total volume) and a 72 hr sample collection interval was 23 % (i.e. fraction of total radioactivity found in urine + faeces + carcass). BPA and its conjugated metabolites were determined by HPLC with fluorescence detection. The limit of detection (LOD) of the method was 1.5 ng/ml. The disruption by acetone of skin lipid structure and the associated barrier function has been described previously (Zhai et al., 1998) so this exposure condition is a conservative model for the extent of human exposure from thermal paper. Marquet et al (2011) also compared percutaneous fluxes *ex vivo* from rat and human frozen dermatomed skin explants and found the human flux to be approximately 10 % of the rat value under identical conditions using the acetone vehicle.

Comment from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- Distinction between unconjugated and conjugated BPA

Weaknesses:

- Single dose level study
- Single acute dose administration
- Test performed in one sex only
- No information on the use of non-PC cages and of non plastic (e.g. glass) water bottles

Mita L, Baldi A, Diano N, Viggiano E, Portaccio M, Nicolucci C, Grumiro L, Menale C, Mita DG, Spugnini EP, Viceconte R, Citro G, Pierantoni R, Sica V, Marino M, Signorile PG and Bianco M, 2012. Differential accumulation of BPA in some tissues of offspring of Balb-C mice exposed to different BPA doses. Environmental Toxicology and Pharmacology, 33, 9-15.

Pregnant adult Balb-C mice were exposed daily to two different doses of BPA by subcutaneous injection (100 µg/kg bw and 1000 µg/kg bw) beginning on gestational day 1 through the seventh day after delivery. The mothers were sacrificed on postpartum day 21, and the offspring were sacrificed at 3 months of age. Control mice were subjected to the same experimental protocol but received saline injections. The liver, muscles, hindbrain and forebrain of the offspring were dissected and processed using HPLC to assess the level of BPA in the tissues and to determine its dependence on the exposure dose and gender. For comparison, the same tissues were dissected from the mothers and analysed. The authors reported that: (1) the level of BPA that accumulated in a given tissue was dependent on the exposure dose; (2) the rank order of BPA accumulation in the various tissues was dependent on the gender of the offspring; (3) the average BPA concentrations in the liver and muscle of the female offspring were higher than in the males; and (4) the average BPA concentration in the central nervous system (i.e. the hindbrain and forebrain) of the male offspring was higher than in the females.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:

Strengths:
- Phytoestrogen-free diet
- Use of non-PC cages and of glass water bottles

Weaknesses:
- Analytical method (HPLC with fluorescence detection)
- No distinction between unconjugated and conjugated BPA

Overall the CEF Panel noted that the method used in the study was HPLC with UV and fluorescence detection. The authors do not give levels of detection and other information on the precision and sensitivity of the method. No mass spectrometry-based analyses have been performed to confirm that the peaks are attributed to BPA. The measurements took place in the mother 14 days after the last administration and in the pups 2 months and 3 weeks after the administration. Given the half-life of BPA in mice of less than 1 hour after PND 21, it is unlikely that the substance which has been measured is BPA.

Nishikawa M, Iwano H, Yanagisawa R, Koike N, Inoue H and Yokota H, 2010. Placental transfer of conjugated bisphenol A and subsequent reactivation in the rat fetus. Environmental Health Perspectives, 118, 1196-203.

Nishikawa et al. (2010) studied the transfer of glucuronidated BPA across the placenta by uterine perfusion in pregnant SD rats (GD 18.5).

The uterus was perfused with a solution containing 2 µM glucuronidated BPA for 20 min, followed by a 70-min perfusion with a solution not containing glucuronidated BPA, after which the perfusate as well as the fetus, amnion, amniotic fluid, placenta, and uterus were collected. HPLC and LC/TOF-MS were used for the determination of glucuronidated and unconjugated BPA. Almost all of the glucuronidated BPA (113 nmol) was detected in the perfusate leaving the uterine vein. Glucuronidated BPA was also detected in the fetus in a small amount (109 pmol, ~0.09% of the total amount) but not in the amnion, amniotic fluid, placenta, and uterus. Moreover, unconjugated BPA was detected to a small amount in the amniotic fluid (31 pmol) and in the fetus (6 pmol)

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strength:
- Measurements of BPA and BPA-gucuronide independently in all compartments of the fetus
- Analytical method (LC/TOF-MS)

Weaknesses:
- Study design not appropriate to the scope (only one time point late in pregnancy (GD 18.5))

Patterson TA, Twaddle NC, Roegge CS, Callicott RJ, Fisher JW and Doerge DR, 2013. Concurrent determination of bisphenol A pharmacokinetics in maternal and fetal rhesus monkeys. Toxicology and Applied Pharmacology, 267, 41–48.

This paper describes measured concentrations of unconjugated and conjugated BPA in the plasma of rhesus dams and in the plasma of fetus after administration of deuterated BPA (100 µg/kg bw) intravenously and orally to two groups of dams. The concentrations of unconjugated and conjugated BPA were determined in the amniotic fluid and in the placenta by liquid chromatography tandem mass spectrometry (LC-MS-MS). The limit of detection (LOD) for deuterated BPA was 0.2 (0.05 ng/ml) in serum and 0.4 pmol/g (0.1 ng/g) in tissue. The kinetics in the dams were similar to the findings in a

previous study (Doerge et al., 2010). In the fetus, plasma concentrations were several fold lower than in the dams and internal exposure as measured by AUC was 0.43 fold of the exposure in dam given BPA by the intravenous route. Concentrations in the dams being about 45 600 pg/ml 5 minutes after dosing declining to below 22.8 pg/ml 24 hours thereafter. In the fetus, the concentration of 22.8 pg/ml is reached already 8 hours after dosing. In amniotic fluid, concentrations of unconjugated BPA were detectable (less than 22.8 pg/ml) but clearly lower than the conjugated BPA. The concentrations of both, conjugated and unconjugated BPA were several fold lower in the amniotic fluid compared to the fetal plasma. In the placenta, unconjugated BPA concentration was 2.7–fold higher than in the plasma of the dams and the fraction of the concentrations of the conjugated BPA was 4.5–fold. The data show that the fetus is exposed to BPA but to a lower extent than the damns. In the fetus, the ratio of the concentrations of conjugated to unconjugated BPA is 3 to 4 in the first half hour and increases with time to a factor of 300. This is due to the fact that the unconjugated BPA concentration declined with a half life of roughly 5 hours whereas the concentrations of conjugated BPA remained constant within the observation period. This finding indicates fetal metabolism. In three fetusses the concentration in brain was measured. Highly variable results indicated that BPA id distributed into the brain but the concentrations are low.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- Oral administration by gavage
- Analytical method (LC-MS-MS)
- Distinction between unconjugated and conjugated BPA

Overall the CEF Panel considered that the study is reliable concerning the experimental design and the results. It is to be noted that the results were obtained after single dose. However, for unconjugated BPA extrapolation to multiple dosing is feasible and indicates that the fetal exposure to BPA is lower than the exposure of the dam with a factor of roughly 0.5 which is in contrast to Nahar et al. 2012. In addition, no indication is given that the placenta is able to de-conjugated conjugated BPA. The concentrations measured in the amniotic fluid are consistent with the concentrations measured in the rat model from the same group of scientists, but in contrast to the study of Edlow et al. (2012) in amniotic fluid collected from pregnant women where 83–91 % of the conjugated plus unconjugated BPA was unconjugated BPA and concentrations were in the ng/ml range at environmental exposure.

Taylor JA, vom Saal FS, Welshons WV, Drury B, Rottinghaus G, Hunt PA, Toutain P-L, Laffont CM and VandeVoort CA, 2011. Similarity of Bisphenol A Pharmacokinetics in Rhesus Monkeys and Mice: Relevance for Human Exposure. Environmental Health Perspective, 119, 422–430.

Taylor et al. (2011) measured unconjugated and conjugated BPA levels in serum from adult female rhesus monkeys and adult female mice after oral administration of BPA and compared findings in mice and monkeys with prior published data in women. Eleven adult female rhesus macaques were fed 400 µg/kg bw deuterated BPA (dBPA) daily for 7 days. Levels of serum dBPA were analyzed by isotope-dilution liquid chromatography–mass spectrometry (LC-MS, 0.2 ng/ml limit of quantitation) over 24 hr on day 1 and on day 7. The same dose of ^{3}H-BPA was fed to adult female CD-1 mice; other female mice were administered ^{3}H-BPA at doses ranging from 2 to 100 000 µg/kg bw (4 doses). In monkeys, the maximum unconjugated serum dBPA concentration of 4 ng/ml was reached 1 hr after feeding and declined to low levels by 24 hr, with no significant bioaccumulation after seven daily doses. Mice and monkeys cleared unconjugated serum BPA at virtually identical rates. A linear (proportional) relationship between administered dose and serum BPA was observed in mice over a 50 000-fold dose range. The authors considered that the study demonstrates that for conjugated BPA, pharmacokinetics in women, female monkeys, and mice is very similar. The authors further claim that based on similarity of plasma conjugated BPA profiles between humans, mice and monkeys, linear dose-plasma level kinetics and similarity of conjugated over non-conjugated BPA plasma level ratios

between monkeys and mice, it must be assumed that also in humans for comparable exposures to BPA comparable plasma levels of unconjugated BPA must be reached. They argue that kinetics in humans, monkeys and mice are not so different that extrapolation from rodents to humans would be inappropriate.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- Analytical method (LC-MS)
- Use of stainless steel (monkeys) and PP (mice) cages and non plastic water bottles

Weaknesses:

- Phytoestrogen content of diet not reported (soy-based diet for mice)

Overall the CEF Panel noted that the ratio of conjugated/unconjugated BPA determined in this study in both mice and monkeys plasma is about 100, which is consistent with the results of other studies. The study also confirms that in order to get unconjugated BPA levels of ca. 2 ng/ml external exposures are needed in the order of 400 µg/kg bw, or for an adult human being ca. 28 mg per person. The use of stable isotope-labeled BPA reassures that no contamination by ubiquitous BPA had occurred.

Vom Saal FS, VandeVoort CA, Taylor JA, Welshons WV, Toutain PL, Hunt PA. Bisphenol A (BPA) pharmacokinetics with daily oral bolus or continuous exposure via silastic capsules in pregnant rhesus monkeys: Relevance for human exposures, 2014. Reproductive Toxicology, 45, 105-16.

In this study, dBPA and its conjugated metabolites were measured in serum in non-pregnant and pregnant female rhesus monkeys, their fetuses and amniotic fluid by a reliable isotope-dilution LC/MS analysis. dBPA was administered daily by as single oral dose injected into a piece of fruit (400 µg/kg bw/d) or by continuous administration by sc implantation of Silastic capsules (release rate not given). A concentration time profile was obtained in the animals orally exposed whereas only one concentration was measured every other day for 12 days in the animals with continuous administration. Concentration time profiles and single concentrations were measured at different time points in pregnancy. The average serum unconjugated dBPA concentrations were below 1 ng/ml. Using the AUC of the IV route reported previously in rhesus monkeys (i.e. 180 ± 76 nM h/L or 41 ng h/mL) for an IV BPA dose of100 µg/kg bw the authors estimated the absolute bioavailability of dBPA by the oral route to be about 7.3% in the non-pregnant animals and about 5% for pregnant animals at GD 50. At GD 100, when pregnancy was terminated by hysterotomy in a subgroup, the observed serum concentrations of unconjugated dBPA in pregnant females were lower relative to pre-pregnancy values. Lower concentrations in fetal serum were measured than in maternal serum, 1 hour after oral administration, and levels in fetal serum were below LOQ (0.2 ng/ml) at 3 hours after oral administration. No parent BPA was detected in the amniotic fluid whereas conjugated BPA was present although in much lower concentration than in the fetus. In the maternal deciduas and in the fetal placenta dBPA was measured 1 hour after oral administration, and levels of parent compound were below LOQ (0.2 ng/ml) in tissues from one animal and at a similar concentration in the tissues form the second animal at 3 hours after oral administration. It is to be noted that the ratio of conjugated BPA-AUC/unconjugated BPA-AUC was several fold higher after oral administration (between 35 and 62) than the ratio for the continuous s.c. administration (between 1 and 4). This is in line with high first pass metabolism. The observed differences in pharmacokinetics of dBPA, that were evident between pre-pregnancy, early and late pregnancy, reflect changes in maternal physiology during pregnancy with a lower absorption rate and higher renal clearance.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- Use of stable isotope-labeled BPA

- Analytical method (LC-MS-MS)
- Distinction between unconjugated and conjugated BPA
- Oral and sc route of administration (enabling to establish steady state concentration after non-oral administration)

Weaknesses:

- Dose in the sc route study not known
- No details on the kinetic given as continous administration does not allow the elucidation

The CEF Panel considered that this well performed study adds to the toxicokinetic knowledge on BPA. It demonstrates that BPA concentration in fetus is low (lower than the maternal concentration) and is not retained in the fetal tissue as well as in placenta. Furthermore, there is only a very low concentration of unchanged BPA in the amniotic fluid which renders study results in humans doubtful which has measured high concentrations of unchanged BPA in amniotic fluid. It confirms that the fetus is able to conjugate BPA (day 50 and older) and excrete the conjugate into the amniotic fluid.
The oral administration was by way of a fruit which was hoarded in the cheek. This would explain the higher bioavailability in this study (7.3% in non-pregnant and 5% in pregnant animals) compared to the findings of the Doerge group (0.94%) as by hoarding the contact time of the BPA with the buccal mucosa is prolonged and for the absorbed amount first pass is circumvented.
The results are plausible and the use of stable isotope-labelled BPA reassures that no contamination by ubiquitous BPA had occurred.

(iii) In vitro studies

Appraisal of strengths and weaknesses and WoE analysis were not carried out for in vitro studies.

Demierre AL, Peter R, Oberli A and Bourqui-Pittet M, 2012. Dermal penetration of bisphenol A in human skin contributes marginally to total exposure. Toxicology Letters, 213, 305-308.

This study is also described and discussed in Appendix D.

The aim of the study was to determine the dermal penetration rate of BPA in human skin by means of an *in vitro* test method according to the OECD Test Guideline 428. The analysis was done under GLP conditions. Full thick skin obtained from two human cadavers was frozen at -20 °C for up to one year. After thawing, 7 skin sections of 200 µm thickness were cut off from the top and used to perform the study. The applied dose was 1.82 µg ^{14}C-BPA/cm^2 skin under non-occluded conditions. After 24 hours after application 8.6% of the applied dose penetrated into the receptor fluid, whereas 34.9 % of the dose was recovered in the stratum corneum, especially in the outer layers. When stripping the skin, 0.6% of the applied dose was in the skin membrane. The authors considered this latter amount together with that present in the receptor fluid as being bioavailable: hence, the total amount bioavailable after application to skin is according to the authors 9.3%.

The CEF Panel considers the study as appropriately performed from a methodological point of view and study reporting fit for purpose. The CEF Panel agrees with the interpretation of the results by the study authors. Given the good quality of the study and the detail of reporting, the study of Demierre et al. (2012) was used as a reference for comparison with the other studies.

Kaddar N, Harthe C, Dechaud H, Mappus E and Pugeat M, 2008. Cutaneous penetration of bisphenol A in pig skin. Journal of Toxicology and Environmental Health-Part a-Current Issues, 71, 471-473.

This study is also described and discussed in Appendix D.

Kaddar and collaborators (2008) analyzed shaved pig skin from the flanks in a static Franz diffusion cell. Physiological serum was used as vehicle, and ^{14}C-BPA was applied in a concentration of 10 mg/l. The applied surface density was not reported. The experiments were carried out at ~32 °C, either for

24 h with repeated sampling in regular intervals (transfer kinetics experiment) or for 2, 5, and 10 h with single sampling (skin distribution experiment). For the skin distribution experiment, six replicates were used per exposure duration. Additional methodical details (e.g., skin thickness, applied surface concentration) were not reported. Analysis of skin distribution after the longest exposure time of 10 h showed that 5.4% and 8.8% of the applied dose to be in the epidermis and dermis, respectively. The transfer kinetics experiment revealed a lag time of ~3 h and a percutaneous penetration of 4.1% after 24 h.

The CEF Panel consider that the study reporting is insufficient due to the omission of several methodical details including the applied surface density (µg/cm^2) and the skin thickness. As for data interpretation, the percutaneous penetration value of 4.1% is in line with the value of 8.6% reported by the high quality study of Demierre et al. (2012).

Marquet F, Payan JP, Beydon D, Wathier L, Grandclaude MC and Ferrari E, 2011. In vivo and ex vivo percutaneous absorption of [14C]-bisphenol A in rats: a possible extrapolation to human absorption? Archives of Toxicology, 85, 1035-1043.

This study is also described in the previous section on animal studies and discussed in Appendix D.

In this study, percutaneous BPA absorption was measured in vivo (see in animal studies) in the rat and ex vivo both in the rat and humans. Marquet et al. (2011) used a static Franz diffusion cell and analyzed viable and non-viable (frozen) human skin from 6 patients undergoing plastic surgery. The skin was dermatomed to a thickness of 500 µm, and the skin integrity was checked by measuring the transepidermal water loss. Acetone was used as vehicle, and ^{14}C-BPA was applied in a high concentration of 4000 mg/l, resulting in a surface density of 200 µg/cm^2. The receptor fluid consisted of cell culture medium with 2% BSA (BPA solubility ≥300 mg/l). The experiments were conducted at 32 ± 1 °C for 24 h, and receptor-fluid samples were taken on regular intervals. Permeation experiments with 15 non-viable human skin sections revealed a recovery of 95.6% and a maximum percutaneous flux of 0.12 µg/cm^2/h occurring at the end of the incubation period at 23.5 h. The quotient of maximum percutaneous flux and vehicle concentration yielded a permeability coefficient of 3.0×10^{-5} cm/h which was 3.7-fold lower than in Demierre et al. (2012) but still comparable given the differences in vehicle type, surface density, and diffusion-cell design. Additional permeation experiments with non-viable rat skin under identical conditions revealed a ~12-fold higher permeability for rat skin compared to human skin. Finally, the authors used viable human and rat skin to estimate the extent of skin metabolism by measuring the BPA metabolites in the receptor fluid after 24 h of exposure. For both human and rat skin, metabolized BPA accounted for ~3% of the permeant.

In summary the authors reported an approximately 12-fold difference in permeability between rat skin and human skin, with permeability being higher in the rat. In addition, inter- and intra-individual variability of up to tenfold was observed in humans. No accumulation of BPA in the skin was found during exposure. The skin clearance rate following exposure was estimated at 0.4 µg/cm^2/h.

Mazur CS, Kenneke JF, Hess-Wilson JK and Lipscomb JC, 2010. Differences between human and rat intestinal and hepatic bisphenol A glucuronidation and the influence of alamethicin on in vitro kinetic measurements. Drug Metabolism and Disposition, 38, 2232-2238.

Native hepatic microsomes were used from rat and from human liver and intestine to study the enzyme kinetics of glucuronidation of BPA. BPA glucuronidation in liver microsomes were sex dependent. Female rat and female human liver microsmomes had a higher V(max) values than that in males. K(m) for glucuronidation was much higher in female rats than in humans and male rats. The dissimilar K(m) measured for female rat together with inhibition studies suggests that different UDP-glucuronosyltransferase (UGT) enzyme(s) are involved in BPA glucuronidation in rats, UGT2B7 and UGT2B15 being candidates. Human intestinal microsomes (mixed gender) showed little BPA glucuronidation activity compared with those from male rat intestine, which in the presence of alamethicin, a membrane-disrupting agent, exhibited a V(max) that was nearly 30-fold higher than that

for mixed human microsomes. The species- and gender-related metabolic differences observed between rat and human liver and intestine provide key information for delineating BPA pharmacokinetics needed for human health risk assessment.

Mazur CS, Marchitti SA, Dimova M, Kenneke JF, Lumen A and Fisher J. (2012) Human and Rat ABC Transporter Efflux of Bisphenol A and Bisphenol A Glucuronide: Interspecies Comparison and Implications for Pharmacokinetic Assessment. Toxicological Sciences, 128, 317-25

Mazur et al. investigated the interaction of BPA and BPA-G with human and ABC transporters: P-glycoprotein (MDR1), multidrug resistance–associated proteins (MRPs), and breast cancer–resistant protein (BCRP) in insect cells transfected with the transporters. As the transport is energy dependent, using ATP as energy source, ATPase activity can be used to detect whether BPA and BPA-G are substrates for the investigated transporters. Based on high ATPase activity, BPA is likely a substrate for rat mdr1b but not for human MDR1 or rat mdr1a. Results indicate that BPA is a potential substrate for rat mrp2 and human MRP2, BCRP, and MRP3. The metabolite BPA-G demonstrated the highest apparent substrate binding affinity for rat mrp2 and human MRP3 but appeared to be a non-substrate or potential inhibitor for human MRP2, MDR1, and BCRP and for rat mdr1a, mdr1b, and bcrp. Comparison of ABC transporter amino acid sequences revealed differences in putative binding site that may explain the observed differences. For BPA transporter activity was shown for transporters facilitating transport into the bile and into the intestinal lumen (MDR1, human; MDR1b, rat; MRP2, human and rat; BCRP, human and rat). In rat, BPA-G is transported by the same transporters, thus facilitating biliary excretion of BPA-G whereas, in human, due to the basolateral location of MRP3, BPA-G would be transported into hepatic vein and into the intestinal veins draining into the portal vein.

Overall the CEF Panel notes that this in vitro study provides further information on the interspecies differences between human and rodent in toxicokinetics of BPA. Technically, the study is considered to be well performed. However, the CEF Panel noted that the lowest concentration tested was 1.95 µM (444.6 ng/ml) and the concentration showing some effect was 10 µM (2 280 ng/ml). Both concentrations are far above human exposure levels of BPA (Cmax 0.024 ng/ml in humans for an oral dose of 1.5 µg/kg bw obtained by simulation; Cmax 0.1 ng/ml in rats for an oral dose of 100 µg/kg bw).

Mørck TJ, Sorda G, Bechi N, Rasmussen BS, Nielsen JB, Ietta F, Rytting E, Mathiesen L, Paulesu L and Knudsen LE, 2010. Placental transport and in vitro effects of Bisphenol A. Reprod Toxicol, 30, 131-137.
This study is also described and discussed in Appendix D.

Mørck et al. (2010) used a static Franz diffusion cell and analyzed non-viable human skin from breast-surgery patients according to the OECD TG 428. Full thickness skin (800–1000 µm) was used, and the skin integrity was checked by capacitance measurements. A diluted ethanol solution was used as vehicle, and ^{14}C-BPA was applied in a high concentration of 4000 mg/l, resulting in a surface density of 259 µg/cm^2. The receptor fluid consisted of physiological saline solution containing 5% BSA. The experiments were carried out at ~32 °C for 48 h, and receptor-fluid samples were taken in regular time intervals. Experiments with 11 skin sections after 48 h incubation showed a percutaneous penetration 13.0%, a skin deposition of 24.6%, and a recovery of 82.1%. A more detailed analysis of skin deposition showed 7.4% and 17.2% of the applied dose to be in the epidermis and dermis, respectively. Percutaneous penetration was 13.0%.

Trdan Lušin T, Roškar R and Mrhar A, 2012. Evaluation of bisphenol A glucuronidation according to UGT1A1*28 polymorphism by a new LC-MS/MS assay. Toxicology, 292, 33-41.

The glucuronidation of BPA in adult human microsomal preparations was studied with a sensitive analytical method using labeled BPA in LC-MS/MS, which enabled simultaneous determination of aglycone and conjugated BPA. The study was performed in microsomes prepared from liver, kidneys, intestines and lungs. No BPA-glucuronidation could be determined in human lung microsomes. In liver, kidneys and intestines, the microsomal intrinsic clearances were 950, 40 and 24 μL × min^{-1}/mg microsomal protein, corresponding to full tissue intrinsic clearances of 857, 8 and 2 ml × min^{-1}/kg bw, after scaling-up of the microsomal data to full organ weight.

While the liver intrinsic clearance was very high (857 ml min^{-1} kg body $weight^{-1}$), the tissue intrinsic clearances for the kidney and intestine were less than 1 % of liver intrinsic clearance. Since BPA is a UGT1A1 substrate, we postulated that the common UGT1A1*28 polymorphism influences BPA glucuronidation, and consequently, BPA detoxification. Hepatic tissue intrinsic clearances for UGT1A1*1/*1, UGT1A1*1/*28, and UGT1A1*28/*28 microsomes were 1113, 1075, and 284 ml min^{-1} kg body $weight^{-1}$, respectively. The in vitro results show that the liver is the main site of BPA glucuronidation (Km 8.9 μM, Vmax 8.5 nmol min^{-1} mg^{-1}) and BPA metabolism may be significantly influenced by a person's genotype (Km 10.0–13.1 μM, Vmax 3.4–16.2 nmol min^{-1} mg^{-1}).

These authors also investigated the influence of a polymorphism of human UGT1A1 on the metabolism of BPA. Although this is not the most active form of UGT to contribute to the glucuronidation of BPA (which is UGT2B15), it still has significant capacity. For genotyped microsomes containing only wild-type UGT1A1*1, an intrinsic clearance of 1240 μL × min^{-1}/mg microsomal protein was found and for UGT1A1*1/*28 (heterozygous) an intrinsic clearance of 1190 μL × min^{-1}/mg microsomal protein. However, for the homozygous UGT1A1*28/*28, the intrinsic clearance was only 320 μL × min^{-1}/mg microsomal protein. There were no differences in Km values for the two allelic variants studied. Thus for the three different genotypes intrinsic tissue clearances of 1113, 1075 and 284 ml × min^{-1}/kg bw were calculated. The authors reasoned that this polymorphism of UGT1A1 may have toxicological consequences, since the glucuronidation capacity of the liver may be strongly reduced in UGT1A1*28 homozygous individuals.

Polymorphisms have been described for the enzymes relevant for the conjugation of BPA. Since BPA conjugation can be carried out by several enzymes, a single polymorphism in one gene, resulting in a reduced or loss of enzymatic activity of the respective gene product (i.e. the functional enzyme) is not anticipated to result in immediate major changes in the plasma levels of aglycone BPA.

Zalko D, Jacques C, Duplan H, Bruel S and Perdu E, 2011. Viable skin efficiently absorbs and metabolizes bisphenol A. Chemosphere, 82, 424-430.

This study is also described and discussed in Appendix D.

Zalko et al. (2011) examined the diffusion and metabolism of BPA using viable human skin explants from the abdominal region of female donors. The skin was dermatomed to a thickness of 500 μm and then seeded in cell culture inserts, where the explants were maintained at the air/liquid interface with dermal/epidermal feeding by diffusion of nutrients from the culture medium (1.5 ml) across the insert. Ethanol/phosphate buffer 0.1 M pH 7.4 (1:2, v/v) was used as vehicle, and ^{14}C-BPA was applied in a surface density of 2.75 μg/cm^2. The experiments were carried out at 37 °C, and culture media were collected at 24, 48, and 72 h. Experiments with 3 skin sections after 72 h incubation showed a percutaneous penetration 45.6%, a skin deposition of 41.5%, a residual amount of 2.5 % on the skin surface, and a recovery 92.6%.

Additional experiments with viable human skin and pig ear skin were carried out to analyze the extent of skin metabolism. Major skin metabolites were BPA mono-glucuronide and BPA mono-sulfate,

which were reported to account for 73% and 27% of the dose in porcine an human skin after 72 h of incubation (percentages are unclear).

The CEF Panel noted several methodical flaws in the first experimental phase, e.g., use of cell culture inserts as diffusion cells, missing skin integrity check, exposure times largely exceeding 24 h, 33% ethanol solution as vehicle, which negatively impact the reliability of these estimates for *in vitro* skin absorption.

Again, the transferability of these results to the *in vivo* situation in humans is highly questionable. First, there was almost a complete depletion of the permeant on the skin surface. Second, the concentrations of BPA equivalents in the culture medium (i.e. the receptor compartment) reached values well above 1 µM, which is not really the "sink" condition prevailing *in vivo* with serum concentrations for BPA equivalents being generally far below 10 nM. As a consequence, there was no longer a directional transport of the permeant from the donor compartment to the receptor compartment, and a re-uptake of BPA from the culture medium with subsequent metabolization in the skin cannot be excluded. Ignoring these methodical flaws would lead to an overestimation of the extent of *in vivo* skin metabolization.

(iv) PBPK modelling

Appraisal of strengths and weaknesses and WoE analysis were not carried out for these PBPK modelling studies. Also given the very limited number of studies on this topic, the time period for the literature search was extended to earlier than 2010.

Edginton AN and Ritter L, 2009. Predicting plasma concentrations of bisphenol A in children younger than 2 years of age after typical feeding schedules, using a physiologically based toxicokinetic model. Environmental Health Perspectives, 117, 645-52.

The age dependence of the toxicokinetics of BPA and its glucuronidated metabolite, BPA-Glu, has been evaluated using a coupled BPA–BPA-Glu physiologically based toxicokinetic (PBTK) model. Using information on the concentration-time profile and urinary excretion of the main metabolite BPA-Glu gathered from the study in adult humans by Völkel et al. (2005) clearance has been modeled by optimization procedures. Based on age dependence of physiologic parameters relevant for absorption, distribution, metabolism, and excretion the kinetic of BPA was age-adjusted. In the model the metabolism of BPA was modeled with a single metabolite, namely BPA-Glu and metabolism to BPA-sulfate has not been taken into consideration. The average steady-state BPA plasma concentration in newborns has been simulated to be 11 times greater than that in adults when given the same weight-normalized dose mainly due to reduced metabolic clearance mirroring the lower expression of the enzyme relevant for glucuronidation, namely UTG 2B 15, in the newborn. Because of the rapid development of the glucuronidation process, this ratio dropped to 2 by 3 months of age. Because of uncertainty in on the hepatic BPA intrinsic clearance, these values represent preliminary estimates. The model predicts a Cmax-value of 50 nM after an oral dose of 5 mg (in the study of Völkel et al., 2002 between 54.3 and 87.7 µg/kg bw).

Comments from the CEF Panel:
This modelling approach is based on a commercially available modelling tool with a complex structural model. The physiological parameters for organ weights and blood flows are taken from available literature. The metabolism of BPA was modeled with a single metabolite, namely BPA-glu and metabolism to BPA-sulfate has not been taken into consideration as the metabolic clearance of BPA is obtained by an optimisation procedure for describing the time course of BPA-Glu with data from the study by Völkel et al. (2002). For the situation in adults, the influence of this fact is not important as in the adult – at least in persons with an un-impaired glucuronidation capacity - the sulfate pathway does contribute the metabolic clearance to only roughly 10%. For the situation in subjects with impaired glucuronidation, i.e. the newborn sulfate metabolism plays an important role.

Hence, the prediction for the adult with un-impaired glucuronidation capacity but nor for the newborn is acceptable.

Fisher JW, Twaddle NC, Vanlandingham M and Doerge DR, 2011. Pharmacokinetic modeling: Prediction and evaluation of route dependent dosimetry of bisphenol A in monkeys with extrapolation to humans. Toxicology and Applied Pharmacology, 257, 122-136.

A physiologically based pharmacokinetic (PBPK) model was developed for BPA using data from intravenous (iv) and oral bolus doses of 100 µg d6-BPA/kg (Doerge et al., 2010b) in adult rhesus monkeys. This calibrated PBPK adult monkey model for BPA was then evaluated against published monkey kinetic studies with BPA. Using two versions of the adult monkey model based on monkey BPA kinetic data from Doerge et al. (2010) and Taylor et al. (2011), the unconjugated BPA pharmacokinetics were simulated for human oral ingestion of 5 mg d16-BPA per person (Völkel et al., 2002). Völkel et al. were unable to detect the unconjugated BPA in plasma, but were able to detect BPA metabolites. These human model predictions of the unconjugated BPA in plasma were then compared to previously published PBPK model predictions obtained by simulating the Völkel et al. kinetic study. The BPA human models as developed here, using two parameter sets reflecting the two adult monkey studies, both predicted lower unconjugated levels in human serum than the previous human BPA PBPK model predictions. BPA was metabolized at all ages of monkey (PND 5 to adult) by the gut wall and liver. However, the hepatic metabolism of BPA and systemic clearance of its phase II metabolites appear to be slower in younger monkeys than adults. The authors concluded that use of the current non-human primate BPA model parameters provides more confidence in predicting the unconjugated BPA in serum levels in humans after oral ingestion of BPA. The authors further commented that, based on their models, unconjugated BPA may be present in humans at a level of 8.8 nM (~ 2 ng/ml).

The study by Fisher et al may be used to predict BPA and BPA-conjugates plasma levels in humans. The model comprises 7-compartments for unconjugated BPA (covering 5 compartments important for BPA kinetics and 2 compartments representative for target tissues for toxicity) and 1 compartment for BPA-conjugates. The model performance in humans could only be tested for the BPA conjugates, since no adequate kinetics data on unconjugated BPA in humans are available. The model predicted an elimination of > 90 % of an oral dose of 5 mg/person via urine within 12 hrs post dosing. The models also predicted at least 2 orders of magnitude difference in BPA conjugate plasma concentration vs. unconjugated BPA plasma concentration. Unconjugated BPA levels were also slightly less than predicted in previous models in literature (Mielke and Gundert-Remy, 2009; Egington and Ritter, 2009), which is in line with the data from Teeguarden et al (2011).

Mielke H, Gundert-Remy U, 2009. Bisphenol A levels in blood depend on age and exposure. Toxicological Letters, 190, 32-40.

Two approaches are presented to estimate blood concentrations of Bisphenol A (BPA). Simple kinetic principles were applied to calculate steady state plasma concentrations (Css) using the formula Css = f x dose/ ke x VD. F is the fraction absorbed; ke is the first order elimination constant which can be calculated by ke =ln 2/half-life and VD is the volume of distribution. F was taken from the study of Völkel et al. (2005); half-life from data by Tsukioka et al. (2004) (Tsukioka, T., Terasawa, J., Sato, S., Hatayama, Y., Makino, T., Nakazawa, H., 2004. Development of analytical method for determining trace amounts of BPA in urine samples and estimation of exposure to BPA. J. Environ. Chem. 14, 57–63); VD from published data by Sun et al. (2002) (Sun, Y., Nakashima, M.N., Takahashi, M., Kuroda, N., Nakashima, K., 2002. Determination of bisphenol A in rat brain by microdialysis and column switching high-performance liquid chromatography with fluorescence detection. Biomed.Chromatogr. 16, 319–326.),A physiologically based model was used to simulate the blood concentration time profile in several age groups exploring the influence of not yet fully developed metabolic capacity on the blood concentrations in the newborn. The structural model consists of 7 compartments and metabolism is modeled on two pathways glucuronidation and sulfation. The physiological data were

obtained from published sources (Abraham et al. 2005). Metabolism to BPA-G was modeled by upscaling in vitro data on K_M and V_{max} obtained in human hepatozytes and to BPA-S by relating activity to the percentage metabolised. The simple kinetic model gave concordant results with the more elaborated model. The modelling results are in agreement with experimental results [Völkel, W., Colnot, T., Csanady, G.A., Filser, J.G., Dekant, W., 2002. Metabolism and kinetics of bisphenol A in humans at low doses following oral administration. Chem. Res. Toxicol. 15, 1281–1287]. The predictions also agree with published results obtained with a different physiologically based model. According to model simulations, BPA is present in the blood of the normal population at concentrations several orders of magnitude lower than most measurements reported in the literature. At the same external exposure level, the newborn is predicted to have 3 times greater blood concentration than the adult. This is due to the not yet fully developed glucuronidation activity in the newborn, not fully compensated by the unimpaired sulfation pathway. The model predicts a Cmax-value of 50 nM after an oral dose of 5 mg (in the study of Völkel et al., 2002 between 54.3 and 87.7 µg/kg bw).

Comments from the CEF Panel:
As the metabolism of BPA is the main influencing factor for the kinetics the prediction of the model depends critically on the in vitro metabolism data and on its upscaling. The model only describes the kinetics of BPA and does not include the kinetics of BPA-G. The validity of the model could have been increased if it had been extended to BPA-G using available BPA-G experimental data.

Teeguarden JG, Waechter JM Jr, Clewell HJ 3rd, Covington TR and Barton HA, 2005. Evaluation of oral and intravenous route pharmacokinetics, plasma protein binding, and uterine tissue dose metrics of bisphenol A: a physiologically based pharmacokinetic approach. Toxicological Sciences, 85, 823-38.

A physiologically based pharmacokinetic (PBPK) model of BPA pharmacokinetics was developed for rats and for humans. A uterine tissue compartment was included to allow the correlation of simulated estrogen receptor (ER) binding of BPA with increases in uterine wet weight (UWW) in rats. Intravenous- and oral-route blood concentration-time course of BPA and its main metabolite, BPA-glucuronide, were well described by the model. By scaling up the relevant data from rat to human, oral-route plasma and urinary elimination kinetics of BPA-glucuronide (BPA-G) in humans were covered by the model. The model parameters of metabolism were optimised using the data of Völkel et al. 2002. The predicted the concentration time course of BPA-GComparison of metabolic clearance rates derived from fitting rat i.v. and oral-route data implied that intestinal glucuronidation of BPA is significant. In rats, but not humans, terminal elimination rates were strongly influenced by enterohepatic recirculation. The model predicts a Cmax-value of 10 nM after an oral dose of 5 mg (in the study between 54.3 and 87.7 µg/kg bw).

Because of the differences between rat and human concerning biliary excretion and entero-hepatic recirculation of BPA/BPA-G the structural model has some weaknesses. More recent and more appropriate models are now available.

Yang X, Doerge DR and Fisher JW, 2013. Prediction and evaluation of route dependent dosimetry of BPA in rats at different life stages using a physiologically based pharmacokinetic model. Toxicology and Applied Pharmacology, 270, 45-59.

In this study time a physiologically based pharmacokinetic (PBPK) model was developed for in neonatal and adult rats to quantitatively evaluate age-dependent pharmacokinetics of BPA and its phase II metabolites. The PBPK model was calibrated in adult rats using studies on BPA metabolism and excretion in the liver and gastrointestinal tract, and pharmacokinetic data with BPA in adult rats. For immature rats the hepatic and gastrointestinal metabolism of BPA was inferred from studies on the maturation of phase II enzymes coupled with serum time course data in pups. The calibrated model predicted the measured serum concentrations of BPA and BPA conjugates after administration of 100 µg/kg of d6-BPA in adult rats (oral gavage and intravenous administration) and postnatal days 3, 10,

and 21 pups (oral gavage). The observed age-dependent BPA serum concentrations were partially attributed to the immature metabolic capacity of pups. A comparison of the dosimetry of BPA across immature rats and monkeys suggests that dose adjustments would be necessary to extrapolate toxicity studies from neonatal rats to infant humans.

Reproductive and Developmental effects

(i) Human studies

BPA effects on adult reproduction

Bloom MS, vom Saal FS, Kim D, Taylor JA, Lamb JD and Fujimoto VY, 2011a. Serum unconjugated bisphenol A concentrations in men may influence embryo quality indicators during in vitro fertilization. Environmental Toxicology and Pharmacology, 32, 319-323.

This study was a cross-sectional analysis of a subsample of a prospective cohort study of metals and assisted reproductive technologies (SMART). In the current study the authors studied the associations between serum unconjugated BPA concentrations and indicators of embryo quality in 27 couples undergoing *in vitro* fertilization. Unconjugated BPA was collected according to established procedures and measured by HPLC with electrochemical detection (limit of detection, LOD 0.3 ng/ml). The measures used as indicators of embryo quality were: embryo cell number (ECN) and embryo fragmentation score (EFS). The models were adjusted for female and male unconjugated BPA, age, ethnicity and day of embryo transfer for ECN. The authors reported that inverse associations were suggested for male BPA with ECN (OR=0.70, p=0.069) and EFS (OR=0.54, p=0.009), but not for women. Although the study is suggestive of a negative influence of male BPA exposure on human embryo quality the authors acknowledged that the limited sample size and scope of the study make the results preliminary.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- Quality control, including blanks

Weaknesses:

- Cross-sectional design
- Small sample size
- Serum BPA measurement (invalid exposure measurement)
- Single exposure measurements
- Confounding by diet or by concurring exposure factors (contamination through medical treatment during IVF) not considered
- Generalisability to the overall population

Overall, the CEF Panel considers that this study has main limitations, e.g. the cross sectional design, the small sample size and the use of serum BPA, which is not considered a valid measure of BPA exposure. Furthermore, as the study is part of an on-going study of metals, this confounding factor should have been taken into account. Potential contamination through medical treatment in IVF is likely to occur. A considerable number of BPA values in male (48%) were below the LOD.

This paper is included in the WoE table because of its relevance to one or more review questions addressed there.

Bloom MS, Kim D, Vom Saal FS, Taylor JA, Cheng G, Lamb JD and Fujimoto VY, 2011b. Bisphenol A exposure reduces the estradiol response to gonadotropin stimulation during in vitro fertilization. Fertility and Sterility, 96, 672-677.

The authors examined whether serum unconjugated BPA was associated with follicular response to exogenous ovarian stimulation in a cross sectional study of a small group of 44 women undergoing IVF treatment. Serum unconjugated BPA was determined by HPLC with electrochemical detection (LOD 0.3 ng/ml). The main outcome measures were peak 17β-oestradiol (E2) concentrations, E2 normalized for mature-sized follicles at the time of the hCG trigger and the number of oocytes retrieved during IVF (=antral follicle count, AFC).

The results showed an inverse association between serum unconjugated BPA and E2 concentration and E2 normalized for mature sized follicles, but no association between unconjugated BPA and ovarian reserve variables. The results were indicative of a negative association and warrant further studies.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- Quality control, including blanks

Weaknesses:

- Cross-sectional design
- Small sample size
- Serum BPA measurement (invalid exposure measurement)
- Single exposure measurements
- Confounding by diet or by concurring exposure factors (contamination through medical treatment during IVF) not considered
- Generalisability to the overall population

Overall, the CEF Panel considers that the study has main limitations, e.g. the cross sectional design, the small sample size limiting the statistical power of the study and the use of serum BPA, which is not considered a valid measure of BPA exposure. In addition, potential contamination through medical treatment in IVF is likely to occur. The CEF Panel also noted that this study used the same BPA exposure data as the study by Fujimoto et al. (2010), addressing different outcome measures (Fujimoto et al., 2010). Concerning the results, BPA correlations were far weaker than physiological correlations (see Supplemental Figure 2). Given these overall limitations, this preliminary study is not considered as informative on BPA toxicity.

This paper is included in the WoE table because of its relevance to one or more review questions addressed there.

Buttke DE, Sircar K and Martin C, 2012. Exposures to endocrine-disrupting chemicals and age of menarche in adolescent girls in NHANES (2003-2008). Environmental Health Perspectives 120, 1613-1618.

The study examined the relationship between urinary endocrine-disrupting chemical concentrations (parabenes, BPA, triclosan, benzophenones, and dochlorophenols) and the age of menarche in adolescent girls. The study sample included female participants 12-16 years of age who had completed the reproductive health questionnaire and laboratory examination for the Centers for Disease Control and Prevention's National Health and Nutrition Examination Survey (NHANES) for years 2003-2008 (2005-2008 for analyses of phthalates and parabens). Urinary BPA was measured by by on line solid phase extraction (SPE) coupled to liquid chromatography isotope dilution tandem mass spectrometry (LC-MS-MS, LOD 0.40 ng/ml). Exposures were assessed based on creatinine-corrected natural log

urine concentrations of selected environmental chemicals and metabolites found in at least 75% of samples in the study sample. The analysis of urinary total BPA and age of menarche included n=441. Body mass index, family income-to-poverty ratio, race/ethnicity, mother's smoking status during pregnancy and birth weight were evaluated as potential confounders. The weighted mean age of menarche was 12.0 years of age. The geometric mean urinary total BPA concentration was 2.25 µg/g creatinine. Accounting for BMI and race/ethnicity, 2,5-dichlorophenol (2,5-DCP) and summed environmental phenols (2,5-DCP and 2,4-DCP) were inversely associated with age of menarche [hazard ratios of 1.10; 95% confidence interval (CI): 1.01, 1.19 and 1.09; 95% CI: 1.01, 1.19, respectively]. Other exposures (total parabens, bisphenol A, triclosan, benzophenone-3, total phthalates, and 2,4-DCP) were not significantly associated with age of menarche. Hazard ratio for BPA was 0.94 (95% CI: 0.80, 1.10).

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- Analytical method (SPE LC-MS-MS)
- Quality control, including blanks and quality assurance procedures

Weaknesses:

- Cross-sectional design
- Single exposure measurements
- Single spot urine BPA measurement
- Confounding by diet not considered

Overall, the CEF Panel notes that this study evaluated simultaneous exposure to several endocrine-disrupting chemicals in relation to age of menarche in adolescent girls. Urinary total BPA was not associated with age of menarche. Relevant confounders were evaluated, but no dietary variables were included. The cross-sectional design limits the interpretation of the results.

This paper is included in the WoE table because of its relevance to one or more review questions addressed there.

Ehrlich S, Williams PL, Missmer SA, Flaws JA, Berry KF, Calafat AM, Ye X, Petrozza JC, Wright D and Hauser R, 2012a. Urinary Bisphenol A Concentrations and Implantation Failure among Women Undergoing *In Vitro* Fertilization. Environmental Health Perspectives, 120, 978-983.

In a study in Boston, USA, 137 women undergoing 180 IVF cycles were studied to investigate the potential link between BPA and reproductive outcomes early in the IVF process. In this detailed study 1 or 2 spot urine samples, the timing of which were determined by clinic visits rather than biological hypothesis, were analysed for BPA by on line solid phase extraction (SPE) coupled to isotopic dilution liquid chromatography tandem mass spectrometry (LC-MS-MS, LOD 0.4 µg/l), yielding results similar to those seen in many other studies. Data were analysed for confounders, covariates and used a BPA quantiles approach. Broadly, there was an increased odds of implantation failure with higher quartiles of urinary BPA quartiles: 1.02, 1.60, 2.11 for quartiles 2, 3 and 4 respectively compared to quartile 1 and a suggestive positive linear dose response association with implantation failure (p-trend 0.06). .

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- Prospective design
- Urine, container specified (PP cups)
- Repeated measurements (≤ 2)

- Analytical method (SPE LC-MS-MS)
- Quality control, including blanks

Weaknesses:

- Short time frame (only days for exposure measurements per treatment cycle)
- Single spot urine BPA measurements
- Confounding by diet or by concurring exposure factors (contamination through medical treatment during IVF) not considered
- Generalisability to the overall population

Overall, the CEF Panel considers that this study is well-performed and is suggestive that higher level of total BPA is associated with reduced ovarian response in women. However, the generalisability of the results is uncertain for the population other than IVF couples. Also women undergoing IVF are likely to be exposed to BPA from medical plastics during an IVF cycle. In addition, the presence of female factor infertility may be associated with ovarian abnormalities affecting sensitivity to exogenous chemicals.

This paper is included in the WoE table because of its relevance to one or more review questions addressed there.

Ehrlich S, Williams PL, Missmer SA, Flaws JA, Ye X, Calafat AM, Petrozza JC, Wright D and Hauser R, 2012b. Urinary bisphenol A concentrations and early reproductive health outcomes among women undergoing IVF. Human Reproduction, 27, 3583-3592.

Associations between urinary BPA concentrations and early reproductive outcomes were studied among 174 women aged 18-45 years undergoing 237 IVF cycles at a fertility center in Boston, USA. The study was a follow up of Bloom et al. (2011b), who previously reported an association between urinary BPA and decreased ovarian response (peak serum oestradiol (E2) and oocyte count at the time of retrieval) in women undergoing IVF. Total urinary BPA (after enzymatic hydrolysis) was determined by on line solid phase extraction (SPE) coupled to isotopic dilution liquid chromatography tandem mass spectrometry (LC-MS-MS, LOD 0.4 µg/l). The early reproductive outcomes examined were measures of ovarian response: oocyte maturation (metaphase II), fertilization, embryo quality and cleavage rate. Correlation among the multiple IVF cycles in the same woman were used for generalised estimating equations. The geometric mean (SD) for urinary BPA concentrations was 1.50 (2.22) µg/l. After adjustment for age and other potential confounders (Day 3 serum FSH, smoking, BMI), there was a linear dose-response association between increased urinary BPA concentrations and decreased number of oocytes (overall and mature), decreased number of normally fertilized oocytes and decreased E(2) levels (mean decreases of 40, 253 and 471 pg/ml for urinary BPA quartiles 2, 3 and 4, when compared with the lowest quartile, respectively; p-value for trend=0.001). The mean number of oocytes and normally fertilized oocytes decreased by 24 and 27%, respectively, for the highest versus the lowest quartile of urinary BPA (trend test $p<0.001$ and 0.002, respectively). Women with urinary BPA above the lowest quartile had decreased blastocyst formation (trend test P-value=0.08). The results from this extended study, using IVF as a model to study early reproductive health outcomes in humans, indicate a negative dose-response association between urinary BPA concentrations and serum peak E2 and oocyte yield.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- Prospective design
- Urine, container specified (PP cups)
- Repeated measurements (≤ 2)
- Analytical method (SPE LC-MS-MS)
- Quality control, including blanks

- Multiple outcome assessment

Weaknesses:

- Short time frame (only days for exposure measurements per treatment cycle)
- Single spot urine BPA measurements
- Confounding by diet or by concurring exposure factors (contamination through medical treatment during IVF) not considered
- Generalisability to the overall population

Overall, the CEF Panel considers that the scientific soundness of the study was acceptable. According to the authors themselves, potential limitations include exposure misclassification due to the very short half-life of BPA and its high variability over time and uncertainty about the generalisability of the results to the general population of women conceiving naturally and limited sample. In addition, the CEF Panel notes that no dietary confounders were considered. The significance of the new scientific information is limited to the portion of population that is infertile, not necessarily representing the general population.

This paper is included in the WOE table because of its relevance to one or more review questions addressed there.

Fujimoto VY, Kim D, Vom Saal FS, Lamb JD, Taylor JA and Bloom MS, 2011. Serum unconjugated bisphenol A concentrations in women may adversely influence oocyte quality during in vitro fertilization. Fertility and Sterility, 95, 1816-1819.

The association between BPA serum levels and oocyte quality during *in vitro* fertilization were investigated. The study sample comprised 31 women undergoing intracytoplasmic sperm injection (ICSI) and 26 couples undergoing ICSI or conventional IVF. Serum unconjugated BPA measured in fasting blood specimen were obtained from women on the day of oocyte retrieval, and from non-fasting blood specimen in men. Unconjugated BPA in serum was measured by HPLC with electrochemical detection (LOD 0.3 ng/ml). Median serum unconjugated BPA concentrations were 2.53 ng/ml in women and 0.34 ng/ml in men. Oocyte maturity was defined as number of oocytes in metaphase II divided by number of oocytes collected. Oocyte fertilization was defined as the proportion of oocytes fertilized. Multiple statistical analyses revealed no association between BPA and oocyte maturation in the whole population, but an inverse association was observed in nine Asian women separately. However, in all women an inverse association between unconjugated serum BPA and normal fertilization was reported, indicating that BPA exposure in female patients may interfere with oocyte quality during IVF.

Comments from the CEF Panel:

The CEF Panel identified the following strengths/weaknesses in the study:

Strengths:

- Quality control, including blanks

Weaknesses:

- Cross-sectional design
- Small sample size
- Serum BPA measurement (invalid exposure measurement)
- Single exposure measurements
- Confounding by diet or by concurring exposure factors (contamination through medical treatment during IVF) not considered
- Generalisability to the overall population

Overall, the CEF Panel considers that that the study has many limitations, e.g. the cross sectional design, the limited sample size and the use of serum BPA, which is not considered a valid measure of

BPA exposure. The CEF Panel noted that the study population (and serum BPA measurements) is the same as used in the studies by Bloom et al. (2011a and 2011b). Moreover, important factors, such as the particular situation of in vitro fertilization and what it includes, were not considered. For example, the quality of oocytes may be affected by hormonal therapy which precedes fertilization. Potential contamination through medical treatment in IVF is also likely to occur. Given the above limitations, the significance of the results of this preliminary study is doubtful.

This paper is included in the WoE table because of its relevance to one or more review questions addressed there.

Galloway T, Cipelli R, Guralnick J, Ferrucci L, Bandinelli S, Corsi AM, Money C, McCormack P and Melzer D, 2010. Daily bisphenol A excretion and associations with sex hormone concentrations: results from the InCHIANTI adult population study. Environmental Health Perspectives, 118, 1603-1608.

This paper was the first to report human exposure to BPA in a large-scale European population. The study comprised 715 adults aged 20 through 74 years in a suburban and rural town population in Italy (the InCHIANTI adult population study). Participants each collected one 24-hour urine sample and total BPA concentration was measured in the 24-h sample (unconjugated plus conjugated) by on line solid phase extraction (SPE) coupled to isotopic dilution liquid chromatography tandem mass spectrometry (LC-MS-MS, LOD <0.5 µg/l, LOQ 0.5 µg/l). The BPA collection and analysis was appropriate. Fasting blood samples were drawn and the outcomes examined were sex-hormones: 17β-oestradiol, total testosterone, sex hormone binding globulin (SHBG) and free testosterone. Models were adjusted for age, study site, smoking, BMI, weight, waist, and urinary creatinine concentration. Other potential confounders were also evaluated. A weak association between urinary BPA and testosterone were found in men, in models adjusted for age and study site (p=0.044), and in models additionally adjusted for smoking, measures of obesity, and urinary creatinine concentrations (β=0.046; 95% CI, 0.015-0.076; p=0.004). No associations were found for other serum hormone measures and no associations were found for the primary outcomes among women. However, a statistically significant association between BPA and SHBG concentrations was found for the 60 premenopausal women (p=0.004). The authors concluded that higher BPA exposure may be associated with endocrine changes in men.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- Large sample size
- Standardised samples (24-h urine collection)
- Analytical method (SPE LC-MS-MS)
- Quality control, including blanks and quality assurance procedures

Weaknesses:

- Cross-sectional design
- Single exposure measurements
- Handling of values below LOQ not reported
- Confounding by concurring exposure factors (concomitant drug treatment) not considered
- Unclear clinical relevance (small effect size in men)
- Inconsistency in the results (significant association between BPA exposure and testosterone but no association with other hormones)

Overall, the CEF Panel considers that the 24-hour urine collection is a better measure of BPA exposure than single spot urine samples and covers to some extent the same time period as the time covered by the blood sampled for hormone concentrations. The association with testosterone was

weak and the clinical relevance of association is not clear. Concomitant drug treatment was not reported.

This paper is included in the WoE table because of its relevance to one or more review questions addressed there.

Kandaraki E, Chatzigeorgiou A, Livadas S, Palioura E, Economou F, Koutsilieris M, Palimeri S, Panidis D and Diamanti-Kandarakis E, 2011. Endocrine Disruptors and Polycystic Ovary Syndrome (PCOS): Elevated Serum Levels of Bisphenol A in Women with PCOS. The Journal of Clinical Endocrinology and Metabolism, 96, 480-484.

Serum BPA was measured in women with polycystic ovarian syndrome (PCOS) and in healthy controls in Greece. Associations between BPA and hormonal/metabolic parameters were examined in women with PCOS (n=71) and healthy controls (n=100), matched by age and body mass index in a University Hospital setting in Greece. The outcome measures were: anthropometric, hormonal, metabolic parameters. BPA levels measured by immunoassay (ELISA, measurement range 0.30-100 ng/ml) were significantly higher in the total PCOS group compared with the controls (1.05±0.56 vs. 0.72±0.37 ng/ml, $p<0.001$). PCOS women, lean (PCOS-L) and overweight (PCOS-OW), had higher BPA levels compared to the corresponding control group lean (C-L) and overweight (C-OW): (PCOS-L=1.13±0.63 vs. C-L=0.70±0.36, $p<0.001$) (PCOS-OW=0.96±0.46 vs. C-OW=0.72±0.39, $p<0.05$). A significant association of testosterone ($r=0.192$, $p<0.05$) and androstenedione ($r=0.257$, $p<0.05$) with BPA was observed. Multiple regression analysis for BPA showed significant correlation with the existence of PCOS ($r=0.497$, $p<0.05$). BPA was also positively correlated with insulin resistance (Matsuda index) in the PCOS group ($r=0.273$, $p<0.05$). The fact that the association between BPA and PCOS remained when women where stratified for BMI strengthened the finding of higher serum BPA in with PCOS in normal ovulating non hyperandrogenemic controls.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- None

Weaknesses:

- Cross-sectional design
- Serum BPA measurement (invalid exposure measurement)
- Single exposure measurements
- Analytical method (ELISA)
- No quality control (e.g., blanks) and quality assurance procedures
- No distinction between unconjugated and conjugated BPA
- Handling of values outside measurement range not reported
- Confounding by diet and concurring exposure factors not considered
- Generalisability to the overall population (other than women with PCOS)

Overall, the CEF Panel considers that the sample size is quite limited, and although the authors reported that cases and controls were matched for age and BMI, no data were presented. Furthermore, serum BPA was measured using a commercial ELISA kit, which does not distinguish between conjugated and unconjugated BPA. In blood, only unconjugated BPA can be considered a valid measure of BPA exposure. The results should be considered as preliminary and need to be confirmed.

This paper is included in the WoE table because of its relevance to one or more review questions addressed there.

Li DK, Zhou Z, Miao M, He Y, Wang J, Ferber J, Herrinton LJ, Gao E and Yuan W, 2011. Urine bisphenol-A (BPA) level in relation to semen quality. Fertility and Sterility, 95, 625-630.

The aim of the study was to study the association between urinary BPA and semen quality in 218 men in China with and without BPA exposure in the workplace. Of 888 men invited only 514 (58%) participated in the study and adequate semen specimens were obtained from 236 men. Valid semen and urine samples were available from 218 men. Total urinary BPA was determined after hydrolysis by HPLC with fluorescence detection (LOD, 0.31 µg/l). For men with occupational exposure to BPA, two urine samples were collected at the workplace, one sample pre-shift and one post-shift. For men without occupational exposure only one urine sample was collected. Semen quality was determined using six common semen quality parameters: volume, total sperm count, concentration, vitality, motility (forward movement [grades A + B]), and morphology. After adjustment for potential confounders using linear regression, increasing urine BPA level was statistically significantly associated with (i) decreased sperm concentration, (ii) decreased total sperm count, (iii) decreased sperm vitality, and (iv) decreased sperm motility. Compared with men who did not have detectable urine BPA levels, those with detectable urine BPA had more than three times the risk of a lowered sperm concentration and lower sperm vitality, more than four times the risk of lower sperm count, and more than twice the risk of lower sperm motility. Urinary BPA levels were not associated with semen volume or abnormal sperm morphology. The association was noted both in men highly exposed to BPA (at the workplace) and men environmentally exposed to lower doses of BPA.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- Repeated measurements (n=2, workers)
- Standardised samples (pre-shift and post-shift spot urine samples)

Weaknesses:

- Cross-sectional studydesign
- Selection bias of the study population (58% participation rate, without explanation)
- Single exposure measurements (for men without occupational exposure)
- Single spot urine BPA measurement
- No quality control (e.g., blanks) and quality assurance procedures
- No distinction between unconjugated and conjugated BPA
- Confounding by diet not considered
- Occupational exposure (via inhalation)

Overall, the CEF Panel considers that the measurements of sperm quality involved only 218 individuals. Of 888 men who were invited, only 58% (514) participated in the study, without their reasons being known (fertility problem, age, etc.), which may constitute a selection bias. BPA occupational exposure mainly occurs via inhalation route, which is of limited value for the general population, orally exposed to lower doses of BPA. Another limitation when considering occupational exposure to BPA is the potential concurrent exposure to other chemicals and heavy metals (evaluated by interview). The cross-sectional design of the study limits the relevance of the results and no casual inference can be drawn.

This paper is included in the WoE table because of its relevance to one or more review questions addressed there.

Tarantino G, Valentino R, Di Somma C, D'Esposito V, Passaretti F, Pizza G, Brancato V, Orio F, Formisano P, Colao A and Savastano S, 2013. Bisphenol A in Polycystic Ovary Syndrome and its Association with Liver-Spleen Axis. Clinical Endocrinology, 78, 447-453.

In a cross-sectional analysis comprising cases and controls, the authors examined whether serum BPA levels (measured by immunoassay, ELISA, measurement range 0.30-100 ng/ml) were associated with low-grade chronic inflammation, hepatic steatosis, and hyperandrogenism in women with Polycystic Ovary Syndrome (PCOS) and healthy controls in Naples, Italy. Cases (40 lean and overweight/obese premenopausal women with PCOS) and controls (20 healthy age-matched women) were enrolled in the years 2009 to 2011 at the Federico II University Hospital in Naples. Higher BPA levels in PCOS women were associated with higher grades of insulin resistance, hepatic steatosis, FAI, and inflammation, spleen size showed the best correlation (β=0.379, p=0.007). The main finding of this study was the association between serum BPA levels and hepatic steatosis and the markers of low-grade inflammation in women with PCOS, in particular with spleen size, thus unravelling the presence of the liver-spleen axis in this syndrome.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- None

Weaknesses:

- Cross-sectional design
- Small sample size
- Serum BPA measurement (invalid exposure measurement)
- Single exposure measurements
- Analytical method (ELISA)
- No quality control (e.g., blanks) and quality assurance procedures
- No distinction between unconjugated and conjugated BPA
- Handling of values outside measurement range not reported
- Confounding by diet and concurring exposure factors not considered
- Statistics (unjustified use of non-parametric and parametric models)
- Generalisability to the overall population (other than women with PCOS)

Overall the CEF Panel notes that the study has major limitations, i.e. the statistical power of the study is low, the logic to establish causality between variables is unclear and the use of statistics raises concerns. The authors predominantly used univariate statistics and when they used a multivariate model, BPA was the outcome, not the predictor. The CEF Panel notes several other concerns: (1) controls differed from PCOS patients with regard to several anthropometric parameters (e.g. BMI), therefore authors cannot say that the disease increased the levels of BPA; (2) the method of detection of BPA in serum (an ELISA kit with low specificity) is not acceptable; furthermore, measures in urinary samples would be more adequate from an epidemiological point of view; (3) correlations were non-parametric, but the best-fit straight line was reported. Moreover, the multiple regression model is parametric, and authors do not justify their choices. Overall, the general soundness of the manuscript is quite poor.

This paper is included in the WoE table because of its relevance to one or more review questions addressed there.

Zhou Q, Miao M, Ran M, Ding L, Bai L, Wu T, Yuan W, Gao E, Wang J, Li G and Li DK, 2013. Serum bisphenol-A concentration and sex hormone levels in men. Fertility and Sterility, 100, 478-482.

A cross-sectional study evaluated the association between serum BPA and sex hormone levels in 290 male factory workers in China. The participants comprised 137 workers in a petrochemical factory who were exposed to BPA at the workplace for more than 6 months, and 153 age-matched workers from a tap water factory without occupational exposure to BPA. Blood specimens were collected into EDTA tubes, and serum was stored at -80 °C until analysis. Serum BPA was measured by HPLC with fluorescence detection (LOD 0.39 µg/l) after enzymatic hydrolysis. Serum total testosterone, oestradiol, inhibin B, follicle stimulating hormone, prolactin, sex hormone binding protein, androstenedione and free testosterone were measured. The free androgen index (FAI) was calculated as Tx100/sex hormone binding globulin. The median serum BPA concentrations in exposed and unexposed workers were 3.198 and 0.276 µg/l, respectively. After adjustment for potential confounders using linear regression, increasing serum BPA concentration was statistically significantly associated with decreased androstenedione levels, decreased free testosterone levels, decreased free androgen index, and increased sex hormone-binding globulin levels. Comparison of hormone levels between workers exposed and unexposed to BPA showed similar associations.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- Quality control, including blanks

Weaknesses:

- Cross-sectional design
- Small sample size
- Serum BPA measurement (invalid exposure measurement)
- Single exposure measurements
- No distinction between un conjugated and conjugated BPA
- Confounding by diet or by concurring exposure factors (other than occupational exposures) not considered
- Occupational exposure (via inhalation)

Overall, the CEF Panel notes that this study has main limitations, e.g. the cross-sectional design, the relatively small sample size and the use of serum BPA, which is not considered a valid measure of BPA exposure. BPA occupational exposure mainly occurs via inhalation route, which is of limited value for the general population, orally exposed to lower doses of BPA. The study did not evaluate other occupational exposures or diet.

This paper is included in the WoE table because of its relevance to one or more review questions addressed there.

BPA effects on gestational/birth outcomes

Cantonwine D, Meeker JD, Hu H, Sánchez BN, Lamadrid-Figueroa H, Mercado-García A, Fortenberry GZ, Calafat AM and Téllez-Rojo MM, 2010. Bisphenol A exposure in Mexico City and risk of prematurity: a pilot nested case control study. Environmental Health, 9, 62.

The aim of this study was to examine urinary BPA concentrations during the last trimester of pregnancy and the risk of preterm delivery among a small subset (case-control design) of 60 women participating in a pregnancy cohort in Mexico City. Morning spot urine samples (second morning void) were collected for 518 non-smoking cohort participants. Of these, 30 cases were selected among participants who delivered prior to or during gestational week 37 and 30 controls were selected among participants with 38 or more completed gestational weeks. Total urinary BPA (free plus conjugated species) was determined by on line solid phase extraction (SPE) coupled to liquid chromatography tandem mass spectrometry (LC-MS-MS, LOD 0.4 ng/ml) at the Centers for Disease Control and Prevention (CDC). Among the cases, 12 delivered preterm (<37 weeks) and 18 in week 37. BPA was

detected in 80.0% (n=48) of the urine samples; total concentrations ranged from <0.4 µg/L to 6.7 µg/L; uncorrected geometric mean was 1.52 µg/L. The adjusted odds ratio of delivering less than or equal to 37 weeks in relation to specific gravity adjusted third trimester BPA concentration was 1.91 (95% CI 0.93, 3.91, p=0.08). When cases were further restricted to births occurring prior to the 37th week (n=12), the odds ratio for specific-gravity adjusted BPA was larger and statistically significant (p<0.05).

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- Standardised samples (second morning spot samples)
- Analytical method (SPE LC-MS-MS)
- Quality controls, including blanks and quality assurance procedures

Weaknesses:

- Cross-sectional design
- Small sample size
- Single exposure measurements
- Single spot urine BPA measurement
- Invalid/imprecise outcome assessment
- Confounding by diet and concurring exposure factors not considered

Overall the CEF Panel notes that this study is limited by the small sample size and single spot urine samples. The association did only reach statistical significance when urinary BPA was adjusted for specific gravity and when cases were restricted to delivery prior to week 37. An additional weakness was that gestational length was estimated by date of maternally-recalled last menstrual period, which is an unreliable measure. It should be noted that the urinary BPA concentrations in the pregnant women in Mexico City were similar to urinary concentrations in the US and other developed countries. The results suggested that urinary BPA were higher in women who delivered less than or equal to 37 weeks of gestation, but due to study limitations the results can only be regarded as preliminary.

This paper is included in the WoE table because of its relevance to one or more review questions addressed there.

Chevrier J, Gunier R, Bradman A, Holland NT, Calafat AM, Eskenazi B and Harley KG, 2012. Maternal Urinary Bisphenol A during Pregnancy and Maternal and Neonatal Thyroid Function in the CHAMACOS Study. Environmental Health Perspectives, 121, 138-144.

This study evaluated whether exposure to BPA during pregnancy was related to thyroid hormone levels in pregnant women and neonates. Spot urine samples for measuring BPA was collected during the first and second half of pregnancy in 476 women participating in the CHAMACOS study in the agricultural Salinas Valley California (immigrant Mexican-American population), USA. Total urinary BPA (free plus conjugated species) was determined by on line solid phase extraction (SPE) coupled to liquid chromatography tandem mass spectrometry (LC-MS-MS, LOD 0.4 µg/l) at the Centers for Disease Control and Prevention (CDC) in Atlanta. The outcomes were: free thyroxine (T4), total T4 and thyroid-stimulating hormone (TSH) were measured during pregnancy and TSH was measured in neonates. The association between the average of the two BPA measurements and maternal thyroid hormone levels was not statistically significant. Of the two BPA measurements, only the measurement taken closest in time to the TH measurement was significantly associated with a reduction in total T4 (β=-0.13 µg/dL per log2 unit; 95% CI=-0.25, 0.00). The average of the maternal BPA concentrations was associated with reduced TSH in boys (-9.9% per log2 unit; 95% CI=-15.9%, -3.5%) but not in girls. Among boys, the relation was stronger when BPA was measured in the third trimester of pregnancy and decreased with time between BPA and TH measurements. The results suggest that

exposure to BPA during pregnancy was related to reduced total T4 in pregnant women and decreased TSH in male neonates.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- Prospective design
- Urine, container specified (BPA-free urine cups)
- Repeated measurements (n=2)
- Analytical method (LC-MS-MS)
- Quality controls, including blanks and quality assurance procedures

Weaknesses:

- Single spot urine BPA measurement
- Confounding by diet (except iodine) and concurring exposure factors not considered
- Unclear clinical relevance (association between BPA and T4 only observed in urine samples taken during the second half of pregnancy)
- Generalisability to the overall population (low-income Mexican American population)

Overall the CEF Panel notes that this study considered many relevant confounders, including iodine nutrition, but no other contaminants were considered. The study population is likely to be exposed to other chemicals in the agricultural Salinas Valley. Spot urine samples from participants were collected during the first (12.4±3.8 weeks gestation) and second half (26.2±2.2 weeks gestation) of pregnancy. The authors did not include the time between the last BPA measurement and birth in their multivariate analysis. Although statistical significant associations were reported, the clinical relevance of the findings is not clear. The authors reported that almost all values of maternal and neonatal TH levels were not pathological (only 1 abnormal value of TSH among neonates). No measure of BPA in the urine of neonates was taken. The association between maternal BPA and total T4 was stronger when measured closer together relative to further apart in time, suggesting a transient effect of BPA or alternatively, a developmental window of susceptibility. The prospective design of the study strengthens the finding. Others strengths are the fair sample size, accurate statistical analysis and a general sound discussion. In particular, the use of urinary BPA as continuous variable after normalization for creatinine is an important merit. Results were a bit overestimated, in particular the association between total T4 and urinary BPA in pregnant women. No measure of BPA in the urine of neonates was considered, and the study is not conclusive. However, although associations were weak, the clinical relevance of the study may be a cause of concern.

This paper is included in the WoE table because of its relevance to one or more review questions addressed there.

Choi H, Kim J, Im Y, Lee S and Kim Y, 2012. The association between some endocrine disruptors and hypospadias in biological samples. Journal of Environmental Science and Health, Part A: Toxic Hazardous Substances and Environmental Engineering, 47, 2173-2179.

The levels of endocrine disruptors in the urine and plasma of control (n=80), patient (n=80) and patient' mother (n=40) groups were measured and assayed in this study. The selected target compounds were five phthalates (DEHP, DBP, MEHP, MBP and PA), 2 alkylphenols (n-NP and t-OP) and BPA, determined by gas chromatography-mass spectrometry (GC-MS, range given for LOD and LOQ in urine and plasma) after enzymatic hydrolysis. The mean urinary total BPA was 19.8 ng/ml in controls, 51.6 ng/ml in hypospadias and 5.31 ng/ml in mothers. The mean plasma BPA was 2.62 ng/ml in controls, 18.3 ng/ml in hypospadias and 9.04 ng/ml in mothers. Urinary BPA in children was not associated with hypospadias, whereas plasma BPA was higher in children with hypospadias than in controls (p= 7.22e-10). No relationship was seen for levels of BPA in urine or plasma of the mothers and hypospadias.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:
- Urine, container specified (glass)
- Analytical method (GC-MS)

Weaknesses:
- Plasma BPA measurement (invalid exposure measurement)
- Single exposure measurements
- Single spot urine BPA measurement
- No quality control and quality assurance procedures
- No distinction between unconjugated and conjugated BPA
- Confounding by diet or by concurring exposure factors not reported
- Insufficient study reporting (number of patients not clearly reported in the tables)
- Statistics (use correlations instead of odd ratios)
- Unreliable outcome (extremely high urinary BPA concentrations)

Overall, the CEF Panel notes that this study has several limitations: (i) the limited description of sampling, (ii) the use of serum BPA which is not considered a valid measure of BPA exposure, (iii) the lack of distinction between unconjugated and conjugated BPA in plasma, (iv) the extremely high urinary BPA concentrations, and (v) the statistical handling and reporting. Concerning statistics, correlations were reported, whereas odds ratios (hypospadias yes/no) would have been more appropriate. Diagnosis of hypospadias and the numbers of patients reported in the tables was not clearly defined, and if all are included it should say so. There is no detail on handling of possible confounding factors. The lack of associations in this study is undisputable subject to above limitations.

This paper is included in the WoE table because of its relevance to one or more review questions addressed there.

Chou WC, Chen JL, Lin CF, Chen YC, Shih FC and Chuang CY, 2011. Biomonitoring of bisphenol A concentrations in maternal and umbilical cord blood in regard to birth outcomes and adipokine expression: a birth cohort study in Taiwan. Environmental Health, 10, 94.

This was a cross-sectional study which analysed BPA in maternal and umbilical cord blood samples in 97 mother-newborn pairs in a birth cohort in Taiwan, and its association with birth outcomes. Unconjugated BPA was determined by HPLC with UV detection (LOD 0.13 ng/ml). The outcomes examined were: (i) low birth weight (LBW) defined as birth weight <2600 g, (ii) small for gestational age (SGA) defined as birth weight <10th percentile, compared with the birth weight distribution in the same gestational week and gender in Taiwan, (iii) high leptin (HLP-9 defined as leptin>90th percentile in cord blood, and (iv) low adiponectin (LAD) defined as adiponectin <10th percentile. Geometric mean BPA was 2.51 ng/ml in maternal blood and 1.06 ng/ml in umbilical cord blood. In male neonates only, high maternal BPA (upper quartile) was associated with increased risk of low birth weight babies, small for gestational age babies (SGA) and adverse action of leptin and adiponectin.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:
- Container specified (plastic-free)
- Quality control, including blanks and quality assurance procedures

Weaknesses:
- Cross-sectional design
- Blood/plasma and cord blood BPA measurement (invalid exposure measurement)
- Single exposure measurements

- Confounding by diet and concurring exposure factors not considered
- Inconsistent results amongst different studies

Overall, the CEF Panel notes that this study has several methodological limitations, including the cross-sectional design and the excessive categorization of continuous variables. Serum/plasma and cord blood BPA measurements cannot be considered a valid measure of BPA exposure. The CEF Panel also notes that results are not consistent in abstract and paper. The results regarding maternal serum BPA and adverse birth outcomes were weak and can only be regarded as preliminary results. Very little separates groups characterised by high and low BPA exposure, and the groups were small. The results are only interesting for comparing values for maternal/cord pairs. The association with SGA is not convincing.

This paper is included in the WoE table because of its relevance to one or more review questions addressed there.

Fénichel P, Déchaux H, Harthe C, Gal J, Ferrari P, Pacini P, Wagner-Mahler K, Pugeat M and Brucker-Davis F, 2012. Unconjugated bisphenol A cord blood levels in boys with descended or undescended testes. Human Reproduction, 27, 983-990.

The authors tested whether the concentration of unconjugated BPA measured in cord blood differed between male infants with undescended testis (UDT, n=46) and matched controls (n=106). In addition, the authors examined associations between unconjugated BPA and 11 steroid and polypeptide hormones in the same cord blood samples (e.g. testosterone and inhibin B) and between cord blood unconjugated BPA and a range of xenobiotics measured in maternal milk. Blood was collected into BPA free tubes from the umbilical cord following placental expulsion. Samples were checked for non-contamination. Unconjugated BPA was measured using a radioimmunoassay (RIA). There were no differences in unconjugated BPA or any of the hormones between the cryptorchid (UTD) (mean: 1.12 ng/ml) and control boys (mean: 1.26 ng/ml). Unconjugated cord blood BPA was considered as a continuous variable. The levels are in line with other studies. In addition to the unconjugated BPA measurements by RIA (LOD 0.8 ng/ml), GC-MS measurement was obtained for a subsample of the blood samples, and the correlation between the methods were reported (r=0.85).

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- Container specified (BPA-free)
- Quality control, including blanks
- Consistency in results among different studies

Weaknesses:

- Case-control study
- Cord blood BPA measurement (invalid exposure assessment)
- Single exposure measurements
- Analytical method (RIA, no correlation with GC-MS data for values in the low range)
- Confounding by diet or by concurring exposure factors not reported

Overall, the CEF Panel notes that this study is well powered for the cryptorchid group. The use of radioimmunoassay (RIA) for measuring unconjugated BPA is not appropriate and the correlation with the additional BPA measured by GC-MS in a subsample is not convincing for values in the low range of BPA concentrations. Many variable parameters were compared in the 2 groups, which were not separated by maternal or neonatal characteristics. The main finding was that unconjugated cord blood BPA at term does not explain cryptorchidism. The statistical modelling was appropriate.

This paper is included in the WoE table because of its relevance to one or more review questions addressed there.

Jung H, Hong Y, Lee D, Pang K and Kim Y, 2013. The association between some endocrine disruptors in human plasma and the occurrence of congenital hypothyroidism. Environmental Toxicology and Pharmacology, 35, 278-283.

This study investigated the association between plasma concentrations of endocrine disruptors (including BPA) and the occurrence of congenital hypothyroidism in a case-control study in 59 mother-infant pairs in South Korea. They determined the plasma levels in infants with congenital hypothyroidism (n=39) and normal infants (n=20) of the following target compounds: two alkylphenolic compounds, bisphenol A, five phthalates, and three isoflavones. Plasma BPA was determined by was determined by gas chromatography-mass spectrometry (GC–MS, LOD 0.18 ng/ml, LOQ 0.60 ng/ml) after enzymatic hydrolysis. There was no difference in plasma BPA concentrations in patients (mean 2.93±4.14 ng/ml) and controls (mean 4.06±7.86 ng/ml), p=0.2201.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- Analytical method (GC-MS)

Weaknesses:

- Case-control study
- Plasma BPA measurement (invalid exposure measurement)
- Single exposure measurements
- No quality control and quality assurance procedures
- No distinction between unconjugated and conjugated BPA
- Confounding by diet not considered
- Statistics (parametric tests applied to variables not normally distributed)

Overall, the CEF Panel notes that this study determined BPA in plasma, which is not considered a valid measure of BPA exposure. PPlasma BPA concentration did not differ between cases and controls. Although plasma BPA concentrations in patient and controls were not normally distributed, differences between the two groups were assessed by parametric tests.

This paper is included in the WoE table because of its relevance to one or more review questions addressed there.

Lee BE, Park H, Hong YC, Ha M, Kim Y, Chang N, Kim BN, Kim YJ, Yu SD and Ha EH, 2013a. Prenatal bisphenol A and birth outcomes: MOCEH (Mothers and Children's Environmental Health) study. International Journal of Hygiene and Environmental Health.

A study in 757 mother-child pairs in Korea examined the relationship between prenatal BPA exposure and birth outcomes, including birth weight, birth length, and ponderal index considering gender difference. Participants comprised pregnant women participating in a multi-center birth cohort study, Mothers and Children's Environmental Health (MOCEH), which was established in Korea in 2006. Total urinary BPA was measured after hydrolysis by liquid chromatography tandem mass spectrometry (LOD 0.12-0.28 ng/ml). Women were included who had their urinary BPA level measured during the third trimester, as well as information on birth outcome, prior medical history, psychosocial status, health behaviour, environmental exposure as well as socio-demographic characteristics. Furthermore, urinary BPA concentrations were also measured in the first trimester in a subsample of the study populations (number not presented). Regression analysis was performed to assess the effect of BPA on birth outcome. No associations were found for urinary BPA measured during early pregnancy and birth outcomes. For late pregnancy, the geometric mean concentration of

BPA was 1.29 µg/l (1.87 µg/g creatinine) during late pregnancy. Contrary to a number of other studies, urinary BPA concentrations were higher in women with a higher income level. In unadjusted analysis, the correlation between urinary BPA and birth weight was r=0.06, p=0.08, and the correlation with ponderal index was r=0.11, p=0.003. In adjusted analysis, the second tertile of maternal BPA exposure was associated with an increase in birth weight, relative to the first tertile (p=0.04). This relationship was more pronounced in male neonates. Furthermore, prenatal exposure to BPA was associated with an increase of ponderal index in the all neonates and especially in female neonates: all neonates, second vs first BPA tertile was borderline significant (p=0.07) and third vs first BPA tertile was significantly associated (p=0.02). In female neonates, BPA association with ponderal index was significant for second vs first tertile (p=0.003), but not for third vs first tertile (p=0.22).

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- Prospective design
- Analytical method (LC-MS-MS)
- Quality control, including blanks

Weaknesses:

- Single exposure measurements
- Single spot urine BPA measurement
- No distinction between unconjugated and conjugated BPA
- Confounding by diet and concurring exposure factors not reported
- Inconsistent results amongst different studies

Overall, the CEF Panel notes that this multi-center birth cohort study showed weak indications that prenatal BPA exposure (maternal urinary BPA concentrations in third trimester) was associated with increased birth weight and ponderal index, but the associations differed by sex and was generally stronger for the second vs the first tertile of BPA exposure than for the third vs the first tertile. The study took into consideration a wide range of potential confounding factors. The authors state that nutritional and environmental factors were evaluated, but it is not clear how this was handled. Furthermore, the study is limited by single spot urine samples in late pregnancy, which may attenuate potential associations. Gestational length was estimated by date of maternally-recalled last menstrual period, which is an unreliable measure. The gender differences regarding prenatal BPA exposure and fetal growth measures are potentially interesting but more studies are needed to elaborate prenatal BPA exposure and fetal growth.

This paper is included in the WoE table because of its relevance to one or more review questions addressed there.

Miao M, Yuan W, He Y, Zhou Z, Wang J, Gao E, Li G, Li DK, 2011b. In utero exposure to bisphenol-A and anogenital distance of male offspring. Birth Defects Research. Part A, Clinical and Molecular Teratology, 91, 867-872.

In utero exposure to BPA modelled according to 6 categories of paternal or maternal occupational or non-occupational exposure were examined in relation to anogenital distance (AGD) in boys (n=153). BPA exposure was assessed combining air sample monitoring at the workplace, employment history, and change in work environment. BPA exposure was divided into 6 categories according to paternal or maternal occupational or non-occupational exposure. Urinary BPA (measured by HPLC with fluorescence detection, LOD 0.31 ng/ml) was assessed to verify the validity of classification of BPA exposure. The results showed that higher BPA exposure was associated with reduced AGD in boys. This study is the first epidemiological evidence that parental exposure to BPA in the workplace during pregnancy may adversely affect male genital development.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- Container specified (BPA-free)

Weaknesses:

- Case-control study
- Small sample size
- Invalid/imprecise BPA exposure assessment (air monitoring; combination of paternal and maternal exposure)
- Single exposure measurements
- Single spot urine BPA measurement
- No quality control and quality assurance procedures
- No distinction between unconjugated and conjugated BPA
- Confounding by diet or by concurring exposure factors not considered
- Statistics (too many categories)
- Occupational exposure (via inhalation)

Overall, the CEF Panel notes that there is uncertainty related to BPA exposure association. BPA occupational exposure mainly occurs via inhalation route, which is of limited value for the general population, orally exposed to lower doses of BPA. In addition, it is not clear how paternal occupational exposure was transmitted to pregnant wife. Although the sample size is relatively small, BPA exposure was divided into six groups (too many categories). Occupational exposure to BPA may coincide with exposure also to other chemicals related to occupational exposure of factory workers. The value of AGD as a masculinization index is becoming more apparent, especially where studies to link to semen parameters would need to last at least 18 years. Samples size was very small and the value of the time weighted average (TWA) is not that clear. The analysis was interesting but needs to be expanded with BPA quantitation in maternal samples collected during pregnancy.

This paper is included in the WoE table because of its relevance to one or more review questions addressed there.

Miao M, Yuan W, Zhu G, He X and Li DK, 2011a. In Utero Exposure to Bisphenol-A and its Effect on Birth Weight of Offspring. Reproductive Toxicology, 32, 64-68.

Retrospective recall of BPA occupationally exposed and unexposed couples and birth weight of the offspring in China. BPA exposure was assessed by combining work place air sample monitoring, employment history, and change in work environment. Of 587 children, 93 came from families in which mother was occupationally exposed to BPA, 50 came from families in which the father was occupationally exposed (father exposure was considered as indirect exposure to mother) and 444 came from families without occupational exposure to BPA. Birth weight and gestational lengths, as well as maternal height, weight and smoking habits were obtained by an in-person interview of the mother. Parental BPA exposure level during the index pregnancy was determined through personal air sampling measurements and exposure history. Urinary BPA (measured by HPLC with fluorescence detection, LOD 0.31 ng/ml) was assessed to verify the validity of classification of BPA exposure. Current urinary BPA analyses confirmed that urine BPA levels showed a gradient reduction from exposed women (direct fetal exposure) to spouses of exposed male workers (indirect fetal exposure through paternal exposure) to unexposed women. The geometric mean (95% CI) of maternal current urine BPA was 15.98 (9.11–28.02), 2.22 (1.49–3.31) and 0.56 (0.70–0.88) μg/g creatinine in currently exposed mothers, spouses of exposed fathers and unexposed mothers (including unexposed mothers and spouses of unexposed fathers), respectively. After controlling for maternal age at birth, maternal weight before pregnancy, calendar year of birth, maternal education, family income and gravidity, parental exposure to BPA in the workplace during pregnancy was correlated with decreased birth

weight in offspring: compared with offspring from the families without parental BPA exposure in the workplace. Birth weight of offspring with paternal BPA exposure was 90.75 g less on average (p=0.10), and 168.40 g less for those with maternal BPA exposure (p=0.02). The association remained largely the same when analyses were restricted to term births. Likewise, reduced birth weight with increasing BPA exposure was found when the exposure was modelled as an 8 hour weighted time average.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- Prospective design
- Container specified (BPA-free)

Weaknesses:

- Long recall period (up to 16 years)
- Invalid/imprecise BPA exposure assessment (air monitoring; combination of paternal and maternal exposure)
- Single exposure measurements
- Single spot urine BPA measurement
- No quality control and quality assurance procedures
- No distinction between unconjugated and conjugated BPA
- Confounding by diet or by concurring exposure factors not considered
- Inconsistent results amongst different studies
- Occupational exposure (via inhalation)

Overall, the CEF Panel notes that this study claims to provide human epidemiological evidence that parental exposure to BPA in the workplace during pregnancy may be associated with decreased birth weight in the offspring. However, the relationship was weak and the biological significance of the observed decrease is not clear. Furthermore, occupational exposure to BPA may coincide with exposure also to other chemicals related to occupational exposure of factory workers, which may also influence fetal growth. The consideration of paternal exposure as indirect exposure of the mother is not clear and the authors also acknowledged that misclassification derived from recall error could not be ruled out, as the long recall period was up to 16 years. The study is limited by a small sample size in the exposed group. Due to the retrospective nature of the study, a weighted time average, rather than maternal urine BPA level, was used to classify the exposure dosage during the index pregnancy. Further, inaccurate classification of BPA exposure categories could be caused by exposure to BPA from consumer products and sources not considered in the study. The analysis was interesting but needs to be expanded with BPA quantitation in maternal samples collected during pregnancy.

This paper is included in the WoE table because of its relevance to one or more review questions addressed there.

Philippat C, Mortamais M, Chevrier C, Petit C, Calafat AM, Ye X, Silva MJ, Brambilla C, Pin I, Charles MA, Cordier S and Slama R, 2012. Exposure to Phthalates and Phenols during Pregnancy and Offspring Size at Birth. Environmental health Perspectives, 120, 464-470.

Exposure to 11 phthalates and nine phenols, including BPA, was examined in relation to offspring size at birth in a case-control study of male malformations of the genitalia nested in two French pregnancy cohorts. For phenols, data was available for 191 mother-child pairs, comprising 48 cases and 143 controls. Cases and controls were combined into one study group, with a reweighting approach to correct for overrepresentation of congenital abnormalities. Phthalate- and phenol concentrations were measured in maternal spot urine samples collected at various gestational ages and times of day. Total urinary BPA (free plus conjugated species) was determined by on line solid phase extraction (SPE)

coupled to liquid chromatography tandem mass spectrometry (LC-MS-MS, LOD 0.4 ng/ml) at the Centers for Disease Control and Prevention (CDC) in Atlanta. BPA concentrations were positively associated with head circumference, which increased by 0.3 cm (95% CI: 0.0, 0.7) in association with a 1-unit increase in ln-transformed BPA concentration. When BPA was ranked into tertiles, the increase in head circumference was 0.8 cm in the highest BPA concentration tertile compared with the lowest tertile [95% confidence interval (CI): 0.2, 1.3]. No association was seen for birth length. There was no significant trend for either a monotonic or a non-monotonic association with birth weight, however, the association for birth weight suggested an inverse U-shape association: birth weight increased by 169 g (95% CI: 14, 324) in the second BPA concentration tertile and by 85 g (95% CI: –62, 233) in the third concentration tertile, compared with the first. For the other phenols, birth weight decreased by 77 g (95% CI: –129, –25) and by 49 g (95% CI: –86, –13) in association with a 1-unit increase in ln-transformed 2,4-dichlorophenol (DCP) and 2,5-DCP urinary concentrations, respectively. Benzophenone-3 (BP3) ln-transformed concentrations were positively associated with weight (26 g; 95% CI: –2, 54) and head circumference at birth (0.1 cm; 95% CI: 0.0, 0.2). For phthalate metabolites there was no evidence of associations with birth weight.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- Analytical method (LC-MS-MS)
- Quality control, including blanks and quality assurance procedures

Weaknesses:

- Case-control study design
- Single exposure measurements
- Single spot urine BPA measurement
- Confounding by diet not considered
- Unclear clinical relevance (small effect size)
- Inconsistent results amongst different studies

Overall, the CEF Panel notes that this is a case-control study nested in two French pregnancy cohorts. As also pointed out by the authors, the BPA concentrations were relatively low in the study group, enhancing the analytical uncertainties and hence the potential for exposure misclassification. Other limitations include the choice of study group and small sample size, statistical handling and use of single spot urine samples. Finally, the clinical relevance of the association between BPA exposure and increased head circumference is uncertain. The fairly small numbers (again the issue of how long it takes to collect reasonable numbers of human genital malformations must be considered) and case/control matching are not supportive. The observed BPA effect is not convincing.

This paper is included in the WoE table because of its relevance to one or more review questions addressed there.

Snijder CA, Heederik D, Pierik FH, Hofman A, Jaddoe VW, Koch HM, Longnecker MP and Burdorf A, 2013. Fetal Growth and Prenatal Exposure to Bisphenol A: The Generation R Study Environmental Health Perspectives, 121, 393-398.

This study was embedded in a Dutch, population based cohort study and aimed to investigate the relation of prenatal BPA exposure with intrauterine growth and to evaluate the effect of the number of measurements per subject on observed associations. Spot urine was sampled in early, mid and late pregnancy and total (unconjugated and conjugated) BPA was measured by liquid chromatography tandem mass spectrometry (LC-MS-MS) at two different laboratories (LOD, 0.26 and 0.05 ng/ml, respectively). Ninty-nine had one measurement, 40 had two measurements and 80 had three measurements of urinary BPA. Median BPA ranged from 1.1 to1.9 ng/ml. BPA concentrations were adjusted for creatinine, and examined by quartiles and as a continuous variable. Linear regression

models for repeated measurements of both BPA and fetal growth were used to estimate associations between urinary concentrations of creatinine based BPA (BPA_{CB}) and intrauterine growth. Two outcomes were examined: 1) the SD score of fetal weight per gestational week and 2) the SD score for fetal head circumference per gestational week. The results showed that the relationship between BPA_{CB} and fetal growth was sensitive to the number of BPA measurements per woman. Among 80 women with three BPA measurements, women with BPA_{CB} >4.22 µg/g creatinine (highest quartile) had lower growth rates for fetal weight and head circumference than women with BPA_{CB} <1.54 µg/g creatinine (lowest quartile), with estimated differences in mean values at birth of –683 grams (20.3% of mean) and –3.9 cm (11.5% of mean), respectively. When fewer measurements were available per woman, the exposure-response relationship became progressively attenuated and statistically non-significant.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- Prospective design
- Container specified (BPA-free)
- Repeated measurements (3)
- Analytical method (LC-MS-MS)
- Quality control, including blanks and quality assurance procedures
- Repeated growth measurements

Weaknesses:

- Single spot urine BPA measurements
- Confounding by diet not considered
- Inconsistent results amongst different studies

Overall, the CEF Panel notes that this prospective study is a well-conducted investigation that used repeated measures of BPA exposure and objectively measured outcomes. The statistical modelling is sound and BPA was modelled both by quartiles and on the continuous scale. The study demonstrated that using three measures of BPA (mean of 3 spot urine samples) resulted in more precise effect estimates (narrower confidence intervals) than using fewer BPA measurements, and highlights the importance of including repeated urinary samples for assessment of BPA exposure. Many confounders were considered, but no dietary variables other than alcohol were included.

This paper is included in the WoE table because of its relevance to one or more review questions addressed there.

(ii) Animal studies

Adewale HB, Jefferson WN, Newbold RR, Patisaul HB, 2009. Neonatal Bisphenol-A exposure alters rat reproductive development and ovarian morphology without impairing activation of gonadotrophin-releasing hormone neurons. Biology of Reproduction, 81, 690-699.

Long Evans rats received sc injections of vehicle (10% EtOH and 90% sesame oil) or BPA (50 µg/kg bw per day or 50 mg/kg bw per day [HED pups, 15.5, 15,500 mg BPA/kg bw per day) daily from PND 0 to PND 3. Oestradiol benzoate (EB, 25 µg) and the estrogen receptor alpha (ESR1) agonist (PPT, 1 mg/kg bw per day) were used as positive and negative controls respectively. Cross-fostering, with litters limited to 12 pups/dam (10 dams in all). Upon weaning, the pups were checked daily for day of vaginal opening. The low dose of BPA (50 µg/kg bw per day) showed a significantly earlier day of vaginal opening than the control animals, while the high dose of BPA (50 mg/kg bw per day) had no effect on day of vaginal opening. By 15 weeks after day of vaginal opening, only 33% of the females exposed to the highest dose BPA (n=9) were still cycling, compared to 86% of the females exposed to the low dose of BPA. Females exposed to the high dose of BPA displayed abnormal folliculogenesis, containing multinucleated cells. Ovaries from the females exposed to the low dose of BPA showed all stages of follicular development. Most of the high dose treated BPA animals were acyclic at the time of ovariectomy, and the authors considered it unlikely that these follicles would progress to ovulation.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- Appropriate positive and negative control (EB and PPT)
- Phytoestrogen-free diet
- Good sample size (10-12 female offspring/group)

Weaknesses:

- Type of cages and drinking bottle not reported

Overall neither HED dose was ≤3.6 mg BPA/kg bw per day and this largely well-conducted study found significant advese effects of the high BPA doses used on post-natal reproductive development and function.

This study is not included in the WoE table because niether of the HED BPA doses used are ≤3.6 mg BPA/kg bw per day.

Berger RG, Hancock T and DeCatanzaro D, 2007. Influence of oral and subcutaneous bisphenol-A on intrauterine implantation of fertilized ova in inseminated female mice. Reproductive Toxicology, 23, 138-144.

To examine the effects of BPA on implantation, mice were exposed via either sc injection or two dietary administration routes (not gavage). Subcutaneous injections of BPA (0.016, 0.047, 0.14, 0.45, 1.3, 3.9, 35.2, 105.5 mg/kg bw per day [0.24, 0.71, 2.1, 6.75, 19.5, 58.5, 528, 1582.5 mg/kg bw per day]) in peanut oil were administered daily to adult female primiparous CF-1 mice on gestation days 1-4. Oral administration via either (1) dietary supplement (calculated daily intake: 36, 278, 550, 453 mg BPA/kg bw per day [HED, to dam, 2.49, 18.9, 37.4, 30.8 mg BPA/kg bw per day]) or (2) food contamination (calculated daily intake: 2,223, 2,293 mg BPA/kg bw per day [HED to dam, 151, 156 mg BPA/kg bw per day]) also occurred on GD1-4. Implantation rate, litter size, number of implantation sites and uterine histology (at the highest BPA dose only) were assessed. Although adverse effects were observed at the two highest doses, BPA at HED ≤3.6 mg/kg bw per day had no significant effect on any of the measures assessed. None of the oral doses had any significant effects at HED ≤3.6 mg/kg bw per day.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- Number of doses (≥3)

Weaknesses:

- Small sample size (mostly <8 animals/group)
- Type of drinking bottle not reported
- Study reporting inadequate for the oral studies, very difficult to relate doses to effects to statistical analysis.

Overall there were some barriers to interpretation of the study with the BPA dosages requiring conversion into mg/kg bw per day prior to calculation of the HED levels. In addition, relating the oral BPA doses to specific outcomes was poorly described in the publication. While well performed, the low sample numbers and the analysis of uterine histology only at the highest BPA dose means that the CEF Panel rated this study as preliminary.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Cabaton NJ, Wadia PR, Rubin BS, Zalko D, Schaeberle CM, Askenase MH, Gadbois JL, Tharp AP, Whitt GS, Sonnenschein C, Soto AM. Perinatal exposure to environmentally relevant levels of bisphenol A decreases fertility and fecundity in CD-1 mice. Environmental Health Perspectives, 119, 547-552.

This study is one of the rare forced/continuous breeding studies with BPA and as such potentially important to obtain insights into potential fecundity effects of BPA in humans. CD-1 female mice were exposed to BPA at 0.025, 0.25, 25 µg/kg bw per day [HED: dams 0.38, 3.75, 375 µg/kg bw per day] via subcutaneous osmotic minipumps from GD8-PND16. 10 ng/kg bw per day of DES was used as a positive control. Cages, bedding and water were assessed for estrogenicity by Escreen and the purity, but not the dose level, of BPA confirmed by HPLC-MS. The female F1 pups (1 per litter) at 2 months of age were housed with unexposed breeder males for 32 weeks, with the F2 pups were removed at parturition. The samples size of 18-21 litters/individuals is good but there is some statistical weakness in the detailed analysis of the number of pups delivered to each dam per litter (e.g. failure to show statistical significance of correlation coefficients while showing R2, which are for linear regressions, furthermore the data (Fig. 4) do not look appropriate for linear fits. BPA had no significant effect on the numbers of live or dead pups, the sex ratio of the pups, time to first delivery of F1 dam body weight at 32 weeks. However, the lowest and highest BPA doses were associated with a significantly reduced cumulative number of pups. At the highest BPA dose, numbers of litters and % of females with ≥6 litters were significantly reduced. The authors conclude that there was a dose-dependent effect of BPA on reproductive capacity.

Comments from the CEF Panel:
Sub-cutaneous study via mini-pump

The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- ≥3 dose levels of BPA
- Cages, bedding, water, food content of oestrogens negligible at E-screen.
- Adequate positive controls included (DES)
- Large sample size (18-21 litters/individuals)

Weaknesses:

- Inappropriate statistics (failure to show statistical significance of correlation coefficients while showing R2, which are for linear regressions, furthermore the data (Fig. 4) do not look appropriate for linear fits)

Overall the CEF Panel agreed that cumulative pup numbers were significantly reduced by BPA at the highest and lowest doses and by DES, but not by the intermediate BPA dose. Therefore the effect was not dose-dependent, raising questions about the role of chance given that all 3 BPA doses were considered low, even in terms of HED.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Castro B, Sanchez P, Torres JM, Preda O, Del Moral RG and Ortega E, 2013. Bisphenol A exposure during adulthood alters expression of aromatase and 5alpha-reductase isozymes in rat prostate. PLoS One, 8, e55905.

Adult male Wistar rats were exposed to 4 dose levels of BPA at 25, 50, 300, 600 µg/kg bw per day [HED: Adults males = 650, 1 300, 7 800, 15 600 µg/kg bw per day] for 4 days by sc injection in sesame oil. Controls received sesame oil alone and group size was 8. After harvesting circulating testosterone and oestradiol were determined and qPCR and Western blot performed to determine srd5a1, -a2, -a3 and cyp19a1 transcript and protein levels in the prostate glands. Testosterone increased, oestradiol decreased ans the testosterone/oestradiol ratio was skewed by exposure to BPA at all doses tested. Prostate levels of srd5a1 and a2 were reduced and a3 and cyp19a1 increased at all doses tested.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- Number of doses (≥3)
- Use of non-PC cagesand glass bottles

Weaknesses:

- Animal diet poorly described

Overall the CEF Panel noted that this is a well performed study with reasonable and valid enpoints. The changes described, especially the skewing of the T/E2 ratio and increased aromatase is considered symptomatic of prostate disease. Despite the acute nature of the exposure the study may be of interest although a more prolonged exposure paradigm was not included to check whether the effects persisted or disappeared with time.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Christiansen S, Axelstad M, Boberg J and Hass U, 2013. Low dose effects of BPA on early sexual development of male and female rats. Reproductive Toxicology, 41, 11.

Mated nulliparous young adult Wistar rats were were allocated to three experimental blocks. Day of vaginal plug was GD 1 and GD 23 was called postnatal day1 (PND 1) as the expected day of delivery. Four different dose levels of BPA were administered (0.025, 0.25, 5, 50 mg/kg bw per day) in corn oil [HED: Dams = 0.018, 0.18, 3.6, 36 mg/kg bw per day; Pups = 0.475, 4.75, 95, 950 mg/kg bw per day]. Twenty-two maternal rats were allocated to each treatment group and treatment was by gavage once a day from GD 7 to PND 22, excluding the day of delivery. BPA levels in stock solutions were verified by analysis. Considerable efforts were made to avoid BPA contamination from the rat environments. Animal morpholiogical measures included AGD and nipple retention. On PND 16 and 17 1 male and

1 female pup/litter were harvested and a range of organs weighed. 14-20 male pups and 15-20 female pups were analysed at each treatment dose. Very few statistically significant effects of BPA were observed. Male pup AGD was significantly decreased (7% max) at all except the lowest BPA dose and nipple retention increased at the highest dose (4-fold, but dose-dependent). Female pup AGD was also significantly decreased (9% max) at all doses. Among the organs weighed, the only significant effect was an increase in retroperitoneal fat pad weight in male pups at the highest BPA dose. The authors conclude that BPA below the NOAEL of 5 mg/kg bw per day can affect reproductive development in the rat.

Comments from the CEF Panel:
The CEF Panel identified the following strengths in this study:
Strengths:
- Large sample size
- Number of doses (≥3)
- Oral administration by gavage
- Phytoestrogen-free diet
- Use of non-PC cages and of non plastic bottles (polysulphone bottles)

Weaknesses:
- None

Overall, the CEF Panel noted that the concentrations of BPA in the treatment solutions were verified and that statistical analyses were appropriate. The CEF Panel partly agrees with the authors' conclusion. While the decrease in male AGD is indicative of some impairment of masculinization it is not known from this study whether there is any decrease in subsequent fertility. The decrease in AGD in females is also indicative of an effect of all doses of BPA on genital development, but the reproductive significance of shorted AGD in the females is uncertain since increased AGD in young women is associated with greater follicle numbers (Mendiola et al., 2012). Reduced female AGD is inverse to the expected increase in female AGD associated with ovarian cysts/PCOS in the case of increased androgen action.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

DeCatanzaro D, Berger RG, Guzzo AC, Thorpe JB and Khan A, 2013. Perturbation of male sexual behaviour in mice (*Mus musculus*) with a discrete range of bipshenol-A doses in the context of high- or low- phytoestrogen diet. Food and Chemical Toxicology, 55, 164-171.

Adult female CF0-1 mice were maintained on either a high or low phytoestrogen certified commercial diets. 5 different dose levels of BPA were used in 2 experiments. From GD9 to PND1 the mothers received, in the diet in 1 g of peanut butter, either vehicle (peanut oil) or 0.175, 1.75, 17.5 µg BPA/g peanut butter/day (experiment 1) [HED: Dams = 0.15, 1.5, 15 µg/kg bw per day]) or, with a high phytoestrogen diet only, 17.5, 175, 1750 µg BPA/g peanut butter (experiment 2 [HED: Dams = 15, 150, 1,500 µg/kg bw per day]). Pups were weaned on PND27 and males maintained on the same diet as their mother until PND 60 or 90. Male offspring AGD, reproductive organ weights, capacity to inseminate and urinary hormone levels were measured. 1 male per litter, from 12-20 litters, was used for each analysis. In experiment 1, the 17.5 µg BPA/day + high phytoestrogen was associated with reduced vesicular-coagulating gland weight and increased latency to inseminate, but did not affect body, testis or preputial gland weights or AGD. In experiment 2 none of the BPA doses affected these body/reproductive organ indices and urinary testosterone, oestradiol and creatinine were also unaffected. At the 17.5 µg BPA/day dose there were reductions in intromission number (also at the 175 dose) and ejaculations by around 50%.

Comments from the CEF Panel:
BPA doses were not normalised to body weight

The CEF Panel identified the following strengths and weaknesses in this study:
Strengths:

- Large sample size (n=15 dams/group)
- Number of dose levels (≥3)

Weaknesses:

- None

Overall, the CEF Panel noted that numbers and statistical analysis are appropriate but the BPA doses are not normalised against body weight. It is not evident what the BPA dose in terms of µg/kg bw per day actually were and BPA levels in the rats were not determined. In addition, the erratic appearance of mostly minor effects of BPA only at the high phytoestrogen dose makes the study interesting but difficult to interpret in terms of human risk.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Delclos KB, Camacho L, Lewis SM, Vanlandingham MM, Latendresse JR, Olson GR, Davis KJ, Patton RE, da Costa GG, Woodling KA, Bryant MS, Chidambaram M, Trbojevich R, Juliar BE, Felton RP and Thorn BT, 2014. Toxicity Evaluation of Bisphenol A Administered by Gavage to Sprague Dawley Rats From Gestation Day 6 Through Postnatal Day 90. Toxicological Sciences, 139, 174-197.

In a new study Sprague-Dawley rats were used for a dose-response approach to investigate the effects of BPA on a very wide range of pathological, physiological, endocrine, reproductive and developmental endpoints. Ethinyl oestradiol was used as a positive control of the estrogenic effects of BPA. The dose-matched vehicle control was carboxymethylcellulose, sodium salt. The doses were: (i) BPA 2.5, 8, 25, 80, 260, 840, 2 700, 100 000, 300 000 µg/kg bw per day [HED: Dams = 1.8, 5.76, 18, 57.6, 187, 604, 1 944, 72 000, 216 000 µg/kg bw per day; Pups = 47.5, 152, 475, 1 520, 4 940, 15 960, 51 300, 1 900 000, 5 700 000 ug/kg bwday although dose to dams was used as conservative], (ii) Vehicle, (iii) EE_2 0.5, 5 µg/kg bw per day. The study included a naïve control group and doses were administered by oral gavage. The protocol ands methods, including statistical analysis were of the high quality and robust with treatment, body weight and litter randomisation and appropriate inclusion and exclusion criteria established prior to the start of the study. The target unit for analysis was 20 litters and 18-23 were achieved. F0 females were dosed from GD 6 up to labour onset and pups from PND 1 until tissue harvesting, up to PND 90. There were vehicle effects compared with naïve controls, including reduced male offspring preweaning survival and reduced male AGD and AGDI (7%). BPA doses between 2.5 and 2 700 µg/kg bw per day were considered low doses and these are considered here. A significant increase in vaginal metestrus prevalence was found in both BPA 2.5 ($P<0.05$) and BPA 25.0 ($P<0.01$) relative to the vehicle controls. In addition, a significant change in the oestrous pattern was found for BPA 25.0 µg/kg bw per day ($P<0.01$) relative to controls. Males showed a slightly increased incidence of seminiferous tubule giant cells (5/23 at 2.5 µg/kg bw per day vs 0/20 for vehicle) and delated testis descent (5%, 260 µg/kg bw per day). Reliability of these findings is supported by the extensive effects of the 100 000 and 300 000 µg/kg bw per day BPA doses in both males and females and the lrgely expected effects of the EE_2 positive controls in both sexes.

In conclusion, at low oral doses of BPA, especially below 2 700 µg/kg bw per day, this study provides strong evidence that BPA pre-natal plus post-natal exposure to BPA does not have highly significant effects on rat reproductive development and adult reproductive indices.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- Large sample size
- Number of doses (≥3) (especially in the low dose range)
- Both naïve and vehicle controls available
- Adequate positive controls included
- Oral administration via gavage
- Phytoestrogen-free diet
- Use of non-PC cages
- Study/analysis performed under OECD guideline
- Study/analysis performed under GLP
- BPA measurements in biological samples

Weaknesses:

- BPA contamination of control groups

Overall, the CEF Panel noted that at low oral doses of BPA, especially below 2 700 µg/kg bw per day, this study provides strong evidence that BPA pre-natal plus post-natal exposure to BPA does not have highly significant effects on rat reproductive development and adult reproductive indices.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Dobrzynska MM and Radzikowska J, 2013. Genotoxicity and reproductive toxicity of bisphenol A and X-ray/bisphenol A combination in male mice. Drug and Chemical Toxicology, 36, 19-26.

As stated in the original abstract from the paper: "This study was designed to investigate the effects of 2 weeks of exposure of male mice to bisphenol A (BPA) alone or in a combination with X-rays on the sperm count and quality as well as induction of DNA strand breaks in somatic and germ cells. Pzh:SFIS male mice were exposed to X-rays (0.05 and 0.10 Gy) or BPA (5, 10, 20, and 40 mg/kg) or to a combination of both (0.05 Gy + 5 mg/kg body weight of BPA and 0.10 Gy + 10 mg/kg of BPA). Both X-rays and BPA administered alone decreased sperm count and quality. X-rays induced DNA strand breaks in spleen cells, whereas BPA induced DNA strand breaks in lymphocytes and in cells from spleen, kidneys, and lung and in germ cells. After combined exposure to both agents, sperm count and quality were similar as after exposure to each agent alone and significantly reduced, compared to control. Levels of DNA damage in somatic and germ cells after combined exposure to lower, as well as higher, doses were significantly reduced, compared to the effects of BPA alone. Results confirmed the mutagenic ability of BPA. Combined exposure to X-rays and BPA leads to the prevention of DNA damage in somatic and germ cells of mice."

Comments from the CEF Panel:
The CEF Panel identified the following strengths and weaknesses in this study:
Strengths:

- Number of doses (≥3)

Weaknesses:

- No vehicle controls were tested
- Drinking water consumption (containing BPA) not measured
- Animal diet poorly described
- Animal diet and phytoestrogen content not reported

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

El Ghazzawy IF, Meleis AE, Farghaly EF, Solaiman A, 2011. Histological study of the possible protective effect of pomegranate juice on bisphenol-A induced changes of the caput epididymal epithelium and sperms of adult albino rats. Alexandria Journal of Medicine, 47, 125-137.

In what can only be considered a preliminary and inadequately quantified study, a single very low dose of BPA administered by oral gavage to adult 13-15 week old male albino rats for 8 weeks. Ten rats per group received daily the following treatments by oral gavage: (1) corn oil, (2) corn oil+pomegranate juice, (3) 20 µg BPA/kg bw per day [HED: Adult males = 14.4 µg/kg bw per day], (4) 20 µg BPA/kg bw per day+ pomegranate juice. A quantified reduction in caudal epididymis sperm numbers (1.8-fold lower in BPA only group) was reported and reversed by the anti-oxidant pomegranate juice. Qualitative observations were made about caput epididymis and sperm structure and ultrastructure. There was no apparent attempt to quantify these observations.

Comments from the CEF Panel:
The CEF Panel identified the following strengths and weaknesses in this study:
Strengths:
- Oral administration via gavage
- Use of non-PC cages

Weaknesses:
- Single dose level study
- Animal diet poorly described
- Insufficient studying reporting (no multiple comparisons statistics)

Overal the CEF Panel noted that this is a single- and low-dose study. While the degeneration of the epididymis in BPA-treated animals compared with controls described by the authors seems convincing, based on the light and electron micrographs presented, this does not seem to be biologically plausible at such a low dose, compared with the results of well-conducted multigeneration studies. Statistical analysis was not adequately described. No multi-comparison analysis encompassing the protective effects of pomegranate juice was applied. The oxidative stress hypothesis is based on these apparent protective effects.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Ferguson SA, Law CD, Jr. and Abshire JS, 2011. Developmental treatment with bisphenol A or ethinyl estradiol causes few alterations on early preweaning measures. Toxicological Sciences, 124, 149-160.

Pregnant Sprague-Dawley rats reared in a low exogenous oestrogen environment were gavaged on gestational days 6–21 with 0, 2.5 or 25 µg BPA/kg bw per day [HED: Dams = 1.8, 18 µg/kg bw per day, Pups = 47.5, 475 µg/kg bw per day], or 5.0 or 10.0 µg/kg per day ethinyl oestradiol (EE). Litters were reduced to four males and four females as far as possible, and the pups were then orally treated on postnatal days 1–21 with the same doses. Parameters investigated, starting on postnatal day 1 and onwards were anogenital distance (AGD) and anogenital distance index, developmental landmarks including bilateral ear canal opening, bilateral eye opening, fur development and nipple retention as well as righting reflexes, slant board behaviour. Total brain weights and weights of different anatomical zones were measured. On post natal day 21 serum levels of a number of hormone were measured; thyroxin, triiodothyronine, oestradiol, testosterone, corticosterone and LH as well as leptin and ghrelin. Administration of BPA at 2.5 or 25µg/kg bw per day had no effects on gestational or lactational bodyweight in the dams, nor birth weight of the pups although preweaning body weights were decreased in both sexes relative to the vehicle control group (maximum 10% decrease in low-dose BPA postnatal day 5 females). Administration of EE resulted in significant decreases in gestational and lactational body weight of the dams, also birth weights and preweaning body weights pups. No effect of treatment with these low doses of BPA were observed on anogenital distances and

AGD index, developmental landmarks, measures of serum hormones, and whole/regional brain weights. Developmental landmarks including age at eye opening, bilateral ear canal opening and fur development, and two early behavioral markers, righting reflex and slant board behavior) were not altered by BPA treatment. No effects on hormonal measures or brain weights at weaning were seen. The authors concluded that these low oral doses of BPA were not associated with early alterations in the offspring.

Comments from the CEF Panel:
The CEF Panel identified the following strengths and weaknesses in this study:
Strengths:

- Large sample size
- Adequate positive control included
- Oral administration by gavage
- Phyoestrogen-free diet
- Use of non-PC cages and of non plastic water bottles

Weaknesses:

- None

Overall the CEF Panel noted that this study, by the US FDA, is a robust well controled study, showing no effects of BPA. However, it covers endpoints that have not been highlighted by ANSES as of particular concern, and covers only 2 low dose levels. Care was taken to avoid confounding factors such as not using oily vehicles to avoid their nutritional effects, the cages were made of polysulfone checked for BPA release, very careful randomisation and dosing procedures, BPA of defined purity and hormones measured at a particular period to avoid confounding diurnal variation. Although the positive control ethinyl oestradiol produced significantly decreased gestational and lactational body weight, birth weights and pre-weaning body weights, these and other indices were not altered by BPA. Thus, developmental BPA treatment at 2.5 or 25.0 mg/kg/day appears to have no effects on gestational or lactational body weight, offspring anogenital distance, pre-weaning behaviour or hormone levels and whole and regional brain weights measured at weaning. Interestingly, following direct oral treatment of the offspring on post-natal days 1–21, the naive control group weighed significantly less than the vehicle (aqueous solution of carboxymethylcellulose sodium salt) control group. The reason for this was unclear although it was hypothesized that increased thirst in vehicle control offspring of the current study may have resulted in increased suckling and potentially increased body weight. However, such findings highlight the potential experimental factors that can confound the interpretation of group differences in these neonatal studies.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Honma S, Suzuki A, Buchanan DL, Katsu Y, Watanabe H and Iguchi T, 2002. Low dose effect of in utero exposure to bisphenol A and diethylstilbestrol on female mouse reproduction. Reproductive Toxicology, 16, 117-122.

This study was aimed primarily at the effects of BPA on female reproduction, but also included some data on males. Pregnant ICR-Jci mice were administered BPA at 2,20 µg BPA/kg bw per day [HED: dams 30, 300 µg/kg bw per day] from GD11-17. DES (0.02, 02, 2 µg/kg bw per day) was used as a psotive control. The offspring of both sexes were assessed at PND 0, 11, 22, 60 and females were mated with unexposed males between PND 90 and 120. Body weights, AGD, vaginal histology and vaginal opening were examined. BPA had no significant effect on gestation, numbers of live pups or pup sex ration. In females BPA reduced body weight at PND 22 and 60 (significant but small effects) and increased AGD (2 µg/kg bw per day) at PND 22 although this effect was no longer seen at PND 60. In males BPA significanrtly reduced body weight at PND60 and while AGD was not affected at PND22, at PND60 both BPA doses were associated with significantly increased AGD. Surprisingly, DES had similar effects in both sexes (at 1 or more dose levels). BPA (and DES) significantly advanced the day of vaginal opening, reduced body weight and age at vaginal opening and increased oestrous cycle length. In subgroups of n=10 F1 female mice mated, there were no significant effects of BPA (or DES) on mating, pups/litter or pup sex ratio.

Comments from the CEF Panel:
Some positive control effects were not as expected (DES had similar effects in both sexes (at 1 or more dose levels))

The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- Adequate positive controls included (3 doses of DES)
- Large sample size (9-10 and 41-51 females, 24-35 males respectively per group)

Weaknesses:

- Inappropriate statistics (not clear if data normality was established, use of Student's t-test, analysis of dose-effects bnot stated)
- Animal diet and phytoestrogens content not reported
- Type of cages and drinking bottles not reported

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Horstman KA, Naciff JM, Overmann GJ, Foertsch LM, Richardson BD and Daston GP, 2012. Effects of transplacental 17-alpha-ethynyl estradiol or bisphenol A on the developmental profile of steroidogenic acute regulatory protein in the rat testis. Birth Defects Res B Dev Reprod Toxicol, 95, 318-325.

In this study pregnant Sprague Dawley rats were dosed from gestational day 11 with either 17-alpha-ethynyl oestradiol (EE) in peanut or sesame oil or BPA in dimethyl sulfoxide by subcutaneous injection. Doses of EE were 0.001, 0.1 or 10 µg/kg/day or BPA at 0.02, 0.5, 400 mg/kg per day day [HED: Dams = 0.52, 13, 10, 400 mg/kg bw per day]. Fetal testes were harvested on gestational days 16, 18 or 20. They were studied using quantitative reverse transcriptase PCR for changes in steroidogenic acute regulatory (StAR) protein transcript levels and immunocytochemistry for StAR protein. Neither EE nor BPA exposure caused morphological changes in the developing seminiferous tubules or the interstitial region at gestational days 16–20. However, BPA and EE slightly reduced StAR mRNA and protein levels at gestational day 18 and 20 but only at the highest doses of 10 µg/kg/day EE or 400 mg/kg/day BPA. Immunohistochemistry also demonstrated decreases in StAR protein levels but again only at the highest doses.

Comments from the CEF Panel:

The CEF Panel identified the following strengths and weaknesses in this study:
Strengths:
- Large sample size
- Number of doses (≥3)
- Positive control included
- Use of non-PC cages

Weaknesses:
- Animal diet and phytoestrogen content not reported
- Insufficient study reporting (some details not completely clear)

Overall the CEF Panel noted that, whilst this study demonstrates the potential effects of neonatal exposure to BPA on testicular function of offspring, it seems to be limited to high exposures which are probably not directly relevant to human exposures. Notwithstanding the use of EE group as a positive control, the precise sample sizes were difficult to interpret from the paper and the immunohistochemistry was not very convincing. However, no effects of BPA doses ≤3.6 mg/kg bw per day HED were reported.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Howdeshell KL, Furr J, Lambright CR, Wilson VS, Ryan BC and Gray LE, 2008. Gestational and lactational exposure to ethinyl estradiol, but not bisphenol a, decreases androgen-dependent reproductive organ weights and epididymal sperm abundance in the male long evans hooded rat. Toxicological Sciences, 102, 371-382.

In a well-doncducted study of BPA reprotoxicology, Long Evans rats were dosed with BPA at 2, 20, 200 µg/kg bw per day [HED: 1.44, 14.4, 144 µg/kg/day] by oral gavage from GD7 to PND18 Two arms – main study 13-29 dams/ group (0.05, 0.5, 5, 50 µg/kg bw per day) second extended EE group (0.05, 0.15, 0.5, 1.5, 5, 15, 50 µg/kg bw per day) study 6-14 dams/group, with the same BPA regime for both arms. At PND5 litters were trimmed to n=8 pups max, as far as possible balanced for pup sex (4 male, 4 female). 9-38 litters were used per group and the liutter effect was accounted for. Dams and male offspring were studied, the females from the same experiment having been reported by Ryan et al (2009a). Maternal and pup/weaning body weights and neonatal AGD were measured. Necroscopy was performed after PND150 and PND229 and reproductive tract weights, morphology and histology as well as external genitalia and nipple retention were assessed. Epidyimyal sperm counts and circulating oestradiol, testosterone, corticosterone, total T4, LH and PRL were measured. Apart from rwo minor exceptions BPA had no significant effect on AGD, birth or weaning weights, age or weight at weaning, growth rates, external genitalia, endoc rine measures, reproductive tract size and morphology or any other indeces of reproductive development or function measured. The exceptions were: (i) 1/18 males treated with 20 µg/kg bw per day vs 0/31 control males showed ventral prostate hyperplasia (dose-related in EE treated positive controls) and (ii) an increased incidence of testisular degeneration (without any effect on testis size or sperm numbers) in 7/20 males treated with 200 µg BPA/kg bw per day compared with 1/31 controls. These effects were seen only in the first arm of the study and not in the second arm. In contrast, the positive control had the expected, dose-dependent effects on the parameters measured.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:
- ≥3 dose levels of BPA
- Oral administration via gavage Large sample size (9-38 litters/group)
- Adequate positive controls included (EE)

Weaknesses:
- Use of polycarbonate cages (although new)

- Animal diet and phytoestrogen content poorly descriped (Purina Rat Chow 5008 has high isoflavone content)

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Hunt PA, Lawson C, Gieske M, Murdoch B, Smith H, Marre A, Hassold T, VandeVoort CA, 2012. Bisphenol A alters early oogenesis and follicole formation in the fetal ovary of the rhesus monkey. PNAS, 109 (43), 17525-17530.

Hunt et al. (2012) investigated reproductive parameters in the female fetuses of pregnant Rhesus macaques. Two routes of administration were used: (1) oral in diet, 400 µg BPA/kg bw per day [HED: Dams = 168 µg/kg bw per day] (single daily dose) or (2) subcutaneous implant tested to yield 2.2-3.3 ng unconjugated BPA/ml plasma in non-pregnant females (continuous exposure). Deuterated BPA was used to allow detection. Two exposure windows were investigated for each route: (1) early GD50-100, the onset of meiosis and (2) late GD100-term, the period of follicle formation. Offspring ovaries were studied: oocyte and quantification of multi-oocyte follicles (late exposure window) and meiotic analyses (early exposure window). Only the results for the oral route were considered for evaluation because of the inadequate number of animals in the subcutaneous route (only 2 monkeys in the control group). BPA ≤3.6 mg/kg bw per day HED was associated with a modest but statistically significant increase in the proportion of multi-oocyte secondary or antral follicles but had no significant effect on incidence of meiotic defects reportedly seen in the implant group).

Comments from the CEF Panel:
The CEF Panel identified the following strengths and weaknesses in this study:
Strengths:
- BPA measurement in animal samples
- Use of non-PC cages

Weaknesses:
- Small sample size
- Single dose level study
- BPA concentration and homogenity not guaranteed analytically
- Diet phytoestrogen content not reported

Overall, the CEF Panel notes that the precise significance of the increased incidence of multi-oocyte follicles for subsequent fertility in monkey or human is likely to be adverse but remains to be demonstrated. Low animal numbers meant that only the oral dose groups could be assessed reliably.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Jin P, Wang X, Chang F, Bai Y, Li Y, Zhou R, Chen l, 2013. Low dose Bisphenol A impairs spermatogenesis by suppressing reproductive hormone production and promoting germ cell apoptosis in adult rats. Journal of Biomedical Research, 27 135-144.

Adult male Sprague-Dawley rats in 5 groups of 10 were allocated to treatment groups. A single dose level of BPA was used. Animals were treated as follows: (i) BPA in olive oil at 2 µg/kg bw per day, (ii) testosterone propionate (TP in DMSO) at 0.1 mg/rat per day, (iii) BPA+DMSO, (iv) BPA+TP, (v) the text mentions a baseline group and a vehicle group without specifying which vehicles. BPA was administered by oral gavage, TP by subcutaneous injection. BPA treatment was for 14 days, TP treatment duration was not specified. Epididymal sperm counts and testicular spermatogenesis were assessed by microscopy/histology and TUNEL quantification of seminiferous tubule apoptosis. Circulating LH, FSH and testosterone were determined. Brain (preoptic area) GnRH immunohistochemistry and qPCR was performed, as well testicular Ar, Fas, FasL, Caspase-3 qPCR.

BPA was reported to reduce sperm counts and seminiferous tubule numbers of all stage VII germ cells. Serum and iontra-testicular testosterone were reduced in BPA-exposed animals and the negative effect of BPA on sperm counts was partially reversed by TP, as were numbers of mPSc and 7Sd stage VII germ cells. BPA-exposed rats had lower FSH and increased LH. Following TP replacement the level of LH was lower in the BPA rats than in controls. Preoptic area GnRH expression was reduced in the BPA exposed group. The BPA-exposed seminiferous tubules had an increased apoptotic index that was unaffected by coadministration of TP. Fas, FasL and caspase-3 were increased by BPA exposure. The authors conclude that the dose of BPA impaires spermatogenesis by decreasing reproductive hormones and activating the Fas/FasL pathway.

Comments from the CEF Panel:
The CEF Panel identified the following strengths and weaknesses in this study:
Strengths:
- Oral administration via gavage
- Use of non-polycarbonate (non-PC cages), and use of glass water bottles
- Adequate positive controls included

Weaknesses:
- Single dose level study
- Animal diet poorly described
- Insufficient study reporting (data presentation is unclear)

Overall the CEF Panel noted that the study appears reasonably well performed in places, subject to the significant caveat about the group set up and controls. However, the data presentation is confusing and erratic in places making interpretation difficult and reduces confidence in the quality and validity of the study.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Kato H, Furuhashi T, Tanaka M, Katsu Y, Watanabe H, Ohta Y and Iguchi T, 2006. Effects of bisphenol A given neonatally on reproductive functions of male rats. Reproductive Toxicology, 22, 20-29.

Neonatal BPA effects on male reproduction were studied in Sprague-Dawley male rats administered BPA at 2, 11, 56, 277, 97,000 µg/kg bw per day [HED: neonates 0.62, 3.4, 17.4, 85.9, 30070 mg/kg bw per day]. BPA dosage was by daily subcutaneous injection from PND0 (birth) to PND9. A single dose level of 0.9 mg/kg bw per day of oestradiol was used as a positive control. 1 male per litter from 12 dams was used for each treatment group, adjusting for the litter effect. The treatment regime required analysis and conversion of doses to mean mg/kg bw per day. The sample size was n=8 and therefore adequate although not good and the study was otherwise well conducted and reported. The pups were investigated an PND0 (birth), 10, 21, 42, 63, 84, 105, 126, 140, 150 (necroscopy at PND 10, 35, 150) and preputial separation, semen analysis (numbers, motility and morphology), circulating testosterone, copulatory rate, fertility rate, testicular histology, key testis ranscript expression (e.g. CYP17A1) and reproductive tract weights and morphology were assessed. At the two lowest doses of BPA (i.e. ≤3.6 mg/kg bw per day) and indeed at all BPA doses, there were no significant effects on any parameter measured other than a reduction in the proportional of abnormal sperm, not adverse outcome. The oestradiol-treated males showed expected estrogen mediated adverse changes, such as reduced testis weight. The litters sired by the BPA treated males were no different from controls.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:
- ≥3 dose levels of BPA
- Adequate positive controls included (oestradiol)

Weaknesses:

- Type of cages and drinking bottles not reported

The CEF Panel noted that the authors' statement that the diet (CRF-1, Oriental Yeast Co, Tokyo, Japan) is considered relatively low in phytoestrogen. No adverse effects of BPA were observed although the fertility test was not using a continuous/forced breeding regume.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Kobayashi K, Kubota H, Ohtani K, Hojo R, Miyagawa M, 2012. Lack of effects for dietary exposure of bisphenol A during in utero and lactational periods on reproductive development in rat offspring. The Journal of Toxicological Sciences, 37, 565-573.

In order to investigate the effects of both in-utero and lactational BPA exposure on male and female offspring reproductive development, the authors exposed female Sprague-Dawley rats (n=10/group) to 3 doses of BPA (0.33, 3.3, 33 ppm in diet, equivalent to 0.02, 0.17, 1.65 mg/kg bw per day [HED: Dams = 0.0144, 0.1224, 1.188 mg/kg bw per day] during gestation and lactation, GD 6–PND 21. F1 offspring were examined at 5 weeks and 3 months postnatally and body and organ weights, anogenital distance, reproductive hormones and sperm counts were quantified. The only BPA-related effect in males was a statistically significant decrease in epididymal weights in the 3-month old male animals receiving 1.188 mg BPA/kg body weight/day HED. In females, the two highest BPA doses were associated with significantly reduced AGD and ovary weights at 5 weeks post-natal although these differences had disappeared by the time the animals were 3 months old.

Comments from the CEF Panel:
The CEF Panel identified the following strengths and weaknesses in this study:
Strengths:

- Number of doses (≥3)

Weaknesses:

- BPA exposure assessment (Feed consumption (BPA given by the diet) not measured , BPA concentration and homogeneity in the feed mixture not guaranteed analytically)
- Animal diet and phytoestrogen content not reported
- Insufficient study reporting (some ambiguity about precise dose and/or incidental exposure of pups by maternal diet/water)

Overall the CEF Panel noted that the study was reasonably well performed but the exact exposure of the animals via the diet is unclear, making the study difficult to compare directly to other studies. The litter effect was taken into account.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

LaRocca J, Boyajian A, Brown C, Smith SD, Hixon M, 2011. Effects of in utero exposure to Bisphenol A or diethylstilbestrol on the adult male reproductive system. Birth Defects Research, Part B. Developmental and Reproductive Toxicology, 92, 526-533.

LaRocca et al. (2011) administered 2.5 or 25 µg BPA/kg bw per day [HED: Dams = 0.08, 0.8 µg/kg bw per day] to pregnant mice by oral gavage from GD12-PND21 and the adult male offspring studied. A positive control (DES, 2 µg/kg bw per day) was included. Investigation of the effects of BPA on pregnancy outcome and on reproductive development of male offspring included testis genes expression and morphology and masculinisation (circulating testosterone and AGD). No changes were reported for masculinisation, sperm production or germ cell apoptosis in adult testes after exposure to either chemical. Adult mRNA levels of genes associated with sexual maturation and differentiation,

GATA4 and ID2, were significantly lower only in DES-exposed testes. Overall there was no significant effect of BPA ≤3.6 mg/kg bw per day HED.

Comments from the CEF Panel:
The CEF Panel identified the following strengths and weaknesses in this study:
Strengths:
- Large sample size
- Adequate positve controls included
- Oral administartion by gavage

Weaknesses :
- Animal diet and phyoestrogen content not reported
- Use of polycarbonate cages

Overall, the CEF Panel noted that the study was a reasonably well conducted study in which no effects of BPA were reported on testicular function including expression of genes associated with steroidogenesis, apoptosis, and Sertoli cell maturation, reported by some other authors to be affected by BPA.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Lee SG, Kim JY, Chung JY, Kim YJ, Park JE, Oh S, Yoon YD, Yoo KS, Yoo YH and Kim JM, 2013b. Bisphenol A Exposure during Adulthood Causes Augmentation of Follicular Atresia and Luteal Regression by Decreasing 17beta-Estradiol Synthesis via Downregulation of Aromatase in Rat Ovary. Environmental Health Perspectives, 121, 663-669.

Adult (8 wks old) female Sprague-Dawley rats were administered 2 dose levels of BPA by oral gavage at 0.001 or 0.1 mg/kg bw per day [HED: Adult females = 0.00072 (0.72 µg), 0.072 (72) mg/kg bw per day] for 90 days. The BPA was dissolved in DMSO and the controls received weight matched DMSO in corn oil. A positive control, 0.001 mg oestradiol benzoate/kg bw per day was included. Group size was 30 for each treatment. After 90 days 18 rats/group were harvested on the day of oestrous while the remainer (12/group) were followed for oestrous cycle staging. Uteri, pituitary glands and ovaries were examined and plasma E2, T, LH and FSH levels measured and granulosa cells isolated from one ovary/rat. Ovarian apoptosis was determined by caspase-3 analysis and steroidogenic enzymes measured in theca cells. Circulating E2 and T were reduced by BPA and EB and the duration of the estrus phase increased. Follicular and corpora luteal atresia was increased by BPA although EB only affected luteal atresia as determined by caspase-3 analysis. Theca cell cyp19 was decreased by BPA and StAR decreased by BPA and EB. Circulating and pituitary LH but not FSH were increased by BPA.

Comments from the CEF Panel:
The CEF Panel identified the following strengths and weaknesses in this study:
Strengths:
- Large sample size
- Adequate positive controls included
- Oral administration by gavage

Weaknesses:
- Animal diet and phytoestrogen content not reported

Overall the CEF Panel acknowleges that in a well performed and well powered study both doses of BPA tested decreased circulating E2 and increased ovarian cell apoptosis, likely partly via decreased theca cell steroidogenesis and reduced testosterone which would then explain the increased LH. EB did not produce the same phenotype exactly.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Liu C, Duan W, Li R, Xu S, Zhang L, Chen C, He M, Lu Y, Wu H, Pi H, Luo X, Zhang Y, Zhong M, Yu Z and Zhou Z, 2013. Exposure to bisphenol A disrupts meiotic progression during spermatogenesis in adult rats through estrogen-like activity. Cell Death Dis, 4.

Adult (9 weeks old) male Wistar rats were exposed to 3 BPA dose levels (2, 20, 200 µg/kg bw per day) [HED: Adults = 1.44, 14.4, 144 µg/kg bw per day] by oral gavage. 17 β-oestradiol (E2, 10 µg/kg bw per day) was administered by subcutaneous injection as a positive control. Both were dissolved in ethanol then corn oil. The ER antagonist fulvestrant (ICI) was dissolved in ethanol and used at 500 µg/kg bw per day, 30 mins prior to gavage where relevant. Solvent control, ICI and E2 groups received equivalent cornoil without BPA. Solvent control, BPA and E2 groups were also injected with ethanol in cornoil. Treatment continued for 60 days. Groups included colvent control/s, 3 doses of BPA only, E2 or ICI only, BPA 200+ICI. After 60 days blood samples and gondas were removed with caudal epidymis used for sperm analysis. One testis was used for meiotic chromosol spread and comet assay while the otjer epididymis and testis were used for histology (H&E) and PAS-H staining. No effect of BPA at less thn 200 µg/kg bw per day was observed. No effects of any dose of BPA on sperm characteristics, sperm apoptosis, serum FSH, LH or testosterone were seen although E2 reduced testosterone (>3-fold), sperm couns and epididymal weights. The ER antagonist reversed the effects of the BPA dose of 200 µg/kg bw per day on sperm counts. The latter dose of BPA and E2 increased stages VII and IX sperm and decreased stage VII sperm, an effect blocked by ER antagonism. Both 200 µg BPA/kg bw per day and E2 reduced the percentage of leptotene and zygotene spermatocytes and increased the proportion of pachytene spermatocytes, again blocked by ER antagonist administration. Extensive analysis of germ cell meiosis indicated that 200 µg/kg bw per day of BPA and E2 induced disruption of meiosis and increased germ cell apoptosis which was blocked by ER antagonist. The authors conclude that BPA at 200 µg/kg bw per day disturbs meiosis via estrogenic activity and this may contribute to male infertility.

Comments from the CEF Panel:
The CEF Panel identified the following strengths and weaknesses in this study:
Strengths:
- Adequate positive controls included
- Number of doses (≥3)
- Use of glass water bottles

Weaknesses:
- Animal diet poorly described
- Study design not appropriate to the scope (description of the study design was poor and confusing in terms of exactly what groups received what and which were compared with what controls)
- Inappropriate statistics (only test used was unpaired t-test)

The CEF Panel noted that the description of the study design was poor and unclear in terms of exactly what groups received what and which were compared with what controls. Also animals were provided with a rodent experimental diet in which it was stated that no phytoestrogens could be detected but this was not checked analytically in the study. The statistical analysis was also not adequately described. Nevertheless, the study provides insight into a potential estrogenic action of BPA in the adult male.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Lopez-Casas PP, Mizrak SC, Lopez-Fernandez LA, Paz M, de Rooij DG, del Mazo J, 2012. The effects of different endocrine disruptor defining compound-specific alterations of gene expression profiles in the developing testis. Reproductive Toxicology, 33, 106-115.

Lopez-Casas et al. (2012) exposed CD-1 mice to mono-(2-ethylhexyl)-phthalate, zearalenone, lindane, bisphenol-A (0.16; 16 or 64 mg/kg bw per day [HED: Dams 0.00048, 0.048, 1.92 mg/kg bw per day]) or 17β-oestradiol (E2: 0.006; 0.012 or 0.048 mg/kg bw per day) via oral administration by drinking water to mothers. There were 3 exposure groups (A) during the two weeks before mating; (B) exposure continued until birth or (C) exposure was continued until four weeks after birth. Body weight, testis weight, testicular morphology (including seminiferous tubule and germ cell characteristics and germ cell mitosis), and testis apoptosis were investigated. Global testis gene changes were quantified using mouse OPERON arrays and confirmed by TaqMan arrays. While some morphological effects of MEHP, Lindane and E2 were observed, none were reported for BPA, other than an increase in germ cell apoptosis at the highest dose during the longest exposure. BPA caused some changes in gene expression considered to be more like those caused by E2 than MEHP or lindane.

Comments from the CEF Panel:
The CEF Panel identified the following strengths and weaknesses in this study:
Strengths:

- Number of doses (≥3)

Weaknesses:

- Drinking water consumption (containing BPA) not measured
- Small sample size
- Animal diet and phytoestrogen content not reported
- Insufficient study reporting

Overall, the CEF Panel noted that sample size (n as low as 3) in some groups/analyses was inadequate. It was also difficult to establish what gene changes were due to BPA. Overall, there was no effect of BPA on body weight or testis morphology at ≤5 mg/kg bw per day (≤3.6 mg/kg bw per day HED)

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Markey CM, Wadia PR, Rubin BS, Sonnenschein C and Soto AM, 2005. Long-term effects of fetal exposure to low doses of the Xenoestrogen bisphenol-A in the female mouse genital tract. Biology of Reproduction, 72, 1344-1351.

In order to investigate the effect of in-utero exposure to BPA, pregnant CD-1 mouse dams (8 weeks old) were implanted, from GD9 to PND4, with subcutaneous osmotic pumps containing BPA at 25, 250 ng/kg bw per day [HED: 0.65, 6.5 µg/kg bw per day]. There was no positive control but there was a vehicle (DMSO) control and the day the vaginal plug was observed was GD1. Litters were culled to 10 pups/mother on PND7 and weaned on PND20. The female offspring were sacrificed at 3 months of age (n=6-10/group, 1 pup/litter) on afternoon of prooestrous and only the best cyclers from the BPA groups were studied. The following were then assessed: genital tract morphology and tissue weights BrdU incorporation, apoptosis, ESR1 (Estrogen receptor alpha) and PGR (progesterone receptor) expression. The highesr BPA dose significantly reduced absolute and relative vagina, but not uterus, weight and decreased the absolute volume of the uterine lamina propria. Uterine but not vaginal BrdU incorporation as a marker of DNA systhesis was significantly reduced by the higher BPA dose in the glandular epithelium although apoptosis was not altered. ESR1 and PGR staining and proportion of high intensity staining were increased in the uterine luminal epithelium (and stroma, ESR1). In this well-conducted study the authors conclude that at doses ≤3.6 mg./kg bw per day, in-utero BPA exposure can adversely affect estrogen responsive uterine and vaginal development.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- Feed, cages and bedding content of oestrogens negligible at E-screen

- Use of glass water bottles

Weaknesses:

- Study design (small sample size:, 6/group in some cases)

In addition, there may be some bias in the study since only the "best cycling" mice were selected for study.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Mendoza-Rodriguez CA, Garcia-Guzman M, Baranda-Avila N, Morimoto S, Perrot-Applanat M and Cerbon M, 2011. Administration of bisphenol A to dams during perinatal period modifies molecular and morphological reproductive parameters of the offspring. Reproductive Toxicology, 31, 177-183.

Adult female Wistar rats were individually housed and treated with BPA via drinkiong water to achieve a dose of approximately 1.2 mg/kg bw per day [HED: dams 0.86 mg/kg bw per day] from GD6 – PND21, with control receiving ethanol vehicle in their water. There was no positive control. Offspring body weights were monitored and the males removed at weaning. At 3 months of age thew females were monitored for oestrous cyclicity for 4 weeks and sacrificed on day of oestrous (n=15/group). The uterine horns and ovaries were fixed for histology, TUNEL analysis and ESR1 (estrogen receptor alpha) immunohistochemistry. Serum from the females was analysed for oestradiol and progesterone. BPA had no significant effect on body weight but significantly reduced the proportion of aimals with normal oestrous cycles although the ovaries were morphologically similar between groups. BPA increased uterine luminal epithelial and stromal thickness and increased the proportion on animals with reduced apoptosis significantly. In terms of ESR1 immunoexpression there is some confusion since the figure 5 legend and the image labelling do nbot agree. However, the quantitation presented for immunopositive nuclei indicates a significant reduction in ESR1 nuclear localisation in both lumional and glandular epithelium. The circulating levels of progesterone and oestradiol were not significantly affected by BPA exposure. The authors conclude that perinatal BPA via the dam can disturb the reproductive cycle of female offspring.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- Large sample size (n=15 pups/group)

Weaknesses:

- Single dose level study Study design (small number of litters:n=5 dams/group)
- Inappropriate statistics (litter effect not considered)
- Drinking water consumption (containing BPA) not measured
- Type of cages /bedding not reported
- Animal diet not reported

The CEF Panel noted that there was some uncertainty about the presentation of the ESR1 immunostaining and would need to be convinced that the differences in staining intensity were consistent across all animals in each group rather than simply variations in staining intensity. In terms of recognition of ESR1 positive nuclei, this would be less certain interms of accurate quantitation in the more heavily stained sections. The lack of effect on ovarian histology and steroid hormone profiles is surprising given the decrease in the proportion of animals with normal cycles.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Nah WH, Park MJ, Gye MC, 2012. Effects of early prepubertal exposure to bisphenol A on the onset of puberty, ovarian weights, and oestrous cycle in female mice. Clinical and Experimental Reproductive Medicine, 38, 75-81.

The authors (Nah et al., 2012) investigated the effects of early prepubertal BPA exposure on the onset of puberty and reproductive parameters such as oestrous cycle and reproductive organ weights in female mice (15 females/dose level group). Female mice were injected subcutaneously at postnatal day (PND) 8 with BPA at the dose levels of 0.1, 1, 10, 100 mg/kg in sesame oil [HED: Pups = 0.87, 8.7, 87, 870 mg/kg bw] or with sesame oil alone. Body weight was measured from PND 10 to 70. Vaginal opening and oestrous cycle were monitored from PND 20 to 29. Animals were sacrificed at PND 25, 30, and 70, and the ovary and uterus weights were measured.

An early prepubertal exposure to BPA at PND8 with subcutaneous administration induced a significant decreased body weight from PND 18 to 30 at dose levels 10 and 100 mg/kg. An early opening of the vagina was observed in all BPA groups, with a mean days of the vaginal opening of 26.4±0.43 in the 0.1 mg/kg BPA group, 26.2±0.28 in the 1 mg/kg BPA group, 26.2±0.57 in the 10 mg/kg BPA group, and 25.9± 0.56 in the 100 mg/kg BPA group versus 27.7±0.61 in the control group. The number of days of estrus was significantly decreased at the highest tested dose level namely 100 mg/kg. The number of oestrous cycle after treatment with BPA at PND 8, was slightly decreased from 10 mg/kg bw BPA dose level without statistical significance. The ovarian tissue weights were significantly lower from 0.1 mg/kg bw and higher versus control group at PND25 but the effect then disappeared at later stages. Uterine weights were significantly lower in the higher dose level group (100 mg/kg bw of BPA) at PND 30 only. At the adult stage (PND 70), the ovarian and uterine weights in the BPA treatment groups were not significantly different from the control group.

Comments from the CEF Panel:
The CEF Panel identified the following strengths and weaknesses in this study:
Strengths:
- Number of doses (≥3)

Weaknesses:
- Animal diet and phytoestrogen content not reported
- Insufficient study reporting

Overall the CEF Panel notes that this study indicated that an early prepubertal exposure to BPA at a single day of treatment (PND8) induces an accelerated onset of puberty exhibited with an early opening of the vagina from 0.1 mg/kg accompanied with a decreased number of days of estrus at 100 mg/kg. Only the highest BPA dose had any significant effect on oestrous cyle length or frequency. The ovary weights were also affected at PND 25 and 30 but return to normal at adult age. Treatment started after completion of many key ovarian developmental events such as primordial follicle formation and statistical analysis was weak (final group size was 5). It is unlikely that a single BPA dose at PND 8 could induce the significant differences reported. Also the implications for reproductive functions are unclear, since the effects were transient and no longer seen at adulthood. The authors did not document the purity of the BPA used nor the type of diet provided to the animals, control of the water or type of cages. No positive control was used in this study.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Nakagami A, Negishi T, Kawasaki K, Imai N, Nishida Y, Ihara T, Kuroda Y, Yoshikawa Y and Koyama T, 2009. Alterations in male infant behaviors towards its mother by prenatal exposure to bisphenol A in cynomolgus monkeys (Macaca fascicularis) during early suckling period. Psychoneuroendocrinology, 34, 1189-1197.

Normally cycling adult female cynomolgus monkeys (5-13 years old) were housed in stainless steel cages, mated and at GD20 subcutaneously implanted osmotic minipumps were used to deliver 10 µg

BPA/kg bw per day [HED: 500 µg BPA/kg bw per day]. The vehicle and control were N,N-dimethylacetamide. The minimumps were placed every 28 days on GD 48, 76, 104, 132. There was no positive control. Offspring were housed with their mothers untiol weaning (6-10months). Pregnancy rates, abortion/stillbirth, gestation length and birth and PND168 weights recorded. In addition behavioural studies were performed of maternal-offspring interactions. BPA administration had no significant effect on any pregnancy characterisc measured including abortion/stillbirth, birth weight, PND168 weights and weaning. Behavioural observations were also made.

Comments from the CEF Panel:
The CEF Panel identified the following strengths and weaknesses in this study:
Strengths:
- Large sample size (18/19 mothers/group)
- Use of non-PC cages

Weaknesses:
- Diet and water bottles not adequately described
- Single BPA dose level used
- Study design (Highly variable animal age and parity not stated)

Overall the CEF Panel noted that this study, showed no significant effect of BPA ≤3.6 mg/kg bw per day during gestation on basic pregnancy statistics in the cynomolgus monkey. The statistical analysis for these aspects of the study were satisfactory. Behavioural effects were reported but these wre considered in the neurotoxicity section of the EFSA CEF Panel, 2010 and therefore only basic pregnancy statistics have been considered here.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Nanjappa MK, Simon L and Akingbemi BT, 2012. The industrial chemical bisphenol A (BPA) interferes with proliferative activity and development of steroidogenic capacity in rat Leydig cells. Biology of Reproduction, 86, 135, 131-112.

This study was also evaluated in relation to proliferative effects in the testis as reported in the section on Carcinogenicity. It is included in this section on developmental and reproductive effects because of the reported effects on steroidogenesis.

This *ex-vivo* study describes the effects of developmental exposure of male rats to BPA via gavage of pregnant and lactating Long Evans dams at 2.5 and 25 µg/kg bw from gestational day 12 to postpartum day 21. Although no exposure measurements were performed the authors estimated, based on previous data, that maternal exposures to BPA at 2.5 and 25 µg/kg body weight represent BPA doses to the offspring of about 8 and 80 pg/kg body weight. Perinatal exposure to BPA did not affect litter size, birth weights of pups and pup sex ratio. Body weights, measured at 21, 35 and 90 days of age, were equivalent in BPA-exposed and control animals ($P > 0.05$). Similarly, paired and relative testes weights (proportion to body weights) were not affected by BPA. However, Leydig cell division was stimulated in the prepubertal period and increased Leydig cell numbers were shown in the testes of adult male rats at 90 days.

Comments from the CEF Panel
The CEF Panel identified the following strengths and weaknesses in this study:
Strengths:
- Large sample size
- Oral administration by gavage
- Phytoestrogen-free diet
- Use of non-PC cages and of non plastic water bottles

Weaknesses:

- Test performed in one sex only

Overall, the CEF Panel noted that the biological significance of small statistically differences in the sophisticated measurements made in this study is unclear, in the context of totally normal pregnancies and littering. Particular care has to be taken in extrapolating findings in rat Leydig cells to humans. A detailed review of comparative physiology and pathology indicated that rats are quantitatively far more sensitive to the development of Leydig cell tumours than men as it appears that Leydig cell luteinizing hormone releasing hormone (gonadotropin-releasing hormone) receptors are unique to rats. Rats also have over 10 times more luteinizing hormone receptors than men[8]. However LH (and indeed AGD, a masculinisation read-out) was not measured which is an omission given the findings presented, and the adaptability of the reproductive axis to small changes in driving signals. It is unlikely that this study confirms an adverse effect of BPA exposure on human male reproductive function as being likely or not without further work (e.g. determination of whether these rats are in fact less fertile).

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Newbold RR, Jefferson WN and Padilla-Banks E, 2007. Long-term adverse effects of neonatal exposure to bisphenol A on the murine female reproductive tract. Reproductive Toxicology, 24, 253-258.

Outbred female CD-1 mice were bred and the pups pooled, separated by sex and all female litters of 8 pups cross-fostered. From PND1-5 the pups received daily subcutaneous injections of 10, 100, 1,000 µg/kg bw per day [HED: pups 87, 870, 8,700 µg/kg bw per day]. Corn oil vehicle and corn oil vehicle control, but no positive control, were used. Mice were weaned at PND21 and housed in single sex cages of 4 animals until sacrifice at 18 months of age. Reproductive tractrs, including ovaries, were fixed, H&E stained and exmined by light microscopy. Lesions were further studied in additional sections or stained with Masson's trichrome stain to confirm if leiomyomas were of smooth muscle origin. Neonatal BPA treatment ha dno effect on body weights and only two statistically significant effects were observed, bith at the HED dose of 870 µg/kg bw per day. Firstly, an increase in incidence from control (7/18) to 14/20 animals exhibiting ovarian cysts and secondly an increase in incidence from control (1/18) to 9/20 animals exhibiting cystic endometrial hyperplasia. None of the other morphological abnormalities showed particularly convincing increases in incidence. The authors conclude tht BPA exposure during critical windows of differentiation may have long-term effects on reproductive tract development and disease.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- Large sample size (16-23/group)

Weaknesses:

- Type of cages and water bottles not reported
- Insufficient study reporting
- Inappropriate statistics (p value not adjusted to multiple testing)

The CEF Panel noted that several abnormalities also occurred in the control group and that with the lack of statistical significance, the wight placed on the non-significant findings by the authors is misplaced. In addition, the authors compared their findings with DES but failed to include a DES positive control in their study.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Newbold RR, Jefferson WN and Padilla-Banks E, 2009. Prenatal Exposure to Bisphenol A at Environmentally Relevant Doses Adversely Affects the Murine Female Reproductive Tract Later in Life. Environmental Health Perspectives, 117, 879-885.

To investigate wheteh in utero exposure to BPA causes adverse effect in female reproductive tracts, pregnant CD-1 mice were doses on GD9-16 with 5 dose levels of BPA by daily subcutabeous injection (0.1, 1, 10, 100, 1,000 µg/kg bw per day [HED: dams 0.0068, 0.068, 0.68, 6.8, 68 µg/kg bw per day]). Corn oil vehicle and corn oil vehicle control, but no positive control, were used. All pups were pooled and cross-fostered (4 male, 4 females pups/litter) and at weaning (PND21) the offspring were housed in same-sex groups of 4. At 16-18 months of age the mice were further investigated by necroscopy, the entire reproductive trascts being fixed and evaluated by light microscopy using at least 5-9 serial images for evaluation of each mouse. If required lesions were stained with Trichrome staining. While 16 offspring/group were used for each exposure, not all could be followed up. BPA had no significant effect on litter size and did not affect the frequency of aimals without corpora lutea. Cystic ovaries were seen in all groups, approaching significance at the HED of 0.068 µg/kg bw per day (p=0.05). Reproted uterine abnormalities were not seen more frequently in BPA-exposed mice. In terms of preneoplastic and neoplastic lesions, most BPA dose levels had no statistically significant effects. The HED dose of 0.0068 µg/kg bw per day was associated with a slightly higher incidence of reproductive tract lesions, while the 0.0068 and 0.068 µg/kg bw per day BPA HED doses increased the total number of lesions, which included mesonephric remnants, from 0/16 in control to 5/14 and 4/13 respectively. The authors conclude tht BPA exposure during critical windows of differentiation may have long-term effects on reproductive tract development and disease.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- Large sample size (13-16)

Weaknesses:

- Use of polycarbonate water bottles and polysulphone cages
- Study design (small number of litters :n=5/group)
- Inappropriate statistics (p value not adjusted to multiple testing)

The CEF Panel noted that the adverse effects reported were more or less inversely related to dose of BPA and while some significant effects of BPA on morphological and./or eoplastic abnormalities were reported, the fact that they were only significant at the lowest 2 BPA dose levels only raises questions about the role of chance. In addition, the authors compared their findings with DES but failed to include a DES positive control in their study.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Otsuka H, Sugimoto M, Ikeda S, Kume S, 2012. Effects of bisphenol A administration to pregnant mice on serum Ca and intestinal Ca absorption. Animal Science Journal, 83, 232-237.

This study examined the effects of BPA administration on serum calcium and calcium metabolism of the gut and kidney in mice. BPA (2 mg or 20 mg/kg bw per day [HED: Dams = 0.06, 0.6 mg/kg bw per day]) was administered by gavage in olive oil to pregnant mice (n= 7) from gestational day 6.5 to gestational day 16.5. Controls (n=4 or 5) received vehicle alone or were untreated. On gestational day 17.5 animals were sacrificed, and determinations were made of serum calcium, alkaline phosphatase (ALP) activity in the duodenum and jejunum and vitamin D receptor (VDR) protein expression in the duodenum, jejunum and kidney (using enzyme histochemical and immunohistochemical analyses,

respectively. Expression of mRNA for VDR, calcium binding protein (CaBP-9k), ECaC2, c-fos, VEGF, occludin, junction adherence molecular A (JAM-A) and ALP were examined in specific regions of the small intestine and expression of mRNA for CYP27B1 was examined in the kidney, using semi-quantitative RT-PCR. Serum calcium was significantly decreased in the mice that had received 20 mg BPA/kg bw per day, and slightly but not significantly reduced in the mice receiving 2 mg/kg bw per day. BPA had no effect on ALP activity and VDR expression in the duodenum and jejunum, while expression of mRNA for occludin and JAM-A in the duodenum and ileum and CaBP-9k and active vitamin D synthesis enzyme (CYP27B1) in the kidney were increased in mice treated with 20 mg BPA. No effect of 2 mg BPA/kg bw per day was reported on these various markers of calcium metabolism. The authors concluded that administration of 20 mg BPA/kg bw per day during gestation results in a decrease in serum calcium, which the authors suggest may be partly due to decreased paracellular Ca absorption.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- Oral administration via gavage

Weaknesses:

- Type of cages and drinking bottles not reported
- Animal diet and phytoestrogen content not reported Small sample size (n=4-7 per treatment group)
- Insufficient study reporting (qPCR – no information on whether the HKG (ACTB) had been properly selected)
- Statistical analysis (no indication that normality of distribution was checked prior to using parametric tests)

This study did not include a positive control. Changes in serum calcium and markers of calcium metabolism were only seen at 20 mg BPA/kg/day (well above 3.6 mg/kg bw per day HED), and were of small magnitude.

This study is not included in the WoE table because it is not relevant to any review question.

Pelch KE, Carleton SM, Phillips CL and Nagel SC, 2012. Developmental exposure to xenoestrogens at low doses alters femur length and tensile strength in adult mice. Biology of Reproduction, 86, 69.

The aim of this study was to examine the effect of developmental exposure to low doses of diethylstilboestrol (DES), BPA or ethinyl oestradiol (EE_2) on bone geometry and torsional strength. C57BL/6 mice were given 0.1 µg/kg/day diethylstilboestrol, 10 µg/kg/day BPA [HED: Dams = 150 µg/kg bw per day], 0.01, 0.1, or 1.0 µg/kg/day ethinyl oestradiol or vehicle from gestation day 11 to post-natal day 12 via a mini-osmotic pump in the dam. Femoral geometry and strength were assessed in the offspring at 10 and 13 weeks of age (females) or 23 weeks (males) by µCT scan and torsional strength analysis, respectively. Hydroxyproline was also measured, as an indicator of collagen content. Exposure to DES, BPA or low dose EE_2 increased adult femur length by small increments (approximately 2.5%). Exposure to the highest dose of EE_2 did not alter femur length, which the authors considered provided evidence of a non-monotonic dose-response. Exposure to EE_2 and DES, but not BPA, decreased femur tensile strength, while no changes were seen in bone collagen content. The authors concluded that developmental exposure to environmentally-relevant levels of xenoestrogens may negatively impact bone length and strength in adulthood.

Comments from the CEF Panel:
The CEF Panel identified the following strengths and weaknesses in this study:
Strengths:

- Adequate positive controls included Use of non-PC cages and of non plastic water bottles

Weaknesses:
- Animal age and body weight not reported
- Single dose level study
- Animal diet phytoestrogen content not reported

Overall the CEF Panel noted that only a single dose of BPA was examined, and the mode of administration was subcutaneous infusion by mini-pump, not supported by any evidence of actual exposure. The magnitude of effects on femur length was small and the claim of a NMDR for EE_2 is very tenuous. The study involved a non-standard analysis of bone in a model of uncertain relevance for humans. Only a single dose of BPA was examined, and the magnitude of effects on femur was small: 2.5% decrease in adult femur length (males) and decrease in energy to failure (males and females) with no concomittant effect on tensile strength or collagen content.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Qiu LL, Wang X, Zhang XH, Zhang Z, Gu J, Liu L, Wang Y, Wang X and Wang SL, 2013. Decreased androgen receptor expression may contribute to spermatogenesis failure in rats exposed to low concentration of bisphenol A. Toxicology Letters, 219, 116-124.

Adult male Sprague-Dawley rats (8 weeks) were administered three dose levels of BPA dissolved in ethanol and then cor noil at 0.0005, 0.5, 5 mg/kg bw per day [HED: Adult males = 0.00036, 0.36, 3.6 mg/kg bw per day] for 8 weeks by oral gavage] for 8 weeks by oral gavage. There were 14 rats/group and the control gropup received the same weight-normalised volume of cornoil as the BPA groups. At harvesting cardiac blood and testes (and other organs) were collected and used to determine circulating and intra-testicular testosterone and sperm analysis using CASA. Testicular histology and steroidogenic and spermatogenic transcripts and proteins were analysed. BPA did not affect organ or body weights, serum biochemistry or hepatonephric function. While circulating testosterone was unaffected, BPA reduced intratesticular testosterone at 5 mg/kg bw per day. This dose also reduced sperm numbers although sperm motility was unaffected. This dose also reduced seminiferous tubule epithelian height, numbers of round spermatids and the ratio of round spermatids/Sertoli cells. Of the spermatogenesis-related genes and proteins, TNP1 and ODF were reduced by BPA at 5 mg/kg bw per day, and also at 0.5 mg in the case of TNP1. Of the steroidogenic genes and proteins StAR and CYP11A1 were increased at 5 mg/kg bw per day dose while HSD17B, HSD3B and CYP19A1 were decreased at 0.5 and 5 mg BPA/kg bw per day. Androgen receptor transcript and protein (but not SHBG) were reduced at 0.5 and 5 mg BPA/kg bw per day.

Comments from the CEF Panel:
The CEF Panel identified the following strengths and weaknesses in this study:
Strengths:
- Number of doses (≥3)
- Oral administration via gavage

Weaknesses:
- Animal diet poorly described
- Study design not appropriate to the scope (corn oil to control rats rather than the vehicle, i.e. ethanol further diluted in corn oil)
- Inappropriate statistics (basic analysis only)

Overall the CEF Panel noted that control rats appeared to receive corn oil only rather than ethanol further diluted in corn oil as was the case for the BPA-exposed groups. Statistical analysis was a little basic with no mention of data normality etc. Otherwise the study was well performed with group sizes. Functional effects were only seen at 5 mg BPA/kg bw per day, with some additional transcript/protein effects at 0.5 and 5 mg BPA/kg bw per day. The results do not suggest the occurrence of BPA effects below 3.6 mg/kg bw per day HED.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Rubin BS, Murray MK, Damassa DA, King JC and Soto AM, 2001. Perinatal exposure to low doses of bisphenol A affects body weight, patterns of oestrous cyclicity, and plasma LH levels. Environmental Health Perspectives, 109, 675-680.

The study has already been evaluated in the EFSA opinion of 2006 (EFSA, 2006) and included in the WoE table as a starting point.

Rubin et al. (2001) measured the effect of BPA on the offspring (n=12 -34) of Sprague-Dawley female rats (n=6) exposed to BPA in drinking water at concentrations of 1 mg/l and 10 mg/l (approximately 100, 1,200 µg BPA/kg bw per day [HED: dams 72, 864 µg/kg bw per day]) from day GD 6 throughout lactation. Water consumption was controlled by measuring the amount of water in the bottles each day, and based on the water consumption the exposure was estimated to be approximately 100, 1,200 µg BPA/kg bw per day. Offsrping were periodically assessed: Males neonatally, 3 and 5 months (n=12-18), Females neonatally and 8 and 12-16 months. Body weights (both sexes were tracked and in females, vaginal opening, AGD, vaghinal cytology and circulating LH were assessed. A statistically significant increased body weight of the offspring from PND 4-11 was observed in both sexes. From day 22 and onwards, only females showed an increased body weight, the effect being greater in the HED 72 µg/kg bw per day group than in the HED 864 µg/kg bw per day group. Patterns of ooestrous cyclicity were determined (n=18-28) by daily examination of vaginal cytology at 4 and 6 months of age. A statistically significant and dose-dependent reduction in the percentage of animals with regular cycles and in the mean number of regular 4 or 5-day ooestrous cycles per animal was found at the highest BPA exposure level. There were no significant effects of BPA on litter size, sex ratios, day of vaginal opening, AGD or macroscopic abnormalities of genital tractsAt the higher BPA dose circulating LH was reduced significantly in long-term ovariectomised females although the biological significance of this is uncertain.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- Use of glass water bottles
- Cages and bedding content of oestrogens negligible at E-screen

Weaknesses:

- Animal diet and phytoestrogen content poorly described.
- Study design (small number of litters (n=6))
- Litter effect not taken into account
- Drinking water consumption (containing BPA) not measured
- Insufficient study reproting

The CEF Panel noted that it was likely that there was underestimation of exposure due to an assumed low level of water consumption.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Rubin BS, Lenkowski JR, Schaeberle CM, Vandenberg LN, Ronsheim PM and Soto AM, 2006. Evidence of altered brain sexual differentiation in mice exposed perinatally to low, environmentally relevant levels of Bisphenol A. Endocrinology, 147, 3681-3691.

The study has already been evaluated in the EFSA opinion of 2006 (EFSA, 2006) and included in the WoE table as a starting point.

CD-1 mice were treated on GD8 via osmotic minipumps delivering 50% dimethylsufoxide as vehicle control or BPA at doses of 25, 250 ng BPA/kg bw per day [HED dams: 375, 3,750 ng BPA/kg bw per day] until PND16. Litters were culled to 4 male and 4 female pups/dam and weaned on PND22-24. Pups used for anatomical and behavioural endpoints were processed before PND22-24 and 27-29 (before puberty) respectively while those used for behavioural studies were also initially examined at 6-9 weeks postnatally. Tyrosine hydroxylase positive neurons in the rostral periventricular preoptic area (AVPV) important for controlling ooestrous cyclicity were counted. Behavioural studies were performed.

BPA had no significant effect on pus/litter or sex ratio. The number of sections through the AVPV was significantly higher in control female than control male brains but not in the BPA exposed groups although the number of sections was significantly reduced in BPA_treated females at the highest dose. Similarly, the number of tyrosine hydroxylase positive neurons were significantly different between male and female offspring in the control and lowest BPA dose groups and significantly reduced at the highest BPA dose in females. Behavioural studies revealed reported that male offspring behaved more like female offspring.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- Use of glass water bottles, diet tested negative at E-screen
- Cages and bedding content of oestrogens negligible at E-screen
- Good sample size (10-17 offspring/group) used for behavioural studies

Weaknesses:

- Study design (sometimes small number of offspring (n=4-8/sex/group) for morphological analyses)
- Litter effect not taken into account
- Insufficient study reporting (not clear how many dams were used per group)

The CEF Panel noted that the higher dose of BPA in particular was associated with a reduction in the sexual dimorphism in tyrosine hydroxylase positive neurons of the AVPV, with the changes in neuron numbers being observed in the female offspring. Both BPA doses were ≤HED 3.6 mg/bk bw per day. While behavioural effects were reported HED ≤3.6 mg BPA/kg bw per day, this was already considered as a starting for neurotoxicity in the EFSA 2006 opinion.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Ryan BC, Hotchkiss AK, Crofton KM and Gray LE, 2010. In Utero and Lactational Exposure to Bisphenol A, In Contrast to Ethinyl Estradiol, Does Not Alter Sexually Dimorphic Behavior, Puberty, Fertility, and Anatomy of Female LE Rats. Toxicological Sciences, 114, 133-148.

In a well-doncducted study of BPA reprotoxicology, Long Evans rats were dosed with BPA at 2, 20, 200 µg/kg bw per day [HED: 1.44, 14.4, 144 µg/kg/day] by oral gavage from GD7 to PND18 Two arms – main study 13-29 dams/ group (0.05, 0.5, 5, 50 µg/kg bw per day) second extended EE group (0.05, 0.15, 0.5, 1.5, 5, 15, 50 µg/kg bw per day) study 6-14 dams/group, with the same BPA regime for both arms. At PND5 litters were trimmed to n=8 pups max, as far as possible balanced for pup sex (4 male, 4 female). 9-38 litters were used per group and the liutter effect was accounted for. Dams and female offspring were studied, the males from the same experiment having been reported by Howdeshell et al (2008). Maternal and pup body weights and neonatal AGD were tracked, the timing of vaginal opening and external genitalia and nipples were assessed. A forced/continuous breeding study with a subset of females for at lkeast 4 months (fecundity) was performed, as were some behavioural studies. BPA had no significant effect on AGD, birth or weaning weights, age or weight at

vaginal opening, lordosis quotient, esternal genitalia, number of pups or fertility/fecundity rates and also had no effects on sex-specific behaviour. The positive control had the expected, dose-dependent effects on the parameters measured.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- ≥3 dose levels of BPA
- Oral administration via gavage
- Large sample size (9-38 litters/group)
- Adequate positive control included (EE2)

Weaknesses:

- Use of polycarbonate cages
- Animal diet and phytoestrogen content poorly descriped (Purina Rat Chow 5008 has high isoflavone content)

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Salian S, Doshi T and Vanage G, 2009. Perinatal exposure of rats to bisphenol A affects the fertility of male offspring. Life Sciences 85, 742-752.

The study has already been evaluated in the EFSA opinion of 2010 (EFSA CEF Panel, 2010) and included in the WoE table as a starting point.

Salian et al. (2009) performed a 3 generation-study assessing the effects of very low oral doses of BPA on the fertility of male Holtzman rats. Eight pregnant rats per group were gavaged with BPA (1.2 or 2.4 µg/kg bw per day), a vehicle control or diethylstilbestrol (DES; 10 µg/kg bw per day) from GD 12 to PND 21. Litters were culled to 4/5 male offspring, weaned on PND 22, cohabited (n=24) on PND 75 with unexposed adult females (n=48) to obtain F2 male generation; by the same procedure, F3 male generation was derived. Fertility was assessed in adult F1-3 males by mating them with unexposed females (not forced/continuous breeding). Immunohistochemical localization of steroid receptors was carried out in the testes of F1, F2 and F3 adults. A significant increase in post implantation loss in the F3 offspring (highest BPA dose) and a decrease in litter size in F1-3 offspring at both BPA concentrations was observed, but a dose-response relationship was only evident for the decrease in litter size. Sperm count and motility were significantly reduced in the F1, F2 and F3 male offspring, with a dose related reduction in sperm count. A reduction in testicular expression profiles of steroid receptors was also observed. Data on vehicle alone (sesame oil) vs vehicle plus alcohol diluent is not shown but stated as not different.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- Oral administration via gavage
- Adequate positive control included (DES)

Weaknesses:

- Type of cages not reported
- Insufficient study reporting (the nature of the diet is unclear: prepared “in house” although stated as soy-free).
- Study design (tsmall numbers of mated F0 dams per group (n = 8),
- Litter effect not taken into account

The CEF Panel noted that this study had several shortcomings. The effects on tissue weights were lost when normalised for body weight, except for F3 seminal vesicle weights. The number of resorptions in the controls appears very low with none in the F1 matings and only one single fetus in one female in each of the F2 and F3 litters. Thus, the apparent effect of BPA pre and post implantation embryo loss may be partly due to atypically low resorption rates in the controls. The CEF Panel found the generation persistence of the reported effects of BPA and DES on F1 males into F2 and F3 mle reproductive system striking and surprising, with some effects becoming more sever with each generation. The explanationis not obvious and this adds to the uncertainty about the effects reported. This uncertainty is exacerbated by the retraction of one of the studies cited in Salian et al. (Transgenerational epigenetic imprinting of the male germline by endocrine disruptor exposure during gonadal sex determination. Chang HS, Anway MD, Rekow SS, Skinner MK. Endocrinology. 2006 Dec;147(12):5524-41 retracted by the authors (Retraction. Transgenerational epigenetic imprinting of the male germline by endocrine disruptor exposure during gonadal sex determination. [No authors listed] Endocrinology. 2009 Jun;150(6):2976), which reduces the supporting literature.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Signorile PG, Spugnini EP, Mita L, Mellone P, D'Avino A, Bianco M, Diano N, Caputo L, Rea F, Viceconte R, Portaccio M, Viggiano E, Citro G, Pierantoni R, Sica V, Vincenzi B, Mita DG, Baldi F and Baldi A, 2010. Pre-natal exposure of mice to bisphenol A elicits an endometriosis-like phenotype in female offspring. General and Comparative Endocrinology, 168, 318-325.

In order to investigate whether BPA exposure might be associated with ediometriosis in women, the authors conducted a study in mice. Adult BALB-C female mice were mated and injected subcutaneously with BPA (in 2% ethanol +saline) from GD1-PND7. The BPA doses were 100, 1,000 µg/kg bw per day [HED: dams, 1,500, 15,000 µg/kg bw per day]. There was no positive control. All pups/treatment were pooled and 5 amle and 5 female pups cross-fostered to each dam. After weaning at PND21, the pups were housed in same-sex cages without further treatment and the females were sacrified at 3 months (n=20/group). The pelvic organs were fixed and studied using light microscopy and immunohistochemistry for ESR1 (estrogen receptor alpha) and HOXA-10. Controls for staining were inadequate since they involved simply omitting the primary antibodies. BPA was measured in maternal livers by HLPC-GC-MS. There were no macroscopic defects at either dose of BPA and although all groups contained animals with cystic ovaries, the incidence was significantly higher in the BPA-exposed animals (9-10/20 vs 2/20), even though there was no effect on the number of corpora lutea. The authors report the lowest dose of BPA increases endometrial adenomatous hyperplasia from 2/10 to 5/20 and atypical endometrial hyperplasia from 0/20 to 3/20, but neither was statistically significant. In terms of the "endometriosis-like structures BPAtreatment was associated ith a statistically significant increase from 1/20 to 6/20 and 7/20 animals, Although BPA was not detected in the control offspring livers, in female offspring in the BPA groups were 0.26±0.06 and 0.24±0.06 ng BPA/g of tissue, significantly lower that maternal BPA levels in the two groups (0.74±0.13 and 1.64±0.53 ng BPA/g of tissue).

Comments from the CEF Panel:
BPA doses and BPA exposure levels in the animals are not internally consistent

The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- Feed content of oestrogens negligible at E-screen
- Cages/bedding tested by E-screen and of non plastic water bottles used
- Large sample size(n=20 offspring)

Weakness:

- Study design (small number of litters (n=6 litters)
- Study design not appropriate to the scope (see text below)

- Lack of mechanistic plausibility (10 fold difference between BPA doses administered but no difference in reported internal BPA levels)

The CEF Panel notes that mice do not get endocmetriosis under natural conditions and the authors did not investigate whether BPA enhanced the establishment of human endometrial tissue as a model of endometriosis, rather they examined so-called "endometriosis-like" symptoms. In addition, the histology shown in the publication and immunohistochemistry was of a poor standard. The "endometriosis-like structures might have been endometrial glandular tissue outside the uterus but could equally well be embryonic remnants. The data and images presented by the authors do not enable a conclusive categorisation to be made. Furthermore, the authors used an unreliable method (HPLC with UV-Vis and fluorescence detection), did not report on the quality of the method, the level of detection, or the level of quatification. No mention is made with respect to the reproducibility and precision of the method.

The last administration of BPA to the mothers was on day 7 after delivery. The mothers were sacrified 2 weeks later (= 336 hours later). Hence, given the half-life of some hours, it is implausible that BPA can be detected after more than 80 half-lifes in the mothers. Similarly, the pups were sacrified at month 3 which is 1848 hours after the last dose. It is implausible that BPA would still be present after such long time periods with respect to the half-life of BPA. Furthermore, it is to be noted that the measured concentrations in the two exposed groups are similar despite the fact that the doses differed by a factor of ten.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Signorile PG, Spugnini EP, Citro G, Viceconte R, Vincenzi B, Baldi F and Baldi A, 2012. Endocrine disruptors in utero cause ovarian damages linked to endometriosis. Frontiers in Bioscience (Elite Edition), 4, 1724-1730.

This is part of a previously reported study in which pregnant Balb-c mice were treated from day 1 of gestation to 7 days after delivery with BPA at 100, or 1 000 µg/kg/day subcutaneously [HED: Dams = 1,500, 15,000 µg/kg bw per day].[7] The vehicle was 2% ethanol in physiological saline. This study reported that the ovaries of BPA-treated mice the number of primordial and developing follicles was lower than in the untreated animals and the number of atretic follicles was higher in the treated animals. This was reported to correlate with animals displaying endometriosis-like phenotype previously reported. At the doses of BPA used, the authors concluded that there was a "dose-dependent" effects on primordial and growing follicle numbers (decreased) and numbers of atreitic follicles (increased), indicating the BPA can negatively disturb ovarian follicle characteristics.

Comments from the CEF Panel:
The CEF Panel identified the following strengths and weaknesses in this study:
Strengths:

- Feed content of oestrogens negligible at E-screen
- Cages/bedding tested by E-screen and of non plastic water bottles used
- Large sample size(n=20 offspring)

Weaknesses:

- Study design (small number of litters (n=6 litters)
- Study design not fit for purpose (see text below)
- Lack of mechanistic plausibility (10 fold difference between BPA doses administered but no difference in reported internal BPA levels)

Overall, the CEF Panel notes that the study was inadequately reported, was not open to unambigious reporting and the data were not convincingly presented. Confidence in the findings is therefore low.

Review of the images in the previous study clearly shows that the glandular changes reported in the fatty tissue near the uterus do not represent endometriosis but merely glandular embryonic rests. It should be underlined that endometriosis does not occur naturally in rodents. The other group differences reported in the uterus and ovaries appear to be within the normal range of changes that can be seen in normal laboratory mice of 3 months of age. There was an attempt to stratify BPA effects of ovarian morphology according to evidence of endometriosis-like phenotype, but this is presented in an opaque manner and is not convincing. This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Tan WJ, Huang H, Wang YF, Wong TY, Wang CC and Leung LK, 2013. Bisphenol A differentially activates protein kinase C isoforms in murine placental tissue. Toxicology and Applied Pharmacology, 269, 163-168.

Six to 8 week old adult female ICR mice were administered 3 dose levels of BPA: 2, 20, 200 mg/kg bw per day [HED: Dams = 0.06, 0.6, 6.0 mg/kg bw per day] in ethanol/corn oil. A vehicle control was included. Pregnant mice were treated by daily oral gavage from E13 – E16. Blood samples and tissues were harvested at E17. Placentae were analysed for corticotrophin-releasing hormone (CRH), PKC and CREB transcript/protein and plasma for oestradiol, testosterone and CRH. Between 3 and 5 mice were used for each measurement although the study started with 6-7 mice/group because of premature deliveries, although this was only statistically significant when the all data from the different BPA doses were pooled. Mice exposed to 20 and 200 mg/kg bw per day had significantly elevated E2, testosteron and CRH although only ther highest dose was associated with increased placental crh transcript and cyp198a1 was not affected. Placental CREB protein was increased in all BPA groups as was the PKC zeta/gamma ratio while PKC delta was only affected at the highest dose. The authors conclude that BPA exposure in pregnant mice might increase premature births by disturbing the endocrine and PKC signalling pathways in the placenta.

Comments from the CEF Panel:
The CEF Panel identified the following strengths and weaknesses in this study:
Strengths:

Number of doses (≥3)
- Oral administration via gavage

Weaknesses
- Animal diet poorly described
- Small sample size (small group size (3-5) for most measures other than pregnancy loss)
- Animal diet and phytoestrogen content not reported

Overall the CEF Panel considers this study as underpowered and preliminary although some of the findings were potentially of interest. In particular, it is noted that the assessment of early pregnancy loss used a good number of animals (>15 mice/dose). On the other side, it is also noted that early delivery was assessed in different group to signalling indices and that effect on early delivery was only significant when analysing all BPA groups and including group >3.6 mg/kg bw per day. As such, these findings need to be reproduced in a much better powered study.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Tinwell H, Haseman J, Lefevre PA, Wallis N and Ashby J, 2002. Normal sexual development of two strains of rat exposed in utero to low doses of bisphenol A. Toxicological Sciences, 68, 339-348.

In a study investigating the effects of BPA exposure in-utero on reproductive development, two strains of rat (Sprague-Dawlery and Alderly Park) were exposed by oral gavage to BPA at 20, 100, 50,000 µg/kg bw per day [HED: dams 14.4, 72, 36,000 µg/kg/day]. Exposure was to pregnant females

(n=7/group) from GD6-21. BPA dosing stability and concentration were confirmed by HPLC-MS. On PND5 litters were culled to 5 males and 5 females as far as possible, but with litter size limited to 8. Sample size (6-7 litters/21-33 individuals/group was good. Maternal and then post weaning diets contained 18.5 and 6.5% soya respectively. Vehicle and vehicle control was arachis oil and ethinyl oestradiol at 200 µg/kg bw per day was used as the positive control. Pup body weights and timing of puberty were monitored and the offspring were assessed by necroscopy at PND90 (males) and 98 (females). Livers and reproductive ract organs were assessed and the vagina and uteru or testis and epididymis were examined histologically. BPA had no significant effect on litter size or sex ratio, body weights, AGD in either sex. In feamles BPA had no significant effect on, age (except for highest dose in AP females) or body weight at vaginal opening or age at first estrus or any organ weights. In males thre was no significant effect of BPA on age or body weight at preputial separation, or any organ weights or on histology of the testis or on sperm production (except for the highest BPA dose in Ap males). However, the ethinyl oestradiol positive control proved toxic with 2 dams terminated early due to severe vaginal bleeding and 2 more at parturition because of liuttering difficulties. The number of pups/litters available for assessment were therefore not adequate. Only a few of the expected effects were therefore statistically significant, introducing a flaw into an otherwise very well conducted study.

Comments from the CEF Panel:
Positive control was flawed – toxic

The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- Oral administration via gavage
- ≤3 BPA dose levels
- Large sample size (6-7 litters/21-33 individuals/group)

Weaknesses:

- Use of polycarbonate cages (PC)

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Tiwari D and Vanage G, 2013. Mutagenic effect of bisphenol A on adult rat male germ cells and their fertility. Reproductive Toxicology, 40, 60-68.

Adult Holtzman male rats were divided into groups of 7 and administered 2 dose levels of BPA (10 µg/kg bw per day and 5 mg/kg bw per day) [HED: Adults = 7.2, 3 600 (3.6 mg) µg/kg bw per day]) orally once per day for 6 days. Controls received the sesame oil vehicle. The males were then repeatedly mated (8 times) up to 56 days post-treatment. Mating implantation and lethal mutation rates (determined as a ratio of implants: live implants), sperm production, motility, morphology and apoptosis were determined. The 5 mg/kg bw per day dose reduced implantation/ebryo survival indices during a single (22-28 days) post treatment interval only and there were not effects on mating or gestation indices. The same dose at the same interval increased post-implantation loss but had not statistical effects, as far as could be determined, on the "dominant lethal mutation). Despite the lack of effect of tested fertility, both BPA doses were associated with reduced sperm production, count and motliltiy although the latter only achieved significance at the higher dose. Only the higher dose caused DNA damage to the sperm. The authors conclude that BPA might be a weak germ cell mutagen.

Comments from the CEF Panel
The CEF Panel identified the following strengths and weaknesses in this study:
Strengths:

- Phytoestrogen-free diet (e.g. soy-free diet)

Weaknesses:

- Study design not appropriate to the scope (e.g. limited number of animals, absence of negative and positive controls, only two dose levels employed and lack of rationale for dose selection)

Overall, the the CEF Panel noted that the precise nature of the oral route was not specified. Statistical analysis was well performed. There is no effect, especially at the lower dose on actual breeding success, which is really the key measure. There are subtle effects on sperm and the lethality measure is interesting although not statistically examined. Likely limited implications for human exposure at the human age equivalent of 8 weeks post-natal in the rat (late teens). The BPA dose having an effect was at 3.6 mg/kg bw per day HED. The results were potentially biased by high background/variability for rodent sperm in the alkaline assay.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Toyama Y and Yuasa S, 2004. Effects of neonatal administration of 17 beta-estradiol, beta-estradiol 3-benzoate, or bisphenol A on mouse and rat spermatogenesis. Reproductive Toxicology, 19, 181-188.

Neonatal male Wistar rats and ICR mice were injected subcutaneously with BPA at species dependent doses ranging between 0.1-10 µg/mouse/injection and 1-600 µg/rat/injection on PND1, 3, 5, 7, 9, 11. To determine the relative dosage and calculate the HED, the CEF Panel calculated the doses relatiove to body weight and determine and average dose across these 11 days. Therefore, BPA dosage was as follows: Mice: 0.014, 0.140, 0.700, 1.401 mg/kg bw per day, Rats: 0.046, 0.461, 4.611, 27.666 µg/kg bw per day [HED: Mice: neonates 0.348, 3.48, 6.09, 12.19 mg/kg bw per day; Rats: neonates 14,26, 142.9, 1,429, 8,577]. Positive controls were oestradiol and oestradiol benzoate and there was a solvent (DMSO) vehicle (olive oil) group as controls. Terminal necroscopy was performed every 2 weeks from 2-10 weeks and PND 24 and 31 with a single fertility (n=11-12) test at 12 weeks for mice and 15 weeks for rats (not a forced/continuous reeding test). The testes were weighed (also seminal vesicles and body weight) and processed for transmission electron microscopy while the cauda epididymides were processed for scanning electron microscopy. These tissues were also examined using light microscopy. The highest BPA dose was fatal to rats and excluded. Note that all rat BPA doses were higher than the 3.6 mg/kg bw per day HED cut-off dose and therefore the findings ≤3.6 BPA mg/kg bw per day are limited to the mouse in the following summary. BPA at the lowest dose had no significant effect on any measure. BPA at 3.48 mg/kg bw per day, approaching the cut-off of 3.6 mg/kg bw per day HED had no significant effects on body, testis or seminal vesicle weights and testicular abnormalities appeared at PND21. Multinucleated giant cells and exfoliation of step 8 spermatids were observed with some mature spermatids being deformed, but not at 3.48 mg/kg bw per day. The effects were no longer seen in adulthood and BPA had no significant effect on litter size, pup birthweights, gestation length or pup sex ratio, demonstrating that the modest effects on the sperm did not impair fertility. Findings for the positive controls were reported as similar as for BPA.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- ≥3 BPA dose levels
- Adequate positive controls included (oestradiol & oestradiol benzoate)

Weaknesses:

- Small sample size ((n=5), especially for the controls (n=3-4)
- Inappropriate statistics (litter effect not considered and statistical analysis not described)
- Type of cages and drinking bottles not reported
- Animal diet poorly described and insufficient study reporting

The CEF Panel notes that the 3.48 mg BPA/kg bw per day dose in mice had some mild adverse effects on gross sperm phenotype but not on electron microscopy identified defects seen with the positive controls and the BPA dopses >3.6 mg/kg bw per day.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Veiga-Lopez A, Luense LJ, Christenson LK and Padmanabhan V, 2013. Developmental programming: Gestational bisphenol-A treatment alters trajectory of fetal ovarian gene expression. Endocrinology, 154, 1873-1884.

Adult Suffolk ewes were treated by sc injections with a single dose level of BPA: 0.5 mg BPA/kg bw per day [HED: dams assumed to be 0.5 mg/kg bw per day in the absence of relevant data] from GD 30 to 90 (of 147) and controls received corn oil alone. BPA levels in arterial umbilical blood samples were monitored at GD 90. Ovaries from 4-5 fetuses/group were obtained at GD 65 and 90. Fetal blood BPA was measured using HPLC-ESI-MS/MS using quality assurance methods of BPA blanks and 13C12-BPA spiking and recovery coirrected by isotopic dilution. RNA recovered from the fetal ovaries was analysed for steroidogenic, hormone receptor and ovarian developmental transcript expression and screened via 2 human miRNA Panels (Exiqon, n=3 ovaries/group). Free BPA increased from 0.4 ng/ml in controls to 2.6 ng/ml in BPA exposed fetuses. CYP19A1 and SRD5A1 were reduced at 65 but not 90 GD in BPA exposed ovaries but had no effect on the pattern of transcript changes between GD 65 and GD 90. BPA exposure downregulated 45 miRNA at GD65 but only 11 at GD 90.

Comments from the CEF Panel:
The CEF Panel identified the following strengths and weaknesses in this study:
Strengths:
- BPA measurement in serum

Weaknesses:
- Single dose level study
- Diet phytoestrogen content not reported

Overall, the CEF Panel notes that this was a well-performed study by a very well known group in the field of sheep developmental endocrinology. The consequences of the changes are not obvious and the recovery of the two altered transcripts renders the significance of the effect at GD65 uncertain, especially since far fewer changes were seen at GD90. Similarly, it is not known whether the miRNA changes would have developmental or post-natal consequences. The lack of any morphological data, e.g. germ cell numbers, markedly limits the relevance of these findings.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Xiao S, Diao H, Smith MA, Song X, Ye X, 2011. Preimplantation exposure to bisphenol A (BPA) affects embryo transport, preimplantation embryo development, and uterine receptivity in mice. Reproductive Toxicology, 32, 434-441.

This study examined the effect of BPA on embryo implantation in the mouse. Pregnant C57BL6 female mice received daily subcutaneous injections of BPA in sesame oil to provide doses of 0, 0.025, 0.5, 10, 40, and 100 mg/kg bw per day [HED: Dams = 0.375, 7.5, 150, 600, 1 500 mg/kg bw per day], from gestation days 0.5–3.5. Additionally, the presence and location of progesterone receptor (PR) was determined in the 4.5 gestation day uterus using immunohistochemistry. No implantation sites were detected in females receiving 100 mg BPA/kg bw per day on gestation day 4.5, retention of embryos in the oviduct and delayed embryo development were observed on day 3.5. Similarly, no implantation sites were detected on day 4.5 when untreated healthy embryos were transferred to pseudopregnant females treated with 100 mg BPA/kg bw per day. Implantation was delayed in mice treated with 40 mg BPA/kg bw per day. Consequential effects of the delayed implantation included significantly increased gestation periods, reduced litter size and reduced postnatal survival rate.

Altered presence and location of progesterone receptor (PR) was reported in mice treated with either 100 or 40 mg BPA/kg bw per day Similar effects were not observed in the mice receiving 10 mg/kg bw per day or lower. The offspring females (8–12 weeks old) from the dams receiving 40 mg BPA/kg bw per day were also mated and examined for BPA-induced effects on reproductive parameters including embryo implantation; implantation and other parameters investigated were comparable in the offspring from BPA-treated dams was comparable to controls. The authors concluded that high doses of BPA adversely affect processes critical for embryo implantation, including embryo transport, preimplantation embryo development, and establishment of uterine receptivity.

Comments from the CEF Panel
The CEF Panel identified the following strengths and weaknesses in this study:
Strengths:
- Number of doses (≥3)
- Adequate positive controls included
- Use of non-PC cages

Weaknesses:
- Animal diet and phytoestrogen content not reported

Overall the CEF Panel noted that this appears to be a relatively well conducted study by the subcutaneous route. Group size was variable (4-12 or higher). The 100 mg/kg/day dose was mostly lethal, with only the 40 mg/kg/day having effects of real potential interest. There were significant effects on implantation at dose levels of 40 mg BPA/kg bw per day and above, together with increased gestation periods, reduced litter size, reduced postnatal survival rate and continued expression of progesterone receptors (PGR) in the luminal epithelium of the uteri. However, no significant effects were observed in mice receiving ≤3.6 mg BPA/kg bw day HED.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Zhang HQ, Zhang XF, Zhang LJ, Chao HH, Pan B, Feng YM, Li L, Sun XF and Shen W, 2012a. Fetal exposure to bisphenol A affects the primordial follicle formation by inhibiting the meiotic progression of oocytes. Molecular Biology Reports, 39, 5651-5657.

This study was designed to assess the effects of BPA on germ cell cyst breakdown and primordial follicle formation in CD1 mice. Pregnant mice were treated orally using Eppendorf pipettes with BPA in 0.1% DMSO at doses of 0, 0.02, 0.04, 0.08 mg/kg bw per day [HED: Dams = 0.6, 1.2, 1.8, 2.4 µg/kg bw per day] from 12.5-18.5 of pregnancy, Offspring ovaries were variously analysed at 13.5, 15.5, 17.5 and 19.5 dpc and 3, 5, 7 pnd for meiosis progression, bisulphite sequencing, histology and immunohistochemistry for meiosis progression markers. It is not possible to determine the number of animals used. Dose-dependent effects of BPA were observed, with retention of oocytes in nests (cysts) and reduced primordial follicle numbers. However, numbers of oocytes were higher in the pnd 3 ovaries, possibly linked with delayed meiosis progression and decreased levels of increasingly methylated Stra8. Progression to meiosis prophase I of oocytes was delayed in the 0.08 mg/kg/day treated group.

Comments from the CEF Panel
The CEF Panel identified the following strengths and weaknesses in this study:
Strengths:
- Number of doses (≥3)

Weaknesses:
- Animal species and strain not reported
- Animal age and body weight not reported
- Animal diet and phytoestrogen content not reported

Overall, the CEF Panel notes that this represents a very complex immunohistochemical and morphometric evaluation of the tiny ovaries of neonatal mice where the only statistically significant differences reported were in the offspring of mice given 0.08 mg/kg/day. The study may suggest a potential mechanism by which BPA might reduce female fertility. However, for this study to be considered important to understand human ovarian developmental risks associated with BPA it will need to be repeated at a higher standard with issues such as samples size addressed.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Zhang GL, Zhang XF, Feng YM, Li L, Huynh E, Sun XF, Sun ZY and Shen W, 2013. Exposure to bisphenol A results in a decline in mouse spermatogenesis. Reprod Fertil Dev, 25, 847-859.

This study investigated the effects of BPA administered subcutaneously to male CD-1 mice on testicular morphology, sperm quality and morphology and meiotic progression in the germ cells, together, sperm quality and DNA, together with effects on expression of oestrogen receptor alpha (ER-alpha) and gene methylation in the testis. The study also investigated whether these effects on spermatogenesis were reflected in the offspring of BPA-treated mice mated with untreated females. CD-1 mice (n= 30 per group for histochemical examination of testicular morphology and also effects on the F1 generation) were administered 0, 20 or 40 µg/kg bw BPA in 0.1 % DMSO in saline from postnatal day (PND) 3 to PND 21 (3 weeks), PND 35 (5 weeks) or PND 49 (7 weeks). The authors report a range of effects on spermatogenesis including a significant increase in germ cells in the testis at 3 weeks in mice treated with 40 but not 20 µg/kg bw BPA/day, followed by a significant decrease at both 5 and 7 weeks in mice receiving 20 or 40 µg/kg bw per day BPA. The decrease in absolute number of germ cells was accompanied by a decrease in the population of germ cells entering meiosis and parallel changes were reported in differential germ cell types in the testis. BPA-related increases in the diameter of the convoluted seminiferous tubules were reported in mice at 3 weeks, followed by decreases at 5 and 7 weeks. Morphological abnormalities were seen in the sperm of the BPA-treated animals, together with decreased motility. Oestrogen receptor alpha expression was increased in the testis of BPA-treated mice, however, BPA had no effect on DNA methylation of genes such as *Igf2, Igf2r, Peg3* and *H19*, in germ cells. Finally, exposure of male mice to 40 but not 20 µg/kg bw BPA followed by mating with untreated females resulted in a reduction in offspring body weight and size at PND 14, 21 and 35, together with a reported increased rate of dystocia and poor body condition. The authors conclude that BPA impairs spermatogenesis in the CD-1 mouse and affects the development of F1 offspring of these mice.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in this study:
Strengths:
- None

Weaknesses:
- Insufficient study reporting (lack of experimental details)
- Animal numbers/group and weights not given
- Animal diet and phytoestrogen content not reported

Overall, the CEF Panel noted that in the study the dosing period was quite prolonged compared with many other studies (daily dosing for up to 7 weeks) and given the repeated exposure to relatively high levels of unconjugated BPA, the effects on testis and spermatogenesis are probably not unexpected. Based on the descriptions in the paper, the level of confidence in the methodology is not high, e.g. the morphological and morphometric analyses do not appear to have been carried out blind, and the differentiation of the germ cell population into different cell types is reported with what is considered to be spurious accuracy given the methodology described. The lack of effect on DNA methylation of a number of genes is in contrast with effects found by the same authors in mouse oocytes. This paper is included in the WoE table because of its relevance to one or more questions addressed there.

(iii) Excluded in vivo studies

The following studies were excluded from further evaluation because the doses used all exceeded the HED of 3.6 mg BPA/kg bw per day.

Reference	Calculated HED value for administered dose(s) (cut-off: HED > 3.6 mg/kg bw per day)	BPA Treatment
Adewale HB, Jefferson WN, Newbold RR, Patisaul HB, 2009. Neonatal Bisphenol-A exposure alters rat reproductive development and ovarian morphology without impairing activation of gonadotrophin-releasing hormone neurons. Biology of Reproduction, 81, 690-699	HED (pups) = 15.5, 15,500 mg BPA/kg bw per day	Long Evans rats received sc injections of BPA(50 µg/kg bw per day or 50 mg/kg bw per day) daily from PND 0 to PND 3
Aikawa H, Koyama S, Matsuda M, Nakahashi K, Akazome Y and Mori T, 2004. Relief effect of vitamin A on the decreased motility of sperm and the increased incidence of malformed sperm in mice exposed neonatally to bisphenol A. Cell and Tissue Research, 315, 119-124.	HED (offspring) = 4.35 and 435 mg/kg bw per day	Newborn male mice given daily subcutaneous injections of 0.5 or 50 mg BPA
Crawford BR and Decatanzaro D, 2012. Disruption of blastocyst implantation by triclosan in mice: Impacts of repeated and acute doses and combination with bisphenol A. Reproductive Toxicology, 34, 607-613.	HED (Dams) = 930, 1 815 mg/kg bw per day]for Adult female CF-1 mice	Adult female CF-1 mice with N=10-15 animals/group, were administered 61 or 121 mg BPA/kg bw per day on on GD 1-3
Doshi T, D'Souza C and Vanage G, 2013. Aberrant DNA methylation at Igf2-H19 imprinting control region in spermatozoa upon neonatal exposure to bisphenol A and its association with post implantation loss. Molecular Biology Reports, 40, 4747-4757.	[HED (Neonatal rats) = 124 000 µg/kg bw per day].	Holtzman rats were administered by sc injection a single dose of BPA:2.4 µg/30 µl that the authors state corresponds to 400 µg/kg bw per day.
El-Beshbishy HA, Aly HA, El-Shafey M, 2012. Lipoic acid mitigates bisphenol A-induced testicular mitochondrial toxicity in rats. Toxicology and Industrial Health, 29, 875-887.	HED (Adult rats) = 7.2 mg/kg bw per day	Adult male albino rats were given a single BPA dose of 10 mg/kg bw per day (suspended in 0.2 ml olive oil) orally for 14 successive days to 8 animals/group.

Reference	Calculated HED value for administered dose(s) (cut-off: HED > 3.6 mg/kg bw per day)	BPA Treatment
Karavan JR and Pepling ME, 2012. Effects of estrogenic compounds on neonatal oocyte development. Reproductive Toxicology, 34, 51-56.	HED (Neonatal mice) = 43.5, 435 mg/kg bw per day.	Female neonatal CD1 mice were injected subcutaneously on post-natal days 1-4 with BPAin peanut oil at 5 mg mg/kg/day (or 10 µg per pup) or 50 mg/kg/day based on a mean pup body weight (or 100 µg per pup)
Norazit A, Mohamad J, Razak SA, Abdulla MA, Azmil A, Mohd MA, 2012. Effects of Soya Bean Extract, Bisphenol A and 17β-Estradiol on the Testis and Circulating Levels of Testosterone and Estradiol Among Peripubertal Juvenile Male Sprague-Dawley Rats. Sains Malaysiana, 41, 63-69.	HED (Juvenile rats) = 72 mg/kg bw per day	Juvenile Sprague-Dawley male rats (n=6/group) of high dose (100 mg/kg/bw) were administered by oral gavage BPA dissolved in TWEEN80 from PND22 for 21 days. .
Quignot N, Arnaud M, Robidel F, Lecomte A, Tournier M, Cren-Olivé C, Barouki R, Lemazurier E, 2012b. Characterization of endocrine-disrupting chemicals based on hormonal balance disruption in male and female adult rats. Reproductive Toxicology, 33, 339-352.	HED (Adult rats) = 144 mg/kg bw per day	Adult male and female Sprague-Dawley rats were dosed for 2 weeks by oral gavage with 200 mg BPA/kg bw per day with a vehicle control of corn oil.
Tainaka H, Takahashi H, Umezawa M, Tanaka H, Nishimune Y, Oshio S, Takeda K, 2012. Evaluation of the testicular toxicity of prenatal exposure to bisphenol A based on microarray analysis combined with MeSH annotation. The Journal of Toxicological Sciences, 37, 539-548	HED (Dams) = 75, 1,500 mg/kg bw per day	Female ICR mice (n=6/group) received subcutaneous injections of 5 and 50 mg BPA/kg in cornoil on days 7 and 21 of pregnancy.
Salian-Mehta S, Doshi T and Vanage G, 2013. Exposure of neonatal rats to the endocrine disrupter Bisphenol A affects ontogenic expression pattern of testicular steroid receptors and their coregulators. Journal of Applied Toxicology, 34, 307-318.	HED: (Neonates) = 93,000 µg/kg/day	Male Holtzman rats (n=4/group) were given a single dose level of BPA prepared in ethanol and sesame oil (2.4 µg/pup/day which the authors state corresponds roughly to 300 µg/kg bw per day, given a pup weight of 5–6 g. Exposure was PND 1-5 by sc injection and 8 male pups were used per group.

Reference	Calculated HED value for administered dose(s) (cut-off: HED > 3.6 mg/kg bw per day)	BPA Treatment
Salloum BA, Steckler TL, Herkimer C, Lee JS and Padmanabhan V, 2013. Developmental programming: Impact of prenatal exposure to bisphenol-A and methoxychlor on steroid feedbacks in sheep. Toxicology and Applied Pharmacology, 268, 300-308.	Assuming an HEDF of 1:1 for the sheep in the absence of suitable data, this study used a BPA dose above 3.6 mg/kg bw per day, i.e. 5 mg BPA/kg bw per day	Pregnant adult Suffolk ewes were administered a single dose level of BPA of 5 mg BPA/kg bw per day in cotton oil by sc injection from GD30-90 (of 147 days gestation).

(iv) In vitro studies

Appraisal of strengths and weaknesses and WoE analysis were not carried out for in vitro studies.

Brienõ-Enríquez MA, Robles P, Camats-Tarruella N, García-Cruz R, Roig I, Cabero L, Martínez F, Garcia Caldés M (2011) Human meiotic progression and recombination are affected by Bisphenol A exposure during in vitro human oocyte development. Human Reproduction, 26,2807–2818.

The authors studied the effect of $1x10^{-6}$-$3x10^{-5}$M BPA on the meiotic prophase of primary human oocytes. Oocytes survival was decreased at $1x10^{-6}$M BPA. The percentage of oocytes at pachynema decreased at $1x10^{-6}$ M BPA and higher concentrations, indicating that normal oocyte development was disturbed. Furthermore, MLH1 foci, which were used as a marker for crossing over, were increased at and above 10 µM BPA.

Guo J, Yuan W, Qiu L, Zhu W, Wang C, Hu G, Chu Y, Ye L, Xu Y, Ge RS (2012) Inhibition of human and rat 11β-hydroxysteroid dehydrogenases activities by bisphenol A. Toxicology Letters, 215,126-130.

The effects of BPA on the enzymatic activity of microsomal 11β-Hydroxysteroid dehydrogenase (11β-HSD) was studied in human liver and kidney microsomes, rat testis and kidney microsomes and primary rat Leydig cells. Both isoforms, 11β-HSD1 and 11β-HSD2 were studied. An IC_{50} of $1.48x10^{-5}$ and $1.94x10^{-5}$M was calculated for human and rat microsomal 11β-HSD1, respectively. No inhibiton was detected at 10^{-8} and 10^{-7}M. Similarly, BPA decreased the 11β-HSD1 activity in intact primary rat Leydig cells. However, the BPA concentration was not stated. In addition, BPA reduced the activity of both human and rat microsomal 11β-HSD2.

Lee M-S, Lee Y-S, Lee H-H and Song H-Y, 2012c. Human endometrial cell coculture reduces the endocrine disruptor toxicity on mouse embryo development. Journal of Occupational Medicine and Toxicology, 7,7.

The authors studied the effect of 10^{-8}-10^{-4}M BPA on the number of developing mouse embryos at 2-cell stage. Embryos were cultivated for 72 h either in medium, in vehicle or as co-culture on primary human endometrial cells. It was concluded that co-cultivation has a beneficial effect on the survival of embryos at all BPA concentrations investigated. At 10^{-4}M BPA only embryos in the coculture system survived. The description of the experimental set-up is not complete, especially the meaning of "vehicle" in table 1. Data on the effects of E_2, which was used as control are missing. The statistical analysis of the data appear inconclusive. The data indicate that embryo survival is also affected at lower BPA concentrations. However, a statistical evaluation is missing. The study suffers from limited data reporting and statistics.

N'Tumba-Byn T, Moison D, Lacroix M, Lecureuil C, Lesage L, Prud'homme SM, Pozzi-Gaudin S, Frydman R, Benachi A, Livera G, Rouiller-Fabre V, Habert R (2012) Differential effects of bisphenol A and diethylstilbestrol on human, rat and mouse fetal Leydig cell function. PLoS One 7(12): e51579.

The authors studied the effect of 10^{-12}-10^{-5} M BPA on testosterone secretion by fetal human, rat and mice testis. 10^{-5}M. BPA did not affect the morphology of testes in any of the species investigated. However, testosterone secretion of human Leydig cells showed significant reduction, to 70% of control levels, at 10^{-8}M BPA. The strongest effect was detected at 10^{-5}M. At 10^{-12}M BPA had no effect on the human fetal testosterone secretion. The absolute amount of released testosterone was 252±38 pg/h at gestational week 6.5-7.5 and increased more than 50-fold to 13879±4231 pg/h at gestational week 9.5-10.5, but was highly variable between testis fragments. Therefore, the toxicological relevance of the slight BPA effect is difficult to assess. A significant decrease in testosterone secretion only occurred in rat and mouse testis a 10^{-5}M BPA. This decrease was detected in wild type as well as in ERα$^{-/-}$ mice, indicating that the effects are independent of the ERα receptor. Furthermore, a decrease in testis hormone insulin-like3 (INSL3) mRNA was detected at 10^{-8}M BPA in human testis only. In contrast to BPA, DES decreased the testosterone release in rat and mouse testis only. No effects were seen at 10^{-6} and 10^{-5}M DES.

The results indicate that BPA can affect the development of the human fetal testis, at least in terms of testosterone release. However, the results are limited due to the small numbers of human testes.

Ptak A, Gregoraszczuk EL (2012) Bisphenol A induces leptin receptor expression, creating more binding sites for leptin, and activates the JAK/Stat, MAPK/ERK and PI3K/Akt signalling pathways in human ovarian cancer cell. Toxicology Letters, 210, 332-337.

In human ovarian epithelial carcinoma cells (OVCAR-3) BPA increased cell proliferation at $8.7x10^{-10}$M and higher and leptin receptor expression at $3.5x10^{-8}$M and higher concentrations. Inhibitors of JAK/Stat, MAPK/ERK and PI3K/Akt pathways decreased the OVCAR-3 cell proliferation, indicating that these pathways were potentially involved in the BPA effects. Results from co-treatment experiments with leptin (40 ng/ml) and BPA ($3.5x10^{-8}$M) indicate that both agents activate the same intracellular signalling pathways.

Considering the different expression patterns of leptin receptors in explants of epithelial ovarian cancer (reported by others) and OVAR-3 cells the impact of the present findings is unclear.

Quignot N, Desmots S, Barouki R and Lemazurier E, 2012a. A comparison of two human cell lines and two rat gonadal cell primary cultures as in vitro screening tools for aromatase modulation. Toxicology in Vitro, 26, 107-118.

The effect of $1x10^{-7}$–$5x10^{-5}$M BPA on the aromatase mRNA expression and enzyme activity was studied using two human cell lines (H295R and JEG-3), primary rat granulosa cells and rat Leydig cells. No decrease in cell viability was detected up to $5x10^{-5}$M BPA. Aromatase expression was reduced by $1x10^{-5}$M BPA in unstimulated, cAMP or FSH stimulated rat granulosa cells. However, a decrease in activity was detected in the cAMP stimulated cells only. In rat Leydig cells $1x10^{-5}$M BPA resulted in a down-regulation of the aromatase mRNA in unstimulated cells only.

No change in aromatase mRNA expression was detected in the H295R up to $5x\ 10^{-5}$M PBA, while a 1.3 fold increase in activity was detected at and above $2.5x10^{-5}$M BPA. In contrast a decrease in the mRNA and enzyme activity was detected in the JEG-3 cell line at $2.5x10^{-5}$ and $5x10^{-5}$ M BPA.

The data confirmed cell- and species-specific effects of BPA on microsomal aromatase activity. This was not observed at relevant BPA concentration (< 10^{-5}M).

Sheng ZG and Zhu BZ (2011) Low concentrations of bisphenol A induce mouse spermatogonial cell proliferation by G-protein-coupled receptor 30 and estrogen receptor-α. Environmental Health Perspectives, 119, 1775-1780.

The authors studied the effect of 10^{-12}-10^{-5}M BPA on the proliferation of the spermatogonial cell line GC-1. An induction of proliferation/DNA synthesis was observed at all BPA concentrations with a maximal proliferation at 10^{-9}M. Proliferation is signalled through cGMP-dependent protein kinase (PKG) and epidermal growth factor receptor (EGFR). Based on knock-down and inhibitor experiments it was concluded that the ERα receptor was phosphorylated through a cross-talk between ERα and the G-protein coupled receptor 30 (GPR30) and MAPK-ERK.

This is an important mechanistic study on the activation of the ERα via a non-classical pathway.

Ye L, Zhao B, Hu G, Chu Y, Ge RS (2011) Inhibition of human and rat testicular steroidogenic enzyme activities by bisphenol A. Toxicology Letters, 207, 137-142.

The authors studied the effect of 10^{-11}-10^{-4}M BPA on the enzyme activity of the microsomal 11β-Hydroxysteroid dehydrogenase (11β-HSD), 17β-Hydroxysteroid dehydrogenase 3 (17β-HSD3), the CYP17A1 activity of rat and human testis as well as the testosterone release of rat Leydig cells. The IC_{50} for BPA effects were $7.9x10^{-6}$M for 11β-HSD and $2.6x10^{-5}$M for human and rat microsomes, respectively. The IC_{50} of the CYP17A1 were $1.9x10^{-6}$M and $6.5x10^{-5}$M for the human and rat microsomes, respectively. In addition, 10^{-4}M BPA inhibited the human 17β-HSD3 by 50%. BPA did not affect the testosterone release from rat Leydig cells at concentrations from 10^{-11} to 10^{-6} M. At and above 10^{-5} M a decrease in testosterone secretion was detected. The CEF Panel noted that BPA might affect testosterone release of rat/mouse Leydig cells at and above 10^{-5} M (not relevant in vivo).

Neurological, neurodevelopmental and neuroendocrine effects

(i) Human studies

Braun JM, Kalkbrenner AE, Calafat AM, Yolton K, Ye X, Dietrich KN and Lanphear BP, 2011. Impact of early-life bisphenol A exposure on behavior and executive function in children. Pediatrics, 128, 873-882.

Braun et al. used a prospective birth cohort of 244 mothers and their 3-year-old children to characterize prenatal and childhood BPA exposures by using the mean total BPA concentrations (unconjugated plus conjugated) in maternal (16 and 26 weeks of gestation and birth) and child (1, 2, and 3 years of age) urine samples, respectively. Urine samples were collected during home visits directly into polypropylene specimen cups. Total urinary BPA (free plus conjugated BPA) was measured at CDC by on line solid phase extraction (SPE) coupled to isotopic dilution liquid chromatography tandem mass spectrometry (LC-MS-MS, LOD 0.4 µg/l). Individual BPA concentrations were adjusted for dilution using urinary creatinine concentrations. Behaviour and executive function were measured at 3 years by using the Behavior Assessment System for Children 2 (BASC-2) and the Behavior Rating Inventory of Executive Function-Preschool (BRIEF-P). The BASC-2 was considered the main instrument, and is a validated parent-reported assessment of a child's problem behaviours. The authors focused on six subscales: aggression, attention, hyperactivity, depression, anxiety and somatisation. BPA was detected in over 85% of the urine samples from mothers and in over 97% of those from children, and although child BPA levels fell between the ages of 1 to 3, the analyses showed that child BPA concentrations were higher and more variable than those of mothers. Addressing potential confounding factors, the study found that each 10-fold increase in prenatal BPA concentration was associated with defective behavioural (hyperactivity, aggression, anxiety and depression) and emotional regulations (poorer emotional control) mainly in girls. Results: Anxiety scale all: β=7.0 (95% CI 1.7, 12), girls only: β=12 (95% CI 4.7, 20). Results Depression scale: all: β=4.9 (95% CI 0.0, 9.9), girls only: β=11 (95% CI 3.6, 18).

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- Prospective study design
- Urine, container specified (PP cups)
- Repeated measurements (3)
- Standardised samples (urinary creatinine)
- Analytical method (SPE LC-MS-MS)

Weaknesses:

- Small sample size
- No distinction between unconjugated and conjugated BPA
- Confounding by diet not considered
- Unclear clinical relevance (small effect size, conflicting results in boys and girls)
- Imprecise/unreliable outcome (neurobehavioral parameters scored using parent-reported but validated methods)
- Inconsistent results amongst different studies

Overall, the CEF Panel considers that this study is a follow up of a previous study (Braun et al., 2009) which indicated a negative association between prenatal BPA exposures (maternal BPA concentrations at gestational week 16) and externalizing behaviours (hyperactivity and aggression) at 2 years of age, and the associations were more pronounced in girls than in boys. The BASC-2 instrument was used in both in the 2009 and 2011 studies, and the follow-up study corroborated the results of the first study by showing to some degree similar associations and the same sex difference at age 3. The study is strengthened by the inclusion of childhood BPA measurements. No associations

between childhood urinary BPA (different to maternal urinary BPA) concentration and behaviour or executive functions were seen. The study also adjusted for caregiving environment and biomarkers of other environmental toxicants (low molecular weight phthalates). Although well designed, it has some weaknesses: (i) neurobehavioural parameters were scored on the basis of parent report questionnaires (although validated) in the absence of any direct measure of children's neuropsychological development, (ii) use of spot urine samples and (iii) the weak levels of significance.

This paper is included in the WoE table because of its relevance to one or more review questions addressed there.

Harley KG, Gunier RB, Kogut K, Johnson C, Bradman A, Calafat AM and Eskenazi B, 2013a. Prenatal and early childhood bisphenol A concentrations and behavior in school-aged children. Environ Research, 126, 43-50.

This study investigated associations between prenatal and childhood BPA exposure and behavior in school aged children in a prospective study with 292 mother-children pairs in the in the CHAMACOS pregnancy cohort in California. Spot urine samples for measuring maternal BPA exposure collected from mothers at two time points during pregnancy and at age 5 of the children. Total urinary BPA (free plus conjugated BPA) was measured at CDC by on line solid phase extraction (SPE) coupled to isotopic dilution liquid chromatography tandem mass spectrometry (LC-MS-MS, LOD 0.4 µg/l). For women with two urine samples (n=221) the average was used. BPA concentrations were adjusted for dilution using either urinary creatinine or specific gravity. Unadjusted geometric mean BPA was 1.1 and 2.5 µg/l in mothers and children, respectively. At 7 years of age, the Behavior Assessment System for Children 2 (BASC-2) and the Conners' ADHD/DSM-IV Scales (CADS) were interviewer-administered to the mother (due to low literacy rates) and self-administered by the child's teacher. Answers were summed and compared to national norms to generate T-scores standardized for age and sex for three outcomes: inattention, hyperactivity, and ADHD DSM-IV scales. At 9 years of age, ADHD behavior was observed directly using the Connors' Continuous Performance Test (CPT), a computerized test that assesses reaction time, accuracy, and impulse control by having the child press the space bar as quickly as possible when any letter except the letter X appears on the screen. Information about possible confounders was obtained from the mothers through interviews in English or Spanish by trained interviewers. Maternal urinary concentrations of dialkyl phosphate (DAP) metabolites of organophosphate pesticides (DAP metabolites were measured in the same maternal urine samples as BPA) and polybrominated diphenyl ether (PBDE) flame retardants were evaluated among confounding variables due to study participants coming from an agricultural region and because associations between DAP and attention problems have been reported in the study population. BPA concentrations were examined on the continuous scale (logarithmic) and by ranked categories. For *prenatal BPA* exposure the results showed that higher urinary BPA concentrations in mothers during pregnancy were associated with increased internalizing problem behaviors, i.e. anxiety and depression (BASC-2), in their sons at 7 years of age. Each doubling of maternal BPA concentration was associated with an increase in internalizing scores of 1.8 points (95%CI: 0.3, 3.3) by maternal report and 2.5 points (0.7, 4.4) by teacher's report. Prenatal BPA concentrations were not associated with any behaviors measured on the CADS at 7 years or in boys or girls. Similarly, prenatal BPA concentrations were not associated with any behavior at measured by direct observation at age 9 (CPT). For *childhood BPA* exposure the results showed that higher urinary BPA concentrations in the children at age 5 were associated with increased internalizing problems and increased ADHD behaviors in both boys and girls and increased externalizing behaviors, including conduct problems, in girls at age 7 years. Each doubling of urinary BPA concentrations at age 5 in girls was associated with an increase in ADHD score at age 7 of 1.3 (95%CI: 0.4, 2.2) by maternal report and 1.7 (0.3, 3.1) by teacher's report. No associations were seen with BPA concentrations at 5 years and any behavior at age 9 (CPT) in boys or girls.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:

Strengths:

- Prospective study
- Urine, container specified (PP cups)
- Repeated measurements (>1, maternal urine)
- Standardized samples (urinary creatinine and specific gravity)
- Analytical method (SPE LC-MS-MS)
- Quality control, including blanks
- Multiple outcome assessment (maternal and teacher-report at age 7 and direct observation at age 9)

Weaknesses:

- Small sample size
- Single spot urine BPA measurement
- No distinction between unconjugated and conjugated BPA
- Confounding by diet not considered
- Unclear clinical relevance (small effect size, conflicting results in boys and girls)
- Generalisability to the overall population (low-income Mexican American population)
- Inconsistent results amongst different studies

Overall, the CEF Panel considers that this study showed associations between prenatal BPA exposure and behavioral problems in boys, and between childhood BPA exposure and behavioral problems in both boys and girls at age 7 years. However, no associations were found for prenatal or childhood BPA exposure and children's behavior assessed by direct observation at age 9 years. The mothers and children in the study were part of the Center for the Health Assessment of Mothers and Children of Salinas (CHAMACOS) in the agricultural Salinas Valley California, which is a deprived immigrant Mexican-American population. Almost all children were Hispanic, and more than 70% lived below the poverty level. Hence, the generalisability of the results is uncertain. However, the study assessed child behavior by multiple observers at school age and included many relevant confounders, including mother's country of birth, maternal education, marital status, maternal language, child's exact age, HOME score, household income, and number of siblings, maternal depression at 7 years, and maternal pesticide metabolites during pregnancy. The study is strengthened by the prospective design and that the associations were consistent in subgroup and sensitivity analyses. However, the study is limited by not all mothers having two urine samples during pregnancy and a relatively small sample size. No dietary variables were evaluated.

This paper is included in the WoE table because of its relevance to one or more review questions addressed there.

Hong SB, Hong YC, Kim JW, Park EJ, Shin MS, Kim BN, Yoo HJ, Cho IH, Bhang SY and Cho SC, 2013. Bisphenol A in relation to behavior and learning of school-age children. Journal of Child Psychology and Psychiatry, and Allied Disciplines, 54, 890-899.

Urinary BPA concentrations and behavioral and learning characteristics were assessed in a cross-sectional study in a general population of 1 008 children, aged 8–11 years in Korea. Participants were recruited from five different administrative regions of which two were urban cities, two were industrial cities and one was a rural district. Children were invited from two or three schools in each area. Spot urine was collected from each child between 9 and 11 a.m. at school, and total urinary BPA (free plus conjugated BPA) measured liquid chromatography isotopic dilution tandem mass spectrometry (LC-MS-MS, LOD 0.15 µg/l). BPA concentrations were adjusted for dilution using urinary creatinine concentrations. Emotional and behavioural problems of the children were assessed by their parents using the Korean version of the Child Behavior Checklist (CBCL) and the Learning Disability Evaluation Scale (LDES). Blood levels of lead and urinary levels of phthalates and cotinine was also measured and included in the analyses. In addition to the other environmental toxicants, the

analyses adjusted for potential confounding by demographic (age, gender, region, parental education, parental income and child's IQ) and obstetric (maternal age at delivery, gestational age, birth weight) variables and psychiatric family histories. The median Cr-standardized BPA was 1.28 µg/g creatinine (mean 1.32 µg/g creatinine) and median unstandardized BPA was 1.23 µg/l. Higher urinary levels of BPA were positively associated with the CBCL total problems score and negatively associated with the learning quotient from the LDES. The linear association with the CBCL anxiety/depression score and the quadratic association with the LDES listening score were significant after correction for multiple comparisons, and the authors concluded that the results suggested a nonmonotonic relationship.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- Standardized samples (urinary creatinine)
- Analytical method (LC-MS-MS)

Weaknesses:

- Cross-sectional study design
- Single spot urine BPA measurement
- No distinction between unconjugated and conjugated BPA
- Confounding by diet not considered
- Inconsistent results amongst different studies

Overall, the CEF Panel considers that the main limitation of this study is the cross-sectional design. Therefore, the results cannot be used to infer that BPA affects behavior and learning of school-age children. A range of confounders were taken into account, but no dietary variables were considered.

This paper is included in the WoE table because of its relevance to one or more review questions addressed there.

Miodovnik A, Engel SM, Zhu C, Ye X, Soorya LV, Silva MJ, Calafat AM and Wolff MS, 2011. Endocrine disruptors and childhood social impairment. Neurotoxicology, 32, 261-267.

This study investigates prenatal exposure to two ubiquitous endocrine disruptors, the phthalate esters and BPA, and social behaviour in a sample of adolescent inner-city children in New York. Third trimester urines of women enrolled in the Mount Sinai Children's Environmental Health Study between 1998 and 2002 (n=404) were analysed for phthalate metabolites and BPA. Total urinary BPA (free plus conjugated BPA) was measured at CDC by on line solid phase extraction (SPE) coupled to isotopic dilution liquid chromatography tandem mass spectrometry (LC-MS-MS, LOD 0.4 µg/l). BPA concentrations were adjusted for dilution using urinary creatinine concentrations. Mother-child pairs were asked to return for a follow-up assessment when the child was between the ages of 7 and 9 years. At this visit, mothers completed the Social Responsiveness Scale (SRS) (n=137), a quantitative scale for measuring the severity of social impairment related to Autistic Spectrum Disorders (ASD) in the general population. Social responsiveness is based on how the brain processes and responds to external social cues. The SRS is a well-validated quantitative instrument which generates a clinically relevant standardized total score (T-score) as well as subscales for rating e.g. social awareness, social cognition etc. In this study T-scores were calculated separately for males and females. No significant associations between prenatal BPA exposure and T-scores was found (β=1.18, 95% CI -0.75, 3.11), whereas low molecular weight phthalate metabolite concentrations were associated with greater social deficits (T-scores: β=1.53, 95% CI 0.25-2.8), specifically poorer social cognition, social communication and social awareness.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:

Strengths:

- Prospective study design
- Standardized samples (urinary creatinine)
- Analytical method (SPE LC-MS-MS)

Weaknesses:

- Small sample size
- Single spot urine BPA measurement
- No distinction between unconjugated and conjugated BPA
- Confounding by diet not considered
- Imprecise/unreliable outcome (neurobehavioral parameters scored using parent-reported but validated methods)
- Inconsistent results amongst different studies

Overall, the CEF Panel considers that one main limitation of this study is the fact that the urine sample is a single spot sample in third trimester of pregnancy, and may not adequately reflect long-term exposure, but represents only a time point during brain development process. Several factors could contribute to a social behaviour assessed at the age of 7-9 years, and it is difficult to establish a relevant association between prenatal exposure and a long-term effect taking into account of all the possible covariates. The authors examined urinary biomarker concentrations as µg/L as well as after correction for dilution as µg/g creatinine. Exposures were examined on the continuous scale and the statistical handling was good. The present paper, although it does not attempt to estimate any exposure dose by extrapolation from urinary levels of BPA, suggests that there is no association between prenatal BPA exposure and effects on social behaviour of children of 7-9 years old.

This paper is included in the WoE table because of its relevance to one or more review questions addressed there.

Perera F, Vishnevetsky J, Herbstman JB, Calafat AM, Xiong W, Rauh V and Wang S, 2012. Prenatal Bisphenol A Exposure and Child Behavior in an Inner City Cohort. Environmental Health Perspectives, 120, 1190-1194.

This study examined the association between prenatal BPA exposure and child behaviour, adjusting for postnatal BPA exposure in a prospective cohort in New York City comprising a low-income minority population. Pregnant African American and Dominican women were recruited to the study from 1998 through 2003. Inclusion was limited to healthy women aged 18-35 years who did not smoke or use other tobacco products or illicit drugs. Prenatal total BPA was measured in spot urine samples collected from the mother during pregnancy (mean 34 gestational weeks) and from the children between ages of 3 and 4 years. Total urinary BPA (free plus conjugated BPA) was measured at CDC by on line solid phase extraction (SPE) coupled to isotopic dilution liquid chromatography tandem mass spectrometry (LC-MS-MS, LOD 0.4 µg/l). BPA urinary concentrations were adjusted for dilution using specific gravity. Child behavior was assessed using the Child Behavior Check List (CBCL) in children between 3 to 5 years of age. Research workers trained in neurodevelopmental testing oversaw the completion of the CBCL by the mothers. The study sample comprised 198 mother child pairs with complete data on pre- and postnatal BPA measurements, with available data on the outcome and with data on potential confounding variables. The results indicated that prenatal exposure to BPA affected child behavior, particularly in boys. *Prenatal* exposure to BPA was dichotomized (first three quartiles vs. last quartile) and a weighted association (weighted for recent child BPA exposure) was found for high BPA and emotional reactivity (increase, $p<0.01$, OR=1.62 [95%CI:1.13, 2.32]) and aggressive behavior (increase, $p<0.01$, OR=1.29 [CI: 1.0, 1.53]) in boys, and anxiety/depression (decrease, $p<0.05$, OR=0.75 [CI: 0.57, 0.99]) and aggressive behavior (decrease, $p<0.05$, OR=0.82 [CI: 0.70-0.97]) in girls, indicating that girls in the high prenatal BPA exposure group had, on average, fewer reported problems in these areas than girls in the low exposure group. *Postnatal* BPA urinary concentration alone had a significant negative effect only on Emotionally

Reactive within the entire sample OR=0.76 (CI: 0.59, 0.97), p=0.029, and was not associated with the other six sub-scores or the composite scores on internalizing or externalizing problems in the entire sample or in boys and girls separately.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- Prospective study
- Standardized samples (specific gravity)
- Analytical method (SPE LC-MS-MS)

Weaknesses:

- Small sample size
- Single spot urine BPA measurement
- No distinction between unconjugated and conjugated BPA
- Confounding by diet or by concurring exposure factors not considered
- Unclear clinical relevance (small effect size, conflicting results in boys and girls)
- Imprecise/unreliable outcome (neurobehavioral parameters scored using parent-reported but validated methods)
- Generalisability to the overall population (low-income African American and Dominican women)
- Inconsistent results amongst different studies

Overall, the CEF Panel considers that the inclusion of both prenatal and postnatal BPA exposure is an advantage. The statistical analysis is overall acceptable. BPA exposure was dichotomized, and no results for continuous BPA exposure were reported. The associations were weak. The authors examined sex-specific effects, but did not sufficiently explain the opposing effects on behaviour in girls and boys. This is notable, as the observed sex differences were inconsistent with results reported in the two studies by Braun et al. (2009, and 2011), which reported evidence of adverse effects predominantly in girls. The study used density but not creatinine to adjust urinary BPA. The authors assert in the discussion the uncertainty of the actual fetal exposure related to the use of single spot urine samples for measuring BPA.

This paper is included in the WoE table because of its relevance to one or more review questions addressed there.

Yolton K, Xu Y, Strauss D, Altaye M, Calafat AM and Khoury J, 2011. Prenatal exposure to bisphenol A and phthalates and infant neurobehavior. Neurotoxicology and Teratology, 33, 558-566.

This study examined associations between BPA- and phthalates exposures pregnancy and infant neurobehavior at 5 weeks. The study used data from the birth cohort in the Cincinnati area (same as Braun et al., 2009 and 2011) and included 350 mother/infant pairs. BPA and phthalates were measured in spot urine samples collected at gestational weeks 16 and 26. Total urinary BPA (free plus conjugated BPA) was measured at CDC by on line solid phase extraction (SPE) coupled to isotopic dilution liquid chromatography tandem mass spectrometry (LC-MS-MS, LOD 0.4 µg/l). Urinary BPA was adjusted for dilution using urinary creatinine concentrations. Infant neurobehavior was examined by the NICU Network Neurobehavioral Scale (NNNS), a tool suited to reveal changes in the neurobehavioral development of typical infants. The domains on the NNS scale examined in the study included attention, arousal, regulation, handling, movement quality, excitability, lethargy, nonoptimal reflexes, asymmetry, hypotonia, stress/abstinence. Detectable BPA was found in over 90% of the maternal urine samples at two time points (16 and 26 weeks). The paper considered simultaneous exposure to BPA and phthalates, and reported that the correlation between $\log_2$ BPA and $\log_2$ phthalate metabolites (DBP, DEHP) at 16 weeks were r=0.50 and r=0.42, respectively, and at 26

weeks were r=0.28 and r=0.21, respectively. Some significant associations were found for phthalate metabolites at 26 weeks (e.g. DEHP associated with more non-optimal reflexes in males), while no significant associations were found between prenatal BPA exposure and infant neurobehaviour (domains of NNS scale) (p>0.1 for all).

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- Prospective study
- Repeated measurements (2, maternal urine)
- Standardized samples (urinary creatinine)
- Analytical method (SPE LC-MS-MS)

Weaknesses:

- Small sample size
- No distinction between unconjugated and conjugated BPA
- Confounding by diet not considered
- Inconsistent results amongst different studies

Overall, the CEF Panel considers that the results of this study show that BPA levels in urine were not associated with infants' neurobehaviour. The statistical analysis is overall acceptable. PBA and phthalate metabolites were mutually adjusted, and all models were adjusted for creatinine, infant age in days and sex, and confounding by a range of variables were explored, and included demographic and socio-economic variables and blood lead levels. Limitations were acknowledged by the authors and include: (i) neonatal exposure to BPA and phthalates was not considered, (ii) uncertainty in defining gestational age and (iii) use of spot urine samples. The present paper suggests that there is no association between prenatal BPA exposure and infant neurobehavior at 5 weeks.

This paper is included in the WoE table because of its relevance to one or more review questions addressed there.

(ii) Animal studies

(1) Studies examining effects of BPA on anxiety-like behaviour

Diaz Weinstein S, Villafane JJ, Juliano N and Bowman RE, 2013. Adolescent exposure to Bisphenol-A increases anxiety and sucrose preference but impairs spatial memory in rats independent of sex. Brain Research, 1529, 56-65.

This study investigated possible effects of short term BPA exposure on anxiety-like behaviour (open field, OF, and elevated plus maze, EMP), spatial memory (Object placement, OP) and sucrose preference in adolescent Sprague Dawley rats. Seven week old rats were subcutaneously exposed to 40 µg/kg BPA or saline daily for 12 days (n= 9 male, 9 female per group). Body weights were recorded at arrival and at four additional time points during the treatment period. The animals were group housed according to sex and treatment. Behavioural testing were performed on exposure days 6 (EPM, OF), 9 (OP) and 12 (sucrose preference), respectively. Behavioural measures were obtained in real time by experienced persons blinded to the animal treatment. Data were analysed by two-ways ANOVA (sex, treatment) with Fisher′s LSD tests for post-hoc analyses. The study reports that exposure to BPA statistically significantly increased anxiety-like behaviour (EPM, OF), impaired spatial memory (OP) and increased sucrose preference, in both sexes.

Comments from the CEF Panel:

The CEF Panel identified the following strengths/weaknesses in the study:

Strengths:

- Use of glass water bottles
- Blind evaluation of the observed behaviour

Weaknesses

- Single dose level study
- Study design not appropriate to the scope (behavioural tests performed only once with limitation to one trial, subsequent testing in two different tests on the same day)
- Insufficient study reporting (body weights not measured daily in conjunction with treatment; no information on sexual maturation and insufficient information on recording of behavioural testing; animal diet and phytoestrogen content not reported)
- Inappropriate statistics (repeated measures for the same animal are not taken into account; cycling is not adjusted for in the analysis; no multiple comparison statistics - Fisher's protected LSD test applied which does not adequately protect from Type 1 Error increase due to multiple comparisons)

Overall, the CEF Panel noted that the interpretation of the reported increased sucrose preference in BPA-exposed rats is uncertain (the test may have been conducted according to a protocol, but no reference is given). Preferentially, behavioural data should be automatically recorded. The value of the results obtained from the behavioural testing with elevated plus maze and open field is limited. Both of these tests were conducted once only; at the same day, and both were limited to one trial lasting 5- and 6-minutes, respectively. Subsequent testing at the same day with the same animals in two different tests may potentially influence the animal's performance in the second test (open field).

The study limitations are considered to lie in uncertainties related to exposure, control of environmental BPA-exposure, lack of information of sexual maturation, and shortcomings in the statistical analyses. Thus, the underlying cause of the reported statistically significant behavioural differences appears unclear.

This paper is included in the WoE table because of its relevance to one or more questions addressed there.

Ferguson SA, Law CD and Abshire JS, 2012. Developmental treatment with bisphenol A causes few alterations on measures of postweaning activity and learning. Neurotoxicology and Teratology, 34, 598-606.

For study details see (2) Studies examining effects of BPA on learning and memory.

Fujimoto T, Kubo K, NishikawaY, Aou S, 2013. Postnatal exposure to low-dose bisphenol A influences various emotional conditions. The Journal of Toxicological Sciences, 38(45), 539-546.

Female Wistar rats were exposed to 0.1 ppm BPA in the drinking water (equivalent to about 24 µg/kg/bw day) or no BPA (controls) from the day of delivery to lactation day 7 (n=5/group). Glass bottles were used to control for potential environmental contamination and distilled water replaced the tap water during the exposure period. The rats were fed standard chow (CE-2). At delivery, litters were culled to four of each sex. Offspring of both sexes (n=16-20/group) were assessed at 6 weeks of age in an open field (OF) test, at 7 weeks of age in the Elevated Plus Maze (EPM) and at 9 weeks of age in the Forced Swimming test (FST). Data were analysed by 2-way ANOVA (group, sex), followed by Fisher's PLSD test.

In the OF a main effect of BPA treatment was evident only for duration of rearing, suggesting a hyperactivating effect of BPA. In the EPM no main effect of BPA was found, but females exposed to BPA were more active than BPA males. In the FST, BPA increased the time spent in immobility in male offspring ($p < 0.005$) and decreased ($p < 0.05$) latency time to display the depressive-like behavioural response (floating while immobile) in both sexes compared to controls.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:

Strengths:
- Use of glass water bottles

Weaknesses:
- Single dose level study
- Animal diet and phytoestrogen content not reported
- Study design not appropriate to the scope (small sample size, N = 5 dams and litters per treatment group; littermates treated as independent observations)
- Insufficient BPA-exposure assessment (drinking water consumption (containing BPA) not measured)
- Insufficient study reporting (insufficient information on recording of behaviour testing)
- Inappropriate statistics(litter effect not considered; no multiple comparison statistics, the Fisher's protected LSD test applied does not adequately protect from Type 1 Error increase due to multiple comparisons)

Overall, the CEF Panel noted that information on control of environmental contamination of BPA except for water bottles is lacking (i.e. feed, bedding). The study used one BPA dose level administered through drinking water to lactation dams. The authors estimated the BPA intake for dams to be 24 µg/kg bw per day, but data are not shown. Only 5 dams and litters per treatment group were used and no consideration of the litter factor was included in the statistical analysis. The underlying cause of the reported statistically significant behavioural differences appears unclear, e.g. chance findings of multiple testing. Additionally, the animals response in a Forced Swimming test is discussed and decreased latency to immobility might as well be interpreted as being smart (i.e. being picked out of the water).

This paper is included in the WoE table because of its relevance to one or more questions addressed there.

Gioiosa L, Parmigiani S, Vom Saal FS, Palanza P, 2013. The effects of bisphenol A on emotional behavior depend upon the timing of exposure, age and gender in mice. Hormones and Behavior, 63, 598-605.

This study was aimed at investigating the behavioural effects of developmental exposure to a low dose of BPA with respect to the timing of the exposure, maternal environment, sex and age at testing. Starting from the last week of pregnancy (GD 11) to the first postpartum week (PND 8), dams of the CD-1 mouse strain daily spontaneously drank either corn oil (control group, n=27) or a solution containing 10 µg/kg bw per day BPA (n = 15) from a modified syringe. Mice were reared on a soy-based standard diet. At birth, the litters were cross-fostered in order to differentiate between pre- and postnatal exposure: pups prenatally exposed to BPA were not exposed during lactation, and pups postnatally exposed during lactation were not exposed during pregnancy. Offspring of the two sexes underwent three diverse experimental paradigms for anxiety-related behaviours: as juveniles (PND 28-30, n=12-15 group/sex) a novelty test, and at adulthood (PND 70, n=12-15 group/sex) both the free exploratory open field and elevated plus maze (EPM) tests. Data were analysed by a two-way (sex, group) analysis of variance (ANOVA) with Turkey's HSD test for post-hoc comparisons. At both testing ages, the control females exhibited less anxious-like behaviours, were more active and more prone to explore a novel environment than control males. BPA pre- and postnatally exposed females showed a behavioural profile more similar to control males than females. In this study, the direction of the behavioural changes was affected similarly by the pre- and postnatal exposures, although with a greater effect associated with postnatal exposure primarily in females. BPA *per se* had a main effect on free exploratory open field test as both sexes tended to remain near the home area and were less prone to explore the environment. In general, in all the three tests applied significant interactions between BPA and sex were evident, BPA reducing or reversing sex differences in anxiety-like and exploratory behaviours.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:

Strengths:
- Blind collection and analyses of behavioural data

Weaknesses:
- Single dose level study
- Animal diet and phytoestrogen content not reported (soy-based standard diet used)
- Use of polycarbonate cages and bottles (new ones)
- Insufficient BPA-exposure assessment (BPA dose adjusted to body weight seemingly not on a daily)
- Inappropriate statistics(no correction for multiple comparisons applied; comparison between the two exposure windows is not appropriate since the same dose is used for either gestational or lactational exposure – resulting in a very different internal dose).

Overall, the CEF Panel noted that in this study the group size of offspring for behavioural testing was sufficient and the litter effect was considered. Behaviours were recorded by video in the three tests and adult female checked for ooestrous phase after testing.

The CEF Panel also noted that these findings are not consistent with those of Ferguson et al. (2012); who used twice the dose (25 µg/kg bw per day) during GD 6-21 without finding any effects of BPA. Notably, Gioiosa et al. found stronger impact of BPA in postnatally than in prenatally exposed animals (females) although in utero exposure provides the offspring with much higher BPA levels than via lactation through dams' milk.

This paper is included in the WoE table because of its relevance to one or more questions addressed there.

Inagaki T, Frankfurt M and Luine V, 2012. Estrogen-induced memory enhancements are blocked by acute bisphenol A in adult female rats: role of dendritic spines. Endocrinology, 153, 3357-3367.

For study details see (2) Studies examining effects of BPA on learning and memory.

Jasarevic E, Williams SA, Vanda GM, Ellersieck MR, Liao C, Kannan K, Roberts RM, Geary DC, Rosenfeld CS, 2013. Sex and dose-dependent effects of developmental exposure to bisphenol A on anxiety and spatial learning in deer mice (peromyscus maniculatus bairdii) offspring. Hormones and Behavior, 63, 180-189.

This study was aimed at assessing the behavioural effects of different BPA doses administered in food during pregnancy and lactation to outbred deer mice (Peromyscus maniculatus bairdii), a rodent species that exhibits well-defined sex- and steroid-dependent behaviours. Dams were fed with a phytoestrogen-free diet supplemented with either 7% corn oil, ethinyl oestradiol (0.1 ppb), or one of the three doses of BPA (50 mg, 5 mg, 50 µg/kg feed weight) starting from 2 weeks before mating up to the end of the lactation period (weaning age of the offspring). Litters with singleton births were excluded. To obtain sufficient numbers of offspring, some dams were bred more than once.

After weaning, the pups were maintained on control diet until they reached sexual maturity and then as adults assessed for spatial learning capabilities in a modified Barnes Maze and for anxiety-like and exploratory behaviours in an Elevated Plus Maze, EPM. Data obtain from the Barnes maze were analysed as a split plot in space and time followed by Fisher's protected LSD, whereas 2-way (sex, diet) ANOVA was used to analyse EPM data and a Cohen's d was determined for significant pairwise comparisons. Relative to controls, males exposed to the two upper doses of BPA exhibited similar behaviour as ethinyl oestradiol-exposed males in the Barnes maze (i.e. inefficient search strategy, higher latency to escape maze) and in the EPM (i.e. reduced time spent in open arms of the maze and reduced exploratory behaviours). Females exposed to ethinyl oestradiol, but not to BPA, consistently

exhibited masculinized spatial abilities, namely they outperformed males in the Barnes maze acquisition. According to the author, cycling was not checked for because it is poorly characterized in this species. The author measured serum BPA concentrations in controls (below limit of detection) and in dams on the BPA diet (5.48 ± 2.07 ng/ml), which was said to be similar to that observed in humans (referring to the study of Teeguarden et al., 2011).

The authors conclude that developmental exposure to environmentally relevant concentrations of BPA can affect spatial learning and anxiety-like and explorative behaviour in male offspring in a dose-dependent manner, and significantly reduce the sex differences present in this species.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- Adequate positive controls included
- Number of BPA doses (3)
- Use of non-PC cages and water bottles
- BPA exposure measurement in animal samples (serum)

Weaknesses:

- Study design not appropriate to the scope (multiple breeding; use of littermates in testing; study not controlled for reproductive cycling at testing time; unclear exposure time of the respective dams or if the controls were concurrent with the treated animals; possible additional dietary exposure of the offspring during late lactation)
- Insufficient study reporting (no information on normalization for dams' body weight and feed consumption described; maternal amount of feed consumed daily not specified; unclear whether offspring may have been exposed directly through feed during late lactation when incisors have appeared; total number of dams and their general reproductive outcome not given)
- Insufficient BPA-exposure assessment (see line above)
- Inappropriate statistics(the litter effect was not adequately addressed in the statistical analysis, no multiple comparison statistics - Fisher's protected LSD test applied which does not adequately protect from Type 1 Error increase due to multiple comparisons)

Overall, the CEF Panel noted that in this study three BPA doses and a relatively long exposure period were used to mimic chronic exposure (i.e. 2 weeks prior to mating, during gestation and lactation). Pre mating exposure implies that the authors assume BPA has no influence on fertility and mating succeed index. The study shows an acceptable sample size (n=6-9 dams per group). However, there is uncertainty as to the actual BPA doses given to the animals.

It is also noted that the authors reported that free BPA in serum at the highest dose was similar to that found in pregnant women.

This paper is included in the WoE table because of its relevance to one or more questions addressed there.

Jones BA and Watson NV, 2012. Perinatal BPA exposure demasculinizes males in measures of affect but has no effect on water maze learning in adulthood. Hormones and Behavior, 61, 605-610.

The study aimed to evaluate the effects of perinatal BPA exposure on learning and emotional responses in adulthood. Fifteen Long-Evans female rats were mated in house and orally exposed to vehicle, 5, 50, 500, or 5000 μg/kg BPA by spontaneously licking oil from a syringe from gestational day (GD) 7 to lactational day 14 (n= 3 dams/group, except 500 μg/kg group where n= 2 dams). Litters were culled to equal sex ratio of male and female at delivery. Rats were fed standard Purina chow, had

access to tap water (dams to water sacks) and were housed in polysulfone cages. Perinatally exposed offspring (n=12 rat/sex/group) were examined as adults (PND 90-150) for spatial cognition in the Morris Water Maze (MWM), for depression in the Forced Swim Test (FST) and for anxiety-like behaviour in the Elevated Plus Maze (EPM).

No effect of BPA on spatial cognition was observed in the MWM as evaluated by repeated measure analysis of variance (ANOVA) and Tukey's HSD post hoc test.

The EMP data were first analysed by ANOVA within each sex to find effects of BPA dose levels, followed by Turkey HSD post hoc test to find specific group differences. Results showed that the 500 µg/kg females produced more fecal boli than other females, and that the low dose females had higher percentage of open arm entries than control females. Amongst males, the low dose males travelled longer distance than control males.

Secondly, for the EPM data, t-tests were used to compare sex differences within each dose group. In the control group, female were generally more active than males, except for producing facal boli and closed arm enteries.

The FST-data for day 2 were evaluated by use of repeated measure ANOVA with Tukey's post hoc test. ANOVA did not reveal any significant differences across dose within the male groups, nor were any of the female BPA groups significantly different from the controls on any parameter, but paired comparisons indicated that a number of sex differences in controls appeard to varying degree in the different dose groups.

The authors concluded that the sex differences found between controls were eliminated in the lowest BPA dose groups on both the EPM and FST, with a non-monotonic trend.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- Number of BPA doses (4)
- Use of non-PC cages, and of BPA-free water sacks (for dams only)

Weaknesses:

- Study design not appropriate to the scope (small number (2-3) of dams per group, littermates treated as independent observations, females not controlled for cycling at the time of testing)
- Insufficient study reporting (unclear reports for the statistical findings in the figures/tables presented, especially for FST)
- Inappropriate statistics(because of the limited number of litters tested (2-3) per dose group, the samples size is considered underpowered; litter effect not considered, lack of analysis of the two way interaction between treatment and sex, no adjustment of cycling included)

Overall, the CEF Panel noted that a major weakness of this paper resides in the statistics: each dose group contained only 2-3 litters with a total of n=12 males and n=12 females per dose (i.e. eight to twelve rats coming from the same litter in each dose group), but the litter effect was not even mentioned and a positive control was lacking. The CEF Panel identified statistical shortcomings, in particular, the interactions dose x sex were not presented, which would have been useful to evaluate the extent of overall BPA effects. Furthermore, despite some significant effects in the Elevated Plus Maze test, generally the effect of BPA was small at all the tested doses, and the statistical significances of the results appear to be overestimated. A possible U-shaped trend should have been confirmed by a higher number of animals (litters), more dose groups and appropriate statistics (see Section 1.2).

This paper is included in the WoE table because of its relevance to one or more questions addressed there.

Kundakovic M, Gudsnuk K, Franks B, Madrid J, Miller RL, Perera FP, Champagne FA, 2013. Sex-specific epigenetic disruption and behavioral changes following low-dose in utero bisphenol

A exposure. Proceedings of the National Academy of Sciences of the United States of America, 110, 9956-9961.

For study details see (3) Studies examining the effects of BPA on social behaviour.

Matsuda S, Matsuzawa D, Ishii D, Tomizawa H, Sutoh C, Nakazawa K, Amano K, Sajiki J, Shimizu E, 2012. Effects of perinatal exposure to low dose of bisphenol A on anxiety like behavior and dopamine metabolites in brain. Progress in Neuro-Psychopharmacology and Biological Psychiatry, 39, 273-279.

Pregnant C57BL/6J mice were subcutaneously injected with vehicle or 250 ng BPA/kg bw per day from gestational day (GD) 10 to postnatal day (PND) 20. Every third day from GD 11 to PND 21 the dams were weighed. BPA dose was calculated using 30 grams of expected mouse body weight and the injection volume was 100 microliters. The vehicle contained 0.01% methanol in phosphate buffered saline (pH 7.4). Mice had free access to laboratory chow (CE-2, CLEA Japan, Inc., Tokyo) and tap water. Litters were culled to six pups (3 female and 3 male) at PND 2. Offspring was tested in an open-field test (10 min) as juveniles (4 weeks) and as adults (8 weeks) (N = 12-15 per sex and group).

A two-way (sex×group) ANOVA and multiple analyses according to Bonferroni were used for the open field test, DA and DOPAC levels, and the DOPAC/DA ratio, whereas one-way (group) was used for MAO activity. Correlations between anxiety-like behaviour and DA levels or DA turnover were analysed using Pearson's product-moment correlation test.

In males, exposure to BPA significantly decreased the time spent in the centre area of the open field in both juveniles and adults compared to controls ($p < 0.05$). A similar effect was not seen in females. Locomotor activity was not affected by BPA treatment in either males or females (juvenile or adults).

One week after testing, adult offspring (N = 4-6) were anesthetized with CO_2 and brain samples collected. DA and DOPAC levels and the DOPAC/DA ratio were assessed in the dorsal hippocampus (HIP), amygdala (AMY), and medulla oblongata (MED) by use of high-performance liquid chromatography (HPLC). BPA significantly altered DA turnover only in adult males. Thus, males were investigated for the activity of monoamine oxidase (MAO)-B, the enzyme that metabolizes DA into DOPAC, and which was reduced in the MED area. The authors conclude that these results suggest that an increase in anxiety-like behaviour induced by perinatal exposure to BPA may be related to decreases in DA metabolites in the brain.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- none

Weaknesses:

- Single dose level study
- Study design not appropriate to the scope (dosing not daily adjusted to body weight; the sample sizes for neurochemical assessment were limited (N=4-6)
- Insufficient study reporting (number of dams lacking, general reproductive outcome and information on check for cycling in female offspring not given; type of cages and drinking bottles not reported; animal diet and phytoestrogen content not reported
- Insufficient BPA-exposure assessment (constant during pregnancy and lactation and not adjusted to body weight)

Overall, the CEF Panel noted that parallel examination of neurobiological (dopaminergic markers) and functional end points (behaviour, open field) was performed. However, pregnant mice were given two days of habituation from arriving to the laboratory until dosing started. Anxiety–like behaviour was tested by an open field procedure and measured as reduced time spent in the centre area. A sufficient sample size was used for behavioural testing (n = 8-12), the litter effect is taken into account as pups

from the same litter were not used in the same experiments, and the study attempts to correlate morphological and functional changes. The main limitation of this study lies in the uncertainty regarding exposure, as it appears that dose was not held constant throughout pregnancy and lactation and that only a single dose level was tested, using the subcutaneous route.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Nakamura K, Itoh K, Dai H, Han L, Wang X, Kato S, Sugimoto T, Fushiki S, 2012. Prenatal and lactational exposure to low-doses of bisphenol A alters adult mice behavior. Brain & Development 34 (2012) 57–63

Mated female ICR/Jcl mice were subcutaneously injected vehicle (sesame oil) or 20 µg BPA/kg body weight from the day vaginal plugs was observed until weaning of the offspring at postnatal day (PND) 21 (n=8 dams/group). Litters were culled to 10 pups at delivery. Different offspring subsets of each group were used for early (PND 21-24) and late (postnatal week, P10-13W) behavioural testing.

Offspring were tested early and late (P10W) in open field, early and late (PW11) in elevated plus maze (EPM), and late in Morris water maze (female at P12W and male at P13W). Body weights were recorded the day prior to early and late testing. Data were analysed two-ways ANOVA (treatment x sex) and the Mann-Whitney *U* test was used for post hoc comparisons.

At weaning, BPA-exposed mice had lower body weights than controls (n=39-40/sex per group), but not at start of late testing (P10W) (n=19-20/sex per group). At early and late testing, no group differences were seen in anxiety-like behaviour measured as time spent central areal (open field) or in open arms (EMP). At early open field-testing, female in general spent less time in central area than males. Both at early (EPM) and late (open field) testing, BPA-exposed mice showed motor impairment as the total travelled distance was shorter than controls. At late EMP-testing, females in general travelled shorter distances than males. No group differences were shown for the Morris water maze data. The authors conclude that gestational and lactational BPA exposure causes hypoactivity in adult mice.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:

Strengths:
- None

Weaknesses:
- Single dose level study
- Insufficient study reporting (maternal body weight not given; lack of information on check for cycling in female offspring; no information of environmental BPA-control, i.e. animal diet and phytoestrogen content, type of cages, bedding and drinking bottles not reported)
- Insufficient BPA-exposure assessment (constant during pregnancy and lactation and not adjusted to body weight)
- Inappropriate statistics (litter effect not considered)

The CEF Panel noted that the body weight recordings at weaning indicate that BPA-exposed offspring were more strained than controls, whereas no group differences were seen in adult mice. However, since different subsets of offspring per group were used for early and late testing, the data are not directly comparable. Still, the main limitations of this study are considered to be 1) uncertainty regarding exposure, as it appears that dose was held constant and not adjusted to body weight throughout pregnancy and lactation and that only a single dose level was tested, using the subcutaneous route, and 2) statistical shortcomings as litter effects have been taken into account.

This study is included in the WoE table because of its relevance to one or more review questions addressed .

Patisaul HB, Sullivan AW, Radford ME, Walker DM, Adewale HB, Winnik B, Coughlin JL, Buckley B and Gore AC, 2012. Anxiogenic effects of developmental bisphenol A exposure are associated with gene expression changes in the juvenile rat amygdala and mitigated by soy. PLoS One, 7, e43890.

The present study focused on the effects of perinatal developmental exposure to BPA and/or soy phytoestrogens, respectively, on the ontogeny of sexually dimorphic anxiety-related behaviours in juvenile and adult rats. Transcriptional changes for 48 genes involved in modulation of socio-sexual behaviours and reported sensitive to oestrogens and/or BPA exposure were analysed in the amygdala, a key brain areas for anxiety and fear responses. The mitigating potential of a soy-rich diet on these same endpoints was also analysed.

Mated Wistar dams were exposed to BPA via drinking water (1 mg/L) from gestation (GD 6) during lactation and given soy-based or soy-free diets. A group exposed to ethinyl oestradiol (EE, 50 µg/L) and fed a soy-free diet served as a positive estrogenic control. From weaning at postnatal day (PND) 21 through puberty (PND 40), offspring were directly exposed through drinking water and continued on the diet (+/- soy) their dams were given.

Estimation of dams' exposure to BPA based on water intake without normalization for body weight were 35.2-55.6 µg/day (-/+ soy diet) and 71.8-105.6 µg/day (-/+ soy diet) during gestation and lactation, respectively. Assessment of dams' serum BPA and genistein (GEN), a soy phytoestrogen, indicated that internal maternal dose was within a human-relevant range (referring to the paper by Vandenberg et al. 2007). Positive control dams were estimated to be exposed to about 30 and 1.5 µg EE/day during gestation and lactation, respectively. Offspring were directly exposed to BPA through drinking water from postnatal day (PND) 21-40, about 18-25 µg/day (-/+ soy) (both sexes). Similarly, positive control offspring were estimated to be exposed to about 1 µg EE/day.

Offspring were tested as juveniles PND 24-28 (Light/dark box and Elevated plus maze, EPM) and as adults (EPM) for anxiety-like and exploratory behaviour. Data calculated as percent were analysed by logistic regression, elsewhere three-ways ANOVA (gender, exposure, diet) was used with post-hoc t-tests. Data collected from the EE-group was not incorporated in the overall statistical analysis, but might be compared to a group of interest by a t-test. Juvenile behavioural data showed no sex differences, thus data were collapsed across sexes.

BPA induced anxiogenic-like behaviour in juveniles and loss of sexual dimorphisms in adult exploratory behaviour, but only in the animals reared on the soy-free diet. In the Light/dark box, BPA juveniles (both sexes) fed soy-free diet used significantly higher time to enter the lit chamber compared to soy-free controls (400 vs. 300 sec, $p<0.05$). Similarly, BPA juveniles on the soy-free diet made significantly fewer open arm entries (about 2.5) in the EPM than control animals on the same diet (about 3.6) ($p\leq0.05$). At adulthood, BPA given on soy-free diet did not induce significant anxiogenic-like effects in either sex in the Elevated Plus maze, but BPA may for the parameter latency of open arm entries be able to eliminate the sex-differences seen for the other parameters in this task. Figure 4 in the paper may shows "some consistent sex differences" in all other parameters (F>M) than latency. For latency, no sex difference appears for soy, BPA + soy or BPA + soy-free. The soy-free control has M>F, and the authors concluded that BPA eliminates this difference.

Expression analysis performed in juveniles brains (PND 34) revealed a suite of genes, including a subset known to mediate sociosexual behaviour, associated with BPA-induced juvenile anxiety. Expression of oestrogen receptor beta (Esr2) and two melanocortin receptors (Mc3r, Mc4r) were down-regulated while the significant sex differences in Kiss1 expression was eliminated by BPA exposure. EE exposure did not completely recapitulate the behavioural and transcriptional effects of BPA and soy and mechanisms different from the estrogenic activity of these compounds may be considered. The authors conclude that these results collectively show that the behavioural effects of BPA can manifest during adolescence, but wane in adulthood, and may be mitigated by a soy-diet.

Comments from the CEF Panel
The CEF Panel identified the following strengths/weaknesses in the study:

Strengths:
- Positive control included
- BPA measurement in animal samples (dams' serum)
- Phytoestrogen-free diet (one group)

Weaknesses:
- Single dose level study
- Insufficient BPA-exposure assessment (estimated based on maternal water intake and not normalized to body weight)
- Lack of constant levels of exposure in time (lactational exposure is much lower than the gestational or juvenile exposure).
- Insufficient study reporting (effects of animal breeding schedule not well described, mating was split in four cohorts with no information on distribution of dose groups, insufficient reporting of number of dams, unclear whether parallel behavioural testing of different dose groups of offspring was performed, duration of testing in EMP not given; type of cages and drinking bottles not reported)
- Inappropriate statistics(unclear if litter effect was properly considered).

Overall, the CEF Panel noted that parallel assessment of molecular markers (ER-beta and Kisspeptin1) and functional end points was performed and that the general methods, design and statistical analysis are acceptable.-Cycling was included, and all females were tested in oestrus (when most active). General reproductive outcome (sex ratio) is reported, number of animals in each group and sex are shown in graphs. The sample size was consistent (n=43 in BPA groups and n=53 in BPA+soy group, with a minimum n=29 rats in the EE group). The behavioural results are sound and consistent in the juvenile stage. The protective effects of the soy-enriched diet are interesting although the mechanisms by which such effects are brought about are not explained. No effects of BPA appeared in adults despite a prolonged exposure period compared to the juvenile.
An important limitation was the lack of constant levels of exposure in time (lactational exposure is much lower than the gestational or juvenile exposure). The exposure to BPA was estimated based on water intake and not normalized to body weight for calculation of internal exposure.

This paper is included in the WoE table because of its relevance to one or more questions addressed there.

Viberg H, Fredriksson A, Buratovic S and Eriksson P, 2011. Dose-dependent behavioral disturbances after a single neonatal bisphenol A dose. Toxicology, 290, 187-194.

The paper by Viberg et al. tested in mice of the NMRI strain the effects of neonatal single oral dose of BPA on maturation of spontaneous exploratory behaviour. Pregnant NMRI mice were fed standardised pellets and tap water ad libitum prior to and after delivery. Litters were culled to 10-14 pups within the first 48 hours. 10-day-old pups were exposed to vehicle (10ml 20% fat solution/kg bw) or BPA at 0.32, 3.2 or 4.8 mg/kg administered as single oral doses (gavage) (n=15/group and minimum 3 litters/group). Male mice were used in the behavioural studies: Spontaneous behaviour in a novel home environment was tested for one hour at 2 and 5 months of age (i.e. locomotion, rearing, total activity), and the latter age was followed by subcutaneous injection of nicotine to test for increased activity (nicotine induced behaviour). Additionally, the male pups were tested in an Elevated plus maze at 3 months age, and in Morris water maze at 4 months of age.

Spontaneous and nicotine-induced behavioural data were evaluated by ANOVA using a split-plot design, and for the Elevated plus maze data one-way ANOVA was used. Both these analyses were followed by Tukey's HSD post hoc test. Data from day 1 to day 4 in the Morris water maze test were evaluated by general linear model with Tukey's HSD post hoc test, and data from day 5 was submitted to general linear model test with pairwise group testing using Tukey's HSD post hoc test.

In a novel home environment at 2 months of age, the male pups neonatally exposed to the middle or high dose of BPA (3.2 or 4.8 mg/kg body weight, respectively) showed a statistically significantly decreased activity during the first 20-min period (0-20 min), while during the last 20-min period (40-60 min) a significantly increased activity was evident, compared to the control animals and the lowest dose of BPA (clear dose-response relation). The males exposed to the highest dose of BPA (4.8 mg/kg body weight) were significantly more hypoactive during the first 20-min period (0-20 min) and significantly more hyperactive during the last 20-min period (40-60 min) compared to the males exposed to the middle dose of BPA (3.2 mg/kg body weight). These effects were still present at 5 months of age. Both BPA and control mice responded with increased activity to administration of nicotine, thus showing that the effects of BPA were not mediated by altered functionality of cholinergic system. Neither spatial learning (Morris water maze) nor anxiety-like behaviour (Elevated plus maze) were affected by BPA treatment.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:

Strengths:
- Number of BPA doses (3)

Weaknesses:
- Single acute dose administration
- Small sample size (3-4 litters/group)
- Test performed in one sex only
- Study design not appropriate to the scope (pup body weight recorded only four times during the 6 months study; only male pups behaviourally tested and it appears as the same 12-15 males representing 3-4 litters were used in all four tests; littermates treated as independent observations)
- Insufficient study reporting (number of dams and general reproductive outcome lacking; litters said to be standardised by size (12-14 pups) but sex ratio is not given; type of cages and drinking bottles not reported)
- Inappropriate statistics(because of the limited number of litters tested (3-4), the samples size is considered underpowered; the litter effect is not properly considered in the statistics)
- Phytoestrogen content in animal diet not given

Overall, the CEF Panel noted that information on control of environmental contamination of BPA is lacking (i.e. cages, feed, water bottles, or bedding). The study reports statistically significant hyperactivity and lack of the expected habituation profile in BPA neonatally exposed mice after a single administration. The selection of behavioural tests seems appropriate, however given the unusual profile of BPA exposed mice both at 2 and 5 months, the lack of motor activity impairments in either the Elevated plus maze and the Morris water maze test (testing performed at different ages) is suprising. The deficit shown by BPA-treated male mice is specific to the exploration of a novel environment, but the authors did not provide any mechanistic explanation.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Wolstenholme JT, Taylor JA, Shetty SR, Edwards M, Connelly JJ, Rissman EF. 2011a. Gestational exposure to low dose bisphenol A alters social behavior in juvenile mice. PLoS One, 6, e25448.
For study details, see (3) studies examining the effects of BPA on social behaviour

Wolstenholme JT, Edwards M, Shetty SR, Gatewood JD, Taylor JA, Rissman EF, Connelly JJ, 2012. Gestational exposure to bisphenol A produces transgenerational changes in behaviors and gene expression. Endocrinology, 153, 3828-3838.
For study details, see (3) studies examining the effects of BPA on social behaviour

Wolstenholme JT, Goldby JA and Rissman EF, 2013. Transgenerational effects of prenatal bisphenol A on social recognition. Hormones and Behavior, 64, 833–839
For study details, see (3) studies examining the effects of BPA on social behaviour

Xu X, Tian D, Hong X, Chen L, Xie L, 2011. Sex-specific influence of exposure to bisphenol-A between adolescence and young adulthood on mouse behaviors. Neuropharmacology. 2011 Sep;61(4):565-73. doi: 0.1016/j.neuropharm.2011.04.027. Epub 2011 May 5.

This study addresses behavioural changes in adolescence ICR mice orally (intragastric, ig) exposed to BPA (40, 400 μg/kg/d) or vehicle (sesame oil) for 8 weeks. Body weights were recoded at the start and at the end of the BPA exposure period. Three days after ending of treatment, at the age of 91 days, mice (12 per sex per group) were behaviourally tested for anxiety-like behaviour in the open field (3 minutes) and elevated plus maze (5 minutes), and subsequently for learning and memory in the Morris water maze (4 days acquisition and 5th day probe day) and the step-down passive avoidance task (one training day with 2 minutes habituation and 5 sec foot-shock when stepping down, with retention for stepping down onto the grid floor after 24 h). After the retention test, all mice were tested for sensitivity to foot shock and found sufficiently sensitive.

Two-way ANOVAs (sex x group) were used for the data of the open field, elevated plus maze, step-down passive avoidance, and the probe trial in Morris water maze. Data obtained during the acquisition training of the Morris water maze were analysed by repeated measures mixed ANOVAs (treatment x training day x sex). Additionally, BPA effect on each gender across groups were evaluated separately by 1-way ANOVA (e.g. control males vs. BPA males), as well as sex differences within each group (e.g. control females vs. control males, etc.). Post-hoc Student's t-tests were used for comparisons of individual means. Statistical significance was accepted at $P < 0.05$.

For the open field data, two-ways ANOVA did not find any differences for group or sex, whereas paired comparison within treatment groups found sex difference in controls for rearing and grooming (males more than females). Corresponding sex differences were not found for the BPA groups, and compared to control males, BPA low dose males showed less rearings. In the elevated plus maze, control males had higher number of open arm entries and time spent in open arms (i.e. were more explorative) than control females. Such activities were reduced in BPA males at both dose levels, but increased in females at the highest BPA dose level (i.e. more explorative/decreased anxiety-like behaviour).
In the Morris water maze, no statistically significant differences were found for the probe day. For the four training days, control females showed longer mean escape path-length to the hidden platform (i.e. slower learners) than control males. The lowest dose BPA (40 μg/kg/d) extended the average escape path-length in males at days three and four, but not in females at any dose levels.
In the step-down avoidance task, no sex differences in the latency to step down from the platform 24 h after receiving a footshock were revealed in the controls, but at the lowest BPA dose level due to shortened latency in males compared to control males.
BPA exposure at both 40 and 400 μg/kg/d markedly reduced the body weight of males, and 40 μg /kg/d BPA markedly reduced the body weight of females when compared with the controls of the same sex. No changes were found in the serum sex-hormone levels or in the weights of reproductive organs for either gender.

Comments from the CEF Panel
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:
- Phytoestrogen-free diet

Weaknesses:
- Statistical analysis (multiple comparison statistics not considered)
- Type of cages and drinking bottles not reported

Overall, the CEF Panel noted that four behavioural tests were used in order to reveal effects of BPA on anxiety-like behaviour and on learning and memory, and that the stage of the female cycle was taken into account prior to behavioural testing. However, performing different tests on subsequent days with the same animals may influence the results. Daily administration via gavage provides a controlled BPA exposure, but puts also extra stress on the animals which may influence the results even in a sex specific manner. Whereas some consistency were shown for sex differences in controls in tests for anxiety-like behaviour (male more explorative in open field and elevated plus maze), the mode of action of the reported abolished (or induced) sex difference of BPA is unclear and may be a result of more unspecific effects on both males and females. This may also apply for the reported lack of sex differences for BPA groups in the Morris water maze. The lowest BPA dose seemed to exert consistent reducing learning effect in male in the two learning tests; however this effect is at least questionable as there was no dose-response observed.
Some uncertainty as to the BPA exposure remains since the dose was adjusted only weekly and not daily to animal body weight, and the control of environmental BPA contamination was limited.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Xu X, Hong X, Xie L, Li T, Yang Y, Zhang Q, Zhang G, Liu X, 2012. Gestational and Lactational Exposure to Bisphenol-A Affects Anxiety- and Depression-like Behaviors in Mice, Hormones and Behavior, 62, 480-490.
This study aims to investigate the changes of anxiety- and depression-like behaviours. Pregnant ICR mice were orally exposed to vehicle (sesam oil), BPA 0.4 or 4 mg/kg either from gestational day 7 to 20, or from lactational day 1 to 14. Mice fed soy-free feed with free access to feed and water. After parturition, litters were culled to 8-10 pups/litter.

On PND 49, female offspring underwent bilateral ovariectomy. Adult offspring (N=10 sex/group) were tested for anxiety- and depression-like behaviours in an open field area (PND 56), a dark–light transition (D/LT) task (PND 57), a mirrored maze (MM, PND 58), an elevated plus maze (EPM) tasks (PND 59) and a forced swim (FS) task (PND 60). In the FS task, the time spent immobile is considered indicative of a depression-like behaviour in mice. The other tests are used to measure anxiety-like and explorative behaviours. The remaining mice from 10 litters were used for body weight recording and reproductive organ weight measurements (both PND 56) and for western blot analyses for NMDA (NR1) and AMPA (GluR1) receptors (PND 56, N=5 litters).

Behaviours in all tests were automatically recorded with a computer based video tracking system. Collected behavioural data were analysed by a mixed repeated analysis of variance (ANOVA), including exposure period (gestation and lactation), treatment (0.4, 4 mg/kg/d BPA, control), gender (male or female). Two-way ANOVA was used for the Western blot analysis data.

The results indicated that both gestational and lactational exposures to BPA (both doses) affected body weight at the age of 8 weeks (ps< 0.01, direction of effects depending on period of exposure) and increased anxiety- and depression-like behaviour in mice of both sexes. The results of locomotor activity were inconsistent across tests (e.g. open field, D/LT, MM and EPM). Open field revealed no effect of BPA exposure except for increased grooming frequency in female exposed prenatally to high dose BPA. The prenatally BPA exposed females exhibited an increased anxiety-like state in the D/LT, MM, and EPM tasks, ($0.05 > ps < 0.001$). The postnatally exposed females and the prenatally exposed males exhibited anxiogenic-like behaviour in two tests (D/LT, EMP) whereas the males with lactational exposure exhibited an anxiogenic-like behaviour only in EMP. The results of the FS task showed that gestational exposure (both doses) increased the immobile time in both sexes ($ps < 0.001$), and the same effect was induced by lactational exposure only with 4 mg/kg/d BPA.

Western blot analyses showed that both exposure periods inhibited the expression of the AMPA receptor subunit GluR1 in the hippocampus and amygdala in mice of both sexes, whereas the level of

the NMDA receptor subunit NR1 was increased in the amygdala following gestational exposure but was reduced in the hippocampus of the females with lactational exposure ($p<0.05$). The authors suggest that perinatal exposure to BPA increase anxiety- and depression-like behaviours of adult ICR mice of both sexes but that gestational exposure exhibits a stronger effect than lactational on anxiety-like state in females. Down regulation of AMPA and NMDA receptors in the hippocampus and amygdala may be associated with BPA-induced behavioural changes.

Comments from the CEF Panel
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:
- Phytoestrogen-free diet

Weaknesses:
- Study design not appropriate to the scope (sequence of testing not randomized)
- Insufficient study reporting (oral route not specified)
- Type of cages and drinking bottles not reported
- Statistical analysis (multiple comparison statistics not considered)

Overall, the CEF Panel noted that this study does not detail control of potential environmental sources of exposure, except that soy free feed was used. The study aimed to compare the exposure effects of two developmental windows. The effects of BPA on the end-points considered were about similar irrespectively of exposure period was gestational only or lactational only, although exposure differs by orders of magnitude. The sample size for the behavioural measures was adequate (n=10 per sex per group) and litter effect was considered (1 pup per litter per sex per group). However, sample size for Western blot was limited (n=5), and the sacrificing and brain collection procedures are not detailed. It cannot be excluded that only one week recovery after surgery (ovariectomy) and until test start may have influenced on the results. The use of different behavioural tests and the attempt to relate behaviour with molecular changes at the level of glutamate receptors are considered positive. However, testing different tests on subsequent days may influence on the results. Result for depression-like behaviour in the FS task seems consistent. Similarly, result for reduction of exploration in the EPM and for the anxiogenic-like effects of BPA in females seems to be consistent. The behavioural findings might reasonably be related to the molecular changes reported in the hippocampus and amygdala as there are experimental data indicating that glutamatergic receptors are involved in anxiety/fear responses in rodents.

This paper is included in the WoE table because of its relevance to one or more questions addressed there.

Xu X, Liu X, Zhang Q, Zhang G, Lu Y, Ruan Q, Dong F and Yang Y, 2013a. Sex-specific effects of bisphenol-A on memory and synaptic structural modification in hippocampus of adult mice. Hormones and Behavior, 63, 766-775.
For study details see (2) Studies examining effects of BPA on learning and memory.

(2) Studies examining effects of BPA on learning and memory

Diaz Weinstein S, Villafane JJ, Juliano N and Bowman RE, 2013. Adolescent exposure to Bisphenol-A increases anxiety and sucrose preference but impairs spatial memory in rats independent of sex. Brain Research, 1529, 56-65.
For study details see (1) Studies examining effects of BPA on anxiety-like behaviour

Eilam-Stock T, Serrano P, Frankfurt M, Luine V, 2012. Bisphenol-A impairs memory and reduces dendritic spine density in adult male rats. Behavioral Neuroscience, 126, 175-185.

This paper examined the effects of a single subcutaneous BPA administration on memory and synaptic plasticity in adult (60-70 days old) male Sprague Dawley rats. BPA (40 µg/kg) was administered subcutaneously between training and retention testing in an Object recognition (OR) task and an Object placement (OP) test, respectively, which measure both visual and spatial memory. The two tests were performed 4 days apart and groups were switched between tasks so that controls in the OR test was exposed to BPA in the OP test and vice versa. The authors did not expect lingering effects of BPA due to short half life.

After at time delay of 11 days the rats were exposed to BPA according to the pattern of the OR-groups and underwent a test session. Immediately (40 min) after completion of the learning task, rats were decapitated and brains removed for morphological and chemical analyses. Dendritic spine density in pyramidal cells in CA1 and medial prefrontal cortex (mPFC) were evaluated by Golgi preparations in a subgroup of rats (n=7) receiving the same dose of BPA, while the activity of several proteins involved in memory consolidation processes were examined by Western blotting. The behavioural data were analysed by two-way repeated measures ANOVA (Group x Object) with post hoc one-tailed, paired t test between the time spent with new vs. old objects/placements during the recognition trial in each of the groups (BPA and control). Apical and basal spine densities were analysed by two-way ANOVA (group x area) followed by post hoc t-tests.

In BPA exposed rats, significantly impaired object recognition and detection of spatial novelty were reported ($p<0.05$). In the rats which underwent memory trials, BPA significantly decreased spine density in both areas evaluated. In the additional group, BPA caused no differences in dendritic spine density in any areas and the values were generally lower than in the tested rats. The authors comment that in the absence of memory consolidation processes, the increase in spine density is moderate. Additionally, BPA significantly decreased PSD-95, a synaptic marker, in the hippocampus and increased cytosolic pCREB, a transcription factor, in mPFC. The authors conclude that these findings show that a single dose of BPA may block the formation of new memories by interfering with neural plasticity processes in the adult brain.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- None

Weaknesses:

- Small sample size (n = 6)
- Single acute dose administration
- Single dose level
- Study design not appropriate to the scope (all test animals were exposed to BPA, no control group)
- Testing performed in one sex only (male)
- Animal diet and phytoestrogen content not reported

The CEF Panel noted that the study was performed in adult rats using an acute subcutaneous administration which limit its value in risk assessment, but that a similar experimental protocol is challenging to apply in developing rats due to the complexity of the learning task. Parallel assessment of neurobiological markers (decreased spinogenesis and PSD95) in two different brain areas and functional effects has been done, but the sample size was rather small (n = 6). The study design imply that all animals were exposed to BPA.

This paper is included in the WoE table because of its relevance to one or more questions addressed there.

Ferguson SA, Law CD and Abshire JS, 2012. Developmental treatment with bisphenol A causes few alterations on measures of postweaning activity and learning. Neurotoxicology and Teratology, 34, 598-606.

Pregnant Sprague-Dawley rats were orally exposed (gavage) on gestational days 6–21 with vehicle, 2.5 or 25 µg BPA/kg bw per day in 0.3% carboxymethylcellullose (CMC), or 5.0 or 10.0 µg/kg bw per day ethinyl oestradiol (EE). Additionally, a naïve control was included as control for potential stress induced by oral gavage. The animals were maintained in a low exogenous oestrogen environment. On PND 1, litters were culled to four males and four females, and the pups were then orally treated on postnatal days 1–21 with the same doses as their dams received. Post-weaning, one offspring/sex/litter, providing a number of 11–12/sex/group, were assessed for treatment-related effects in a Novelty preference test (PND 29), Open field test (PND 40-42), Motor coordination (PND 43-44), Barnes maze (PND 47-50), acoustic startle response (PND 54), and Morris water maze (PND 75-79). Behavioural data were analysed by repeated measure analyses (SAS version 9.2, SAS Institute Inc., Cary, NC). For datasets in which there was a repeated measure (e.g. test days 1–5 for water maze endpoints), within-group correlations were modelled using the heterogeneous AR1 correlation structure. For data without normal distributions, a log transform or an ANOVA on ranks (i.e. Kruskal–Wallis ANOVA) was conducted.

Some treatment-related effects were evident in both BPA and EE-treated animals, but differences were also seen between the naïve and vehicle controls. The main finding with BPA was a dose-related effect on open field activity of male offspring, activity being significantly increased relative to vehicle controls, with EE showing a stronger response. In the acoustic startle reflex test, males of the naïve control, 2.5 µg BPA/kg bw per day, 25 µg BPA/kg bw per day, and 10 µg/kg bw per day EE_2 groups exhibited significantly less startle response on trials 1–5 than males of the vehicle control group. However, there were no significant differences between the BPA and EE_2 female groups and the same-sex vehicle control group. No effects of BPA were observed on motor coordination, spatial learning and memory, although EE did have effects on several of these endpoints.

Overall the authors concluded that BPA had few consistent effects on neurobehaviour typically measured in developmental neurotoxicity studies, in line with the results of earlier oral studies investigating the same endpoints. They noted that EE produced some behavioural alterations, although these were not substantial. The study is one of a series of studies by the same group of authors, investigating possible developmental and neurobehavioural effects of low-dose BPA compared with EE.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:

Strengths:
- Large sample size
- Both naïve and vehicle controls available (to account for the handling of the animal)
- Adequate positive controls included
- Use of non-PC cages and of glass water bottles

Weaknesses:
- Study design not appropriate to the scope (only low doses BPA used)

Overall the CEF Panel noted that this robust well conducted study shows very limited effects of BPA on neurobehavioural endpoints, with the positive control EE, showing more marked, but not substantial changes. Direct exposure of the offspring was used in addition to the exposure through maternal dosing, which in light of the low maternal dose used is an advantage, as well as the use of näive controls makes up for the disadvantage the handling may cause. Multiple tests were performed (Novelty preference test (PND 29), Open field test (PND 40-42), Motor coordination (PND 43-44), Barnes maze (PND 47-50), Acoustic startle response (PND 54, and Morris water maze (PND 75-79). As commented by the authors themselves, the BPA-induced hyperactivity seen in male offspring in

the open field test has not been reported in a number of other similar studies (e.g. Stump et al, 2010), and other measures examined in the study such as novelty preference, did not show evidence of BPA-induced hyperactivity. The decreased startle response in the acoustic study compared with vehicle controls was also apparent in the naïve control and the apparent effect may be due to an aberrant heightened response in the vehicle control. The authors comment that none of the behaviours investigated are known to show sexual dimorphism, and BPA has been reported to affect sexual dimorphic behaviours. These have been investigated in this series of studies and will be reported in a further publication.

A limitation of this study is the use of very low BPA doses. The inclusion of higher doses would have added consistency to the negative results of this study.

This paper is included in the WoE table because of its relevance to one or more questions addressed there .

Inagaki T, Frankfurt M and Luine V, 2012. Estrogen-induced memory enhancements are blocked by acute bisphenol A in adult female rats: role of dendritic spines. Endocrinology, 153, 3357-3367.

The aim of this study was to examine effects of acute BPA exposure, alone and in combination with oestrogens (17-α or 17-β estradiol, E2), on E2-induced memory enhancement and synaptic plasticity in ovariectomized (OVX) and gonadally intact, cycling adult female rats. The study tested effects of BPA alone, and in combination with the most effective E2 doses, on recognition memory.

Female Sprague Dawley rat (83 OVX and 18 intact, 3 months of age) were administered BPA at levels from 0.4 µg/kg to 400 µg/kg by subcutaneous administration. A positive control was not included but some groups of animals received BPA co-administrated with 17α- E2 or 17β- E2. Animals were fed low phytoestrogen food. Two types of hippocampal-dependent memory tasks, object placement (OP) and object recognition (OR), were done. Ovariectomized rats (n=6-8 per dose level) received BPA 30 min before a sample trial (viewing objects) and immediately after the sample trial and retention trials were performed 4 h later. Retention trials tested discrimination between old and new objects (visual memory) or locations (place memory). Those tests were done every 10 days for about 2 months. Thereafter, elevated plus maze (EPM) was used to examine whether BPA indirectly may impair recognition memory by increasing anxiety levels of the subjects. Prior to EPM testing, OVX rats were given 20µg/kg 17-βE2, which enhanced OP memory, and 40 µg/kg bw BPA, which did not affect memory but antagonized E2. Spine density and serum E2 level analysis were done 10 days after the final behavioural tests in OVX rats. In intact, cycling rats vehicle or BPA (40 µg/kg) was administrated immediately after T1 and retention tested 2 hour later. Spine density was assessed at times of memory consolidation (30 min) and retention (4 h) after 17β-E2 (20µg/kg) or BPA (40 µg/kg) + 17β-E2 in OVX animals. For memory tests, data were analysed by one-way ANOVA followed by Fisher's least significant differences (LSD) post hoc tests. Ooestrous phase data were tested by two-way ANOVA (treatment, ooestrous cycle). Data for EPM were tested by one-way ANOVA. For spine density, one-way ANOVA with Newman-Keuls post hoc tests were used.

In OVX animals treated with BPA, no statistical significant difference was observed in the spatial OP and non spatial OR memory consolidation test. When given immediately after the sample trial, BPA at 1–400 µg/kg bw, did not alter recognition memory, but doses from 4 µg/kg blocked 17β-E2-dependent increases in OP memory enhancement and from 40µg/kg OR memory consolidation. BPA inhibits 17-αE2-induced OP memory enhancements from 1µg/kg. No inhibition of the 17α-E2-induced OR memory enhancements was observed with BPA treatments. No significant effect on anxiety was observed in the elevated plus maze with E2 (20 µg/kg) nor BPA (40 µg/kg) or in combination. BPA, given to cycling rats at 40 µg/kg (unique dose level tested), reduced OR memory during pro-oestrus when 2 h inter trial delays were given. In prefrontal cortex, BPA did not alter E2-dependent increases in spine density. In the hippocampus, BPA blocked E2 increases in basal spines at 4 h and was

additive with E2 at 30 min. Thus, the authors conclude that doses of BPA alter neural functions dependent on E2 in adult female rats. No statistically significant difference between the serum E2 level analysis were observed between the E2 (20 µg/kg) and the combined E2 (20 µg/kg) and BPA (40 µg/kg) group.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:
- BPA measurement in biological samples (serum)

Weaknesses:
- Single acute dose administration
- Test performed in one sex only
- Insufficient study reporting (study design, doses and number of animals used in the various tests appears unclear; except from feed content, no information of environmental contamination of BPA is given, i.e. cages, water bottles, or bedding)
- Inappropriate statistics (considerations of repeated measures of the same animal were not included in the statistical analyses, neither multiple endpoint within a test)

Overall the CEF Panel noted that handling and administration of the rats in connection with testing may influence the test results.

This paper is included in the WoE table because of its relevance to one or more questions addressed there.

Jang YJ, Park HR, Kim TH, Yang WJ, Lee JJ, Choi SY, Oh SB, Lee E, Park JH, Kim HP, Kim HS, Lee J, 2012. High dose bisphenol A impairs hippocampal neurogenesis in female mice across generations. Toxicology, 296, 73-82.

Pregnant female C57BL/6 mice (F0) were exposed to BPA (purity > 99.7%) in corn oil at 0.1, 1 or 10 mg/kg bw by daily intraperitoneal injection from gestation day 6 to 17. Female offspring (F2) obtained from F1 females of various groups mated with F1 control males were examined for hippocampal neurogenesis (PND 44) and behaviourally tested by the Morris water maze (PND 56-63) and Passive avoidance, the step through test (PND 63-65). F2 mice were 6 weeks old, randomly divided into 4 groups (n=20 or 22 mice/group). Hippocampal neurogenesis was measured in separate groups of mice following treatment with BrdU (100 mg/kg body weight, intraperitoneally, twice a day) during each of the 3 days prior to sacrifice (PND 42-44). Data were analysed by analysis of variance (ANOVA) with Fisher's protected least significant difference (PLSD) procedure.

Exposure of F0 mice to BPA at 10 mg/kg significantly ($p < 0.01$) decreased hippocampal neurogenesis as assessed by measurement of the number of newly generated cells in the hippocampi of F2 female mice (n=5 mice/group). Passive avoidance testing revealed that high-doses BPA (1 mg/kg and 10 mg/kg) significantly ($p < 0.05$) decreased cross-over latency time in F2 mice (n=5 mice/group) in the step through test. However, the MWM (Morris Water Maze) did not show a significant difference between the treated mice versus control group (n=5 mice/group). It was found that levels of phospho-ERK, brain-derived neurotrophic factor (BDNF), and phospho-CREB in hippocampi were significantly lower ($p < 0.05 – 0.01$) in F2 mice at 10 mg/kg (n=5-8 mice/group). The effects of 10 mg/kg BPA on hippocampal neurogenesis were found to correlate with altered DNA methylation, in particular, of the CREB regulated transcription co-activator 1 (Crtc1) generated in F2 mice. The authors conclude that BPA exposure of pregnant mice could adversely affect hippocampal neurogenesis at 10 mg/kg and cognitive function (1 and 10 mg/kg) in future generations by modulating the ERK and BDNF–CREB signalling cascades.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:

Strengths:

- Number of BPA doses (3)

Weaknesses:

- Small sample size
- Maternal administration via ip injection during pregnancy
- Test performed in one sex only
- Low number of animals tested
- Insufficient study reporting (number of females in the F0 generation was not given; animal body weight not given; animal diet and phytoestrogen content not reported; no information of environmental contamination of BPA is given, i.e. cages, water bottles, or bedding)
- Inappropriate statistics(litter effect not considered, no correction for multiple comparisons)

Overall, the CEF Panel noted that the intraperitoneal route of exposure during pregnancy limits the relevance of this study in human risk assessment, since the dose levels are very high when the route of exposure is taken into account. The consideration of litter effect is not clearly addressed by the authors and the size of the group is quite small, usually 5 mice/group. No positive control was included and the number of females in the F0 generation was not given.

This paper is included in the WoE table because of its relevance to one or more questions addressed there.

Jasarevic E, Williams SA, Vanda GM, Ellersieck MR, Liao C, Kannan K, Roberts RM, Geary DC, Rosenfeld CS, 2013. Sex and dose-dependent effects of developmental exposure to bisphenol A on anxiety and spatial learning in deer mice (peromyscus maniculatus bairdii) offspring. Hormones and Behavior, 63, 180-189.

For study details see (1) Studies examining effects of BPA on anxiety-like behaviour.

Jones BA and Watson NV, 2012. Perinatal BPA exposure demasculinizes males in measures of affect but has no effect on water maze learning in adulthood. Hormones and Behavior, 61, 605-610.

For study details see (1) Studies examining effects of BPA on anxiety-like behaviour.

Kim ME, Park HR, Gong EJ, Choi SY, Kim HS and Lee J, 2011. Exposure to bisphenol A appears to impair hippocampal neurogenesis and spatial learning and memory. Food and chemical toxicology, 49, 3383-3389.

C57BL/6J male mice (42-days old) were orally (gavage) exposed to vehicle (corn oil) or BPA 1, 5 and 20 mg/kg bw per day for 2 weeks (n= 5 per group). Neurogenesis (proliferation) was assessed by intraperitoneal administration of bromodeoxyuridine (BrdU) 100 mg/kg bw twice a day for the last 3 consecutive days of BPA treatment, while survival of newly generated cells was assessed in separate groups of mice (n=5) given BrdU for 3 consecutive days prior to the commencement of BPA treatment. No BPA-related effect on body weight or systemic toxicity was reported. At days 56 to 63, learning and memory was assessed in the Morris water maze (7 days of training). Data were analysed by one-way analysis of variance (ANOVA) with Fisher's protected least significant difference (PLSD). While the high dose (20 mg/kg) decreased neurogenesis and significantly impaired spatial learning in the Morris water maze, the lower dose increased neurogenesis ($p<0.01$) and had no effect on learning abilities. No neuronal loss or damage was observed in the hippocampus after BPA treatment of 20 mg/kg as evaluated by examinations of neuron morphologies and determination of neuron density in Nissl stained sections. BPA had no effect on brain-derived neurotrophic factor (BDNF) levels or reactive oxygen species production in the hippocampus.

Comments from the CEF Panel:

The CEF Panel identified the following strengths/weaknesses in the study:

Strengths:

- Number of doses (3)

Weaknesses:

- Small sample size (n = 5- 6 per group)
- Test performed in one sex only (male)
- Insufficient study reporting (unclear number of mice used and whether the investigation of newly generated cells was performed in separate groups of mice or not; animal diet and phytoestrogen content not reported)
- Inappropriate statistics(Fisher's protected LSD test applied does not adequately protect from Type 1 Error increase due to multiple comparisons)

Overall, the CEF Panel noted that in this study effects were seen only at high doses, not at 5 mg /kg bw per day or lower, and even the exposure period was limited adolescent mice at 5 weeks of age are still in a very vulnerable developmental phase (middle-late adolescence). The main limitations of this study is the reduced sample size (n = 5- 6 per group) and shortcomings in the statistical analyses.

This paper is included in the WoE table because of its relevance to one or more questions addressed there.

Matsuda S, Matsuzawa D, Ishii D, Tomizawa H, Sutoh C, Sajiki J and Shimizu E, 2013. Perinatal exposure to bisphenol A enhances contextual fear memory and affects the serotoninergic system in juvenile female mice. Hormonal Behaviour, 63, 709-716.

Pregnant C57BL/6J mice were subcutaneously (SC) injected with vehicle (0.01% methanol in phosphate buffered saline) or 250 ng BPA/kg/day from gestational day (GD) 10 to postnatal day (PND) 20. Every third day from GD 11 to PND 21 the dams were weighed. BPA dose was calculated using 30 gram of expected mouse body weight and the injection volume was 100 microliter. Litters were culled to six pups (3 female and 3 male) at PND 2. When the litter size was less than six, foster dams were used. Four week (juvenile) and nine weeks (adult) old offspring (N=9-12 sex/group) were tested for fear memory or were sacrificed to collect brain tissue.

Serotonin (5-HT) and 5-HIAA were extracted and analysed by use of high-performance liquid chromatography (HPLC) in adult and juvenile mice of both sexes. In brains of juvenile females, the gene expressions of 5-HT metabolite-related enzymes and 5-HT receptors were analysed using quantitative real-time RT PCR. Three-way ANOVA (age, sex, treatment), followed by post hoc Bonferroni analyses, and Student's t-test was used for 5-HIAA and 5-HT and the ratio 5-HIAA/5-HT.

Fear memory was tested by use of a fear conditioning procedure: Mice were habituated to the test chamber for one 20-min session. The following day, after 3-min acclimation period (PRE) mice received three foot shocks (2 s, 0.75 mA, foot shock-interval: 60–120 s) through a metal-grid floor and returned to their home cage 3 min after the last foot shock (POST) (about 10 min session). The next day (TEST), when returned to the chamber for a 3-min session, the number of freezing exhibited was recorded as a measure of fear memory by used of a software program (FreezeFrame). Freezing was converted to a percentage [(freezing observations / total observations) × 100] for the PRE, POST, and TEST periods. Behavioural data were analysed by 3-way ANOVA (age, sex, and treatment) with repeated measures (test) and post hoc Bonferroni tests were performed for multiple comparisons.

Effect of BPA was observed in juvenile females only, which showed higher freezing percentages than the vehicle-exposed mice (41.02 ± 4.94% vs. 25.58 ± 3.40%). Thus, juvenile BPA-exposed females were divided into two groups which were injected SC from PND 25 to PND 28 with vehicle (N=8) and the selective serotonin re-uptake inhibitor sertraline (5 mg/kg, N=11), respectively. Juvenile control females were administered vehicle on the same days. Testing for fear memory was performed as described above and data were analysed by 2-way repeated-measures ANOVA (treatment and test). No main effect of treatment or interaction appeared, but when data for the two BPA groups were collapsed a slight effect of BPA vs. control appeared.

BPA enhanced fear memory, increased serotonin metabolite (5-HIAA) levels and the ratio 5-HIAA/5-HT in brain areas and increased the expression levels of Tph2, Slc6a4, and Maoa mRNA in the hippocampus of juvenile female mice. The authors conclude by suggesting that perinatal exposure to a low dose of BPA may enhance fear memory and the 5-HTergic system in juvenile mice.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:

Strengths
- None

Weaknesses
- Single dose level (250 ng/kg bw per day BPA)
- Insufficient study reporting (number of dams and general reproductive outcome not given; no information about check for cycling in adult female offspring; no information about environmental BPA contamination e.g. feed and bedding)
- Insufficient BPA-exposure assessment (constant during pregnancy and lactation and not adjusted daily to body weight)
- Animal diet and phytoestrogen content not reported
- Use of polycarbonate (PC) cages and PC water bottles

The CEF Panel noted that parallel assessment of molecular markers (5-HT and 5-HIAA) and functional end points (fear memory) has been performed. PC water bottles were used, but the drinking water was checked to be free of BPA. Pregnant mice were given two days of habituation from arriving to the laboratory until dosing started. A sufficient sample size of offspring was used for behavioural testing (n = 9-12), the litter effect is taken into account as pups from the same litter were not used in the same experiments, and the study attempts to correlate neurochemical and functional changes. The CEF Panel considers that the main limitation of this study lies in the uncertainty regarding exposure, as it appears that the daily PBA dose not was adjusted daily to body weight throughout pregnancy and lactation and that only a single dose level was tested, using the subcutaneous route.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Nakamura K, Itoh K, Dai H, Han L, Wang X, Kato S, Sugimoto T, Fushiki S, 2012. Prenatal and lactational exposure to low-doses of bisphenol A alters adult mice behavior. Brain & Development 34 (2012) 57–63
For study details see (1) Studies examining effects of BPA on anxiety-like behaviour.

Viberg H, Fredriksson A, Buratovic S and Eriksson P, 2011. Dose-dependent behavioral disturbances after a single neonatal bisphenol A dose. Toxicology, 290, 187-194.

For study details see (1) Studies examining effects of BPA on anxiety-like behaviour.

Xu XH, Wang YM, Zhang J, Luo QQ, Ye YP and Ruan Q, 2010a. Perinatal exposure to bisphenol-A changes N-methyl-D-aspartate receptor expression in the hippocampus of male rat offspring. Environmental Toxicology and Chemistry, 29, 176-181.

This study has already been reviewed by the EFSA CEF Panel in its opinion of 2010 (EFSA CEF Panel, 2010). The review done is reported below.

Xu et al. (2010a) gavaged groups of 8 to 9 pregnant Sprague-Dawley rats from GD 7 through PND 21 with different doses of BPA (0.05, 0.5, 5, 50, 200 mg/kg bw perday). At birth, pups were culled to eight and pups were sacrificed to examine the hippocampal region on PND 4, 7, 14, 21, and 56 for expression of the N-methyl-D-aspartate receptor (NMDAR) subunits NR1, NR2A and NR2B, of oestrogen receptor beta (ERβ), and of aromatase cytochrome P450 (P450arom) protein. All the determinations were done only in male pups. At the lower doses of 0.05 to 50 mg/kg bw per day, BPA

concentration dependently inhibited the expression of NMDAR subunits. At the highest dose (200 mg/kg bw per day), the effects of BPA on these subunits were different, with a stronger inhibition of NR1 expression and a slighter inhibition of NR2A, NR2B expression when compared with those at the lower dosage of BPA.

According to the authors, these results indicate a change in the composition of NMDARs thereby affecting function of the hippocampus such as synaptic plasticity, learning, and memory. Perinatal exposure to BPA at doses ≥5 mg/kg b.w./day significantly inhibited the expression of ERβ protein in a dose-dependent manner early after birth (on PND 4 and 7). At later time points (from PND 14 to 56) the expression of this protein was significantly decreased in comparison with control values only in the high dose group (200 mg/kg bw per day). Aromatase was dose-dependently (≥0.05 mg/kg bw per day) increased during the first 1-3 postnatal weeks, with no effects being detectable on PND 56.

The Panel considers that biochemical changes are not sufficient to revise the TDI in the absence of a clear link to an adverse outcome (i.e. a functional correlate). In addition, the Panel has identified shortcomings mostly in study reporting that bring into doubt the validity of the study.

Specifically, there is no indication of the number and the identity of the animals used for testing, and on whether the statistical analysis was performed on a "per litter" basis. In addition, only one sex was tested and there was no reporting on the animal housing conditions, the diet administered and the type of bottles used for drinking water.

Xu XH, Zhang J, Wang YM, Ye YP and Luo QQ, 2010b. Perinatal exposure to bisphenol-A impairs learning-memory by concomitant down-regulation of N-methyl-D-aspartate receptors of hippocampus in male offspring mice. Hormones and Behavior, 58, 326-333.

In the ANSES risk assessment of 2013 this oral study by Xu et al. (2010b) in mice was taken as the key study for neurodevelopmental toxicity, where the critical effects were the alteration of memory and learning functions paralleled by a decrease in the expression of glutamate NMDA receptors.

Therefore this study has been revisited in depth by the CEF Panel for the current evaluation.

The purpose of the Xu study was to investigate the effects of perinatal exposure to BPA on learning/memory and its mechanism of action, especially focusing on N-methyl-d-aspartate receptor (NMDAR) and expression of oestrogen receptor beta (ERβ). BPA at 0.05, 0.5, 5 and 50 mg/kg bw per day were given by oral injections to pregnant ICR mice (n=10/group) from gestational day 7 to PND 21. After parturition (postnatal day 0, PND) litter were culled. At weaning, same sex littermates were group housed. At PND 21-25 and 56-60, respectively, male offspring (n=10/group per test time)were tested in the Morris water maze (spatial memory task) for four days with four daily sessions followed by a probe session the fifth day. At PND 26 and 61, respectively, males (n=10/group) were subjected for training session of the step-down passive avoidance task, and retention test sessions were carried out 24 h thereafter (i.e. at PNDs 27 and 62). At PND 21 and 56, respectively, male offspring (n=10/group) were sacrificed and brains collected and prepared for analyses by gel electrophoresis and immunoblotting (Western blot).

One-way analysis of variance (ANOVA) was applied to the data of the step-down passive avoidance test and Western blot. For the Morris water maze, data from the probe trial were analysed by 1-way ANOVA, whereas 2-ways ANOVA was used for analyses of effects of treatment and training. For comparisons between groups, Tukey's test was used.

In male offspring, at both test times BPA dose-dependently extended the escape length to find the hidden platform in the Morris water maze even the swim velocity were the same. At the early test time, the upper two BPA doses were most effective in increasing the escape length, whereas the three upper doses did so at PND 56-60. Data from the probe trials revealed normal memory retention in all groups. However, at the probe trial PND 25, BPA at 0.5 mg/kg bw per day decreased the percentage of time spent in the quadrant where the platform had been during training in male offspring, whereas at PND 60 BPA both at 5 or 5 mg/kg bw per day did so.

The results of step-down passive avoidance test (instrumental conditioning where mice have to inhibit an escape response in order to avoid a punishment) showed that the error frequency to step down from the platform after receiving foot-shock was significantly increased, and the latency of the step-down response onto the grid floor 24h after received foot-shock was reduced by exposure to BPA at 5 and 50 mg/kg bw per day ($P<0.01$) in the PND 26 offspring, and at 50 mg/kg bw per day in the PND 61 offspring ($P<0.01$).

Perinatal exposure to BPA significantly inhibited the expressions of NMDAR subunits NR1, NR2A, and 2B in the hippocampus during the development stage, especially in PND 56 male mice. Dose-response was only seen for subunit NR1 in 21 days old males. The expressions of oestrogen receptor beta (ERbeta) in both PND 21 and PND 56 mice were markedly down-regulated by BPA at 0.5, 5, and 50 mg/kg bw per day. The authors conclude that these results indicate that perinatal exposure to BPA impairs both spatial memory and avoidance memory. The inhibition of expressions of NMDAR subunits and ERbeta in hippocampus during postnatal development stage may be involved.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:
- Number of doses (4)
- Phytoestrogen-free diet

Weaknesses:
- Test performed in one sex only (only male offspring)
- Insufficient study reporting (reproductive outcome not shown, e.g. maternal bw, no pre-weaning body weight data shown; information about type of bedding and water bottles missing; no indication of the number and the identity of the animals used for testing)
- Insufficient BPA-exposure assessment (unknown if constant during pregnancy and lactation and or adjusted daily to body weight, see line above)

This paper is included in the WoE table because of its relevance to one or more questions addressed there.

Xu X, Tian D, Hong X, Chen L, Xie L, 2011. Sex-specific influence of exposure to bisphenol-A between adolescence and young adulthood on mouse behaviors. Neuropharmacology. 2011 Sep;61(4):565-73. doi: 0.1016/j.neuropharm.2011.04.027. Epub 2011 May 5.

For study details, see (1) studies examining the effects of BPA on anxiety-like behaviour.

Xu X, Liu X, Zhang Q, Zhang G, Lu Y, Ruan Q, Dong F and Yang Y, 2013a. Sex-specific effects of bisphenol-A on memory and synaptic structural modification in hippocampus of adult mice. Hormones and Behavior, 63, 766-775.

This study aims to evaluate the effects of long-term exposure to BPA on memory and modification of synaptic structure in hippocampus of adult mice. Adult ICR mice were exposed to BPA (0.4, 4, and 40mg/kg/day) or arachis oil for 12 weeks by oral gavage needle placed at the back of the mouth (22 mice/group). Body weights were recorded at start and end of treatment. Three days after end of BPA exposure, ten mice per group were sacrificed and brains were collected for 1) electron microscopic preparations and morphometric measurement (n=6 mice/group) and 2) tissue preparation, gel electrophoresis and immunoblotting (n=4 mice/group). Additionally, after termination of treatment (22 weeks of age) 10-12 mice per group were behaviourally tested in the open field test for locomotor activity, and subsequently in two learning tasks, the Morris Water Maze and the Passive Avoidance test (step down test).

Data from acquisition training (days 1–4) in the Morris water maze was analysed by three-way repeated analysis of variance (ANOVA) (sex, treatment, day), whereas for the probe trial a two-way repeated ANOVA was applied (sex, treatment). The latter was also used for data of the open field,

step-down passive avoidance task, body weight, the synaptic density and the synaptic interface structure parameters. One-way repeated ANOVA was applied to the data of reproductive organ weight, serum steroids, and Western blot analyses. Multiple comparisons within significant interactions were performed with the Tukey's HSD test.

Results showed that BPA increased the frequency of rearing (0.4 and 4 mg/kg bw per day), grooming (4, and 40 mg/kg bw per day) and time in the central area (40 mg/kg bw per day) of the open-field in males, while BPA at 0.4 mg/kg/day reduced the frequency of rearing in the females. BPA at 0.4 or 40 mg/kg bw per day extended the average escape path length to the hidden platform in Morris water maze task during the training phase but not the probe day, and at 40 mg/kg bw per day shortened the step-down latency 24h after foot-shock of the males, but no changes were found in females. In males, BPA at the lowest and highest dose reduced numeric synaptic density of pyramidal cells in hippocampus CA1 compared to controls, whereas the same doses increased it in BPA females when compared to BPA males. BPA had a negative effect on the structural parameters of synaptic interface, including an enlarged synaptic cleft (all doses) and reduced length of active zone (lowest dose only) and PSD thickness, in the hippocampus of the male mice compared to controls, but not in females. Western blot analyses further indicated that BPA (all doses) down-regulated expressions of synaptic proteins (synapsin I and PSD-95) and synaptic NMDA receptor subunit NR1 and AMPA receptor subunit GluR1 in the hippocampus of the males, but not females. The authors concluded that these results suggest that long-term exposure to low levels of BPA in adulthood sex-specifically impair spatial and passive avoidance memory of mice. These effects may be associated with the higher susceptibility of the hippocampal synaptic plasticity processes, such as remodelling of spinal synapses and the expressions of synaptic proteins (e.g. synapsin I and PSD-95) and NMDA and AMPA receptors, to BPA in the adult male mice.

Comments from the CEF Panel
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:
- Number of BPA doses (3)
- Phytoestrogen-free diet
- Use of non-PC cages and of non plastic water bottles
- Blinded evaluation of data from the open field test, not specified for the MWM or for passive avoidance

Weaknesses:
- Insufficient study reporting (body weight data collected during treatment not shown)

Overall, the CEF Panel noted parallel measurement of synaptic morphology (neural plasticity index) and functional endpoints. The cycle stage of females was checked and females in deviating stage compared to the majority (di-oestrus) were excluded from the data analyses. The prolonged treatment (about 12 weeks) and use of three dose levels of BPA were noted, however the highest dose was very high (40mg/kg bw per day). The effects on learning functions were significant in males only in the spatial learning task (MWM) and only for the intermediate and high doses. The effects of BPA on passive avoidance (an instrumental learning task not involving spatial learning processes) were also limited to males and observed only at the highest dose (40 mg/kg/bw day). The extent of the structural changes in the synapses of the hippocampal CA1 areas was limited. Significant reduction in expression of the major synaptic proteins analysed was found in males only. The learning impairment in males found in the spatial learning task might be possibly linked to the alteration of the synaptic structural/molecular properties.

This paper is included in the WoE table because of its relevance to one or more questions addressed there.

(3) Studies examining the effects of BPA on social behaviour

Kundakovic M, Gudsnuk K, Franks B, Madrid J, Miller RL, Perera FP, Champagne FA, 2013. Sex-specific epigenetic disruption and behavioral changes following low-dose in utero bisphenol A exposure. Proceedings of the National Academy of Sciences of the United States of America, 110, 9956-9961.

Balb C female mice were daily exposed by oral administration to 2, 20, 200 µg/kg BPA or vehicle from mating until gestation day (GD) 19. The breeding design generated a minimum of 12 litters per treatment [vehicle (n = 14), 2 µg/kg (n = 17), 20 µg/kg (n = 15), and 200 µg/kg (n = 12) BPA]. From postpartum days 1 to 6, dam–pup interactions were observed to determine BPA-induced effects on maternal behaviour and the effects of postnatal maternal behaviour on the possible BPA-induced outcomes in offspring.

At weaning (PND 28), six male and six female offspring per treatment [one or two pups per litter from a minimum of five litters per treatment for each sex; total litters, vehicle (n = 8), 2 µg/kg (n = 7), 20 µg/kg (n = 8), and 200 µg/kg (n = 7) BPA], were killed and whole brains were dissected (prefrontal cortex, hippocampus, hypothalamus) for gene expression and DNA methylation analyses. Remaining animals underwent behavioural testing from PND 30 to 70 [male and female offspring from vehicle (n = 8–10), 2 µg/kg (n = 10–12), 20 µg/kg (n = 10), and 200 µg/kg (n = 12) BPA litters]. Testing included: 1) Home-cage social behaviour in offspring between PND 30 and 40 (60 min by observation), 2) an Open field (OF) area at PND 60 (video recording), and 3) Social approach and aggression between same sex-mice PND 70 (15 min).

Data analyses was based on treatment of BPA dosage as a continuous predictor on a logarithmic scale (2, 20, 200 µg dose) and use of multilevel models to look for evidence of a curvilinear (quadratic) effect of dosage level on offspring gene expression and behaviour as well as on maternal behaviours (specifically licking/grooming and archedback nursing) of BPA-treated dams. A complementary two-way ANOVA (sex and treatment) with Tukeys post hoc test for effects of dose was provided in the supporting information to the paper.

In juvenile offspring, results showed that maternal BPA exposure during pregnancy induced sex-specific, dose-dependent (linear and curvilinear) and brain region-specific changes in expression of genes encoding oestrogen receptors (ERs; ERα and ERβ) and oestrogen-related receptor-γ. Changes in ERα and DNA methyltransferase (DNMT) expression in the cortex (males) and hypothalamus (females) were associated with DNA methylation changes in the ERα gene.

Between PND 30 and PND 40, home-cage social behaviour was examined and prenatal BPA treatment led to marginal sex-specific changes (huddling and sniffing). Some changes were observed in both sexes (allogrooming and hopping). The authors find that the prenatal BPA exposure also disrupted sexual dimorphism in play behaviours.

At PND 60 in the open-field test measured as distance travelled, prenatal BPA exposure was associated with a hyperactive phenotype in males at all doses ($p<0.01$, ANOVA) and hypoactive phenotype in females (not statistically significant). BPA exposure at the highest dose increased anxiety-like behaviour in females ($p<0.05$, ANOVA) and decreased anxiety-like behaviour in males (not statistically significant) as measured as time spent in inner area. The authors find the open field test results to be sexually dimorphic, and that BPA treatment reversed sex differences in these behaviours (distance travelled, inner area time).

At PND 70, prenatal BPA exposure affected dyadic social interactions (sniffing and aggressive behaviour) with a same-sex stimulus mouse. For both sexes, BPA had a marginal effect on sniffing but the authors find a differential effect by sex at high doses (reversal of sex differences). For both sexes, the authors find that prenatal BPA treatment increased the probability of being aggressive/ dominant at the highest dose, following a quadratic dose-response curve

The effects measured in this study appeared to be of different extent and direction depending on BPA dose, suggesting that low doses can be more effective than the higher ones (non-monotonic responses). The author concluded that although postnatal maternal care was altered in mothers treated with BPA

during pregnancy, the effects of in utero BPA were not found to be mediated by maternal care, but that increased maternal care partially may attenuate the effects of in utero BPA on DNA methylation.

Comments from the CEF Panel:

The CEF Panel identified the following strengths/weaknesses in the study:

Strengths:

- Number of dose groups (3)
- BPA measured in biological sample (urine)
- Use of non-polycarbonate (non-PC cages)

Weaknesses:

- Insufficient study reporting (ody weight, litter size and sex- ratio is not given; frequency of various postpartum maternal behaviours given without the litter size; the administration to dams is not specified except that it is oral; urine collection and analysis not described; the sacrificing and brain sampling procedures are not detailed; insufficient information concerning the scoring of social/aggressive behaviour; poor description of the social behaviour testing)
- Inappropriate statistics (lack of accounting for multiple observations within each litter [pseudo-replication] in the ANOVA; the application of the multilevel regression model showed a large scatter of data within dose-groups [only four] and thus a large number of different dose-response curves can be fitted to the data so that it is not possible to conclude on the biological relevance for any of them)
- Animal diet and phytoestrogen content not reported.
- Type of drinking bottles not reported

Overall, the CEF Panel noted that in this study three dose levels were used, and that the sample size is acceptable (n = 10 litters for the behavioural experiments, n = 5-6 in each sex for DNA methylation and oestrogen receptors expression in hypothalamus and frontal cortex). The CEF Panel also noted the parallel assessment of relevant molecular markers and behaviour as well as the assessment of potential effects of BPA on maternal behaviour of dams. The open field behaviour was recorded by video whereas for the social measures, data were recorded by observation. Two different tests for social behaviour were used (home cage social behaviour in juveniles and dyadic interaction with a same-sex stimulus mouse at day 70), but poor description of the testing was given. The paper does not address the possible influence of the open field results (motor activity and anxiety-like behaviour) on the social behaviour results, whereas maternal care was taken into account. The CEF Panel aknowledge that the multilevel (hierarchical) statistical models used take litter effect into account. Furthermore, the large scatter within the dose-groups for the behavioural endpoints does not allow concluding on the shape of the dose-response curve and even a conclusion that there is no dose-response would be compatible with the data (see Section 1.2).

This paper is included in the WoE table because of its relevance to one or more questions addressed there.

Wolstenholme JT, Taylor JA, Shetty SR, Edwards M, Connelly JJ, Rissman EF. 2011a. Gestational exposure to low dose bisphenol A alters social behavior in juvenile mice. PLoS One, 6, e25448.

This study hypothesised that gestational exposure to BPA in mice affected social behaviour and expression of selected genes involved in neurobehavioural plasticity.

The authors assigned adult female mice of the C57BL/6J strain before mating to either a phytoestrogen-free chow (n=11) or the same chow supplemented with 1.25 mg BPA per kg diet (n=12). All females consumed their assigned diets (food and water) ad libitum and the authors calculated that during the last 10 days of gestation the dams had an intake of approximately 5 µg of BPA per dam per day, i.e. approximately 140µg BPA/kg bw per day for a 35 g mouse. The authors also adopted a cross-fostering procedure at birth, to avoid any influence of BPA exposure on maternal

care (n=20 foster dams). Offspring (BPA = 21 male/18 female, control = 15 male/13 female) were subjected to Social interaction test on PND 20 (30 minutes, about 12-14 parameters observed), Social preference test on PND 24 (10 minutes, endpoint was time spent with stimulus mouse) and tested in an Elevated plus maze tests (EPM, 10 minutes) on PND 22, while gene expression analysis was performed on embryo brains (n=5/group). Unconjugated BPA was measured by HPLC in pooled serum samples from dams on gestational day 18.5 (4 BPA-dams, 3 controls), and the limit of detection (LOD) was 0.5ng/ml. Two-ways ANOVA (sex, diet) followed by Fisher's exact post hoc test that adjusts significance levels and takes multiple comparisons into account, was used for analyses of behavioural data.

BPA-chow diet increased blood level of BPA (0.43±0.002 ng/ml) when compared to control diet (0.099±0.014 ng/ml), and it was in the range detected in human maternal blood (0.3 to 18.9 ng/ml). Some of the behaviour parameters were observed to be sexually dimorphic, e.g. the non-social items exploring and sitting alone where males were more active than females, and the social items side-by-side sitting where male were spent less time than female. The author suggest that BPA-exposed females, but not males, were more interactive compared to same sex controls because they spent more time side-by-side interaction, less time self-grooming, showed higher frequency of side-by-side behaviours others than grooming as well as of following other mice, and less frequency of self grooming ($p<0.05$). However, BPA did not affect social preference for the stimulus animal in a social preference test. In the Elevated Plus Maze task, anxiety-like behaviour evaluated as time spent in the open arms and closed arms and the number of crosses between arms was similar in the two groups. Gene expression analysis revealed mRNA for the glutamate transporter, Slc1a1, was enhanced by exposure to BPA in female brains and that expression of two of the three DNA methyltransferase genes, Dnmt1 and Dnmt3a, was modulated by BPA. Notably, expression of oestrogen receptors' genes was not affected by BPA, but oxytocin receptor gene (highly responsive to oestrogen modulation and involved in social behaviour) was to some extent reduced in males.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- BPA measurement in animal samples (serum)
- Phytoestrogen-free diet
- Scoring blind to treatment

Weaknesses:

- Single dose level study
- Feed consumption (BPA given by the diet) not measured
- Insufficient study reporting (information of the content of the mixed litters like number of pups and sex ratio is missing;; no information of control of environmental contamination of BPA, i.e. cages and water bottles)
- Insufficient BPA-exposure assessment (estimated on the basis of the daily quantity of food ingested, see line above)
- Inappropriate statistics (litter effect not properly considered)

Overall, the CEF Panel noted that the behavioural analysis included two different social behaviour tasks and a test specifically suited to reveal differences in general anxiety-like behaviour (elevated plus maze). However, the association of the behavioural results at weaning age with the small changes in gene expression found at the fetal stage is weak, and findings do not support novel mechanistic hypotheses. The main result of the social behaviour domain seems to be that females are more affected by gestational BPA exposure than males. The use of foster dams to ensure gestational BPA exposure only is considered challenging as it resulted in mixed litters and in some cases tail clipping of the pups which may have influenced on their social development and biased the subsequent testing of social interaction. Determination of serum levels below LOD was based on estimation by extrapolation of the standard curve to zero. The CEF Panel noted that the study has several methodological limitations.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Wolstenholme JT, Edwards M, Shetty SR, Gatewood JD, Taylor JA, Rissman EF, Connelly JJ, 2012. Gestational exposure to bisphenol A produces transgenerational changes in behaviors and gene expression. Endocrinology, 153, 3828-3838.

This study was aimed at assessing whether BPA exposure during fetal life has transgenerational effects on genes and behaviour in C57BL/6J mice. Female mice (n=5 per group, F0) received phytoestrogen-free chow with or without BPA (5 mg/kg diet, equivalent to approximately 1 mg BPA/kg bw per day based on a conversion factor of 0.2 (EFSA, 2012) seven to ten days before mating and throughout gestation. Subsequent generations (F1 to F4) received standard rodent chow containing phytoestrogens. F1 brother-sister pairs (BPA n=9, control n=8) were used to produce the transgenerational lines. The authors measured unconjugated BPA in three pooled serum samples from the F0 dams (two dams in each pool) and detected levels of 4.6, 3.9 and 2.0 ng/ml respectively, which the authors noted is comparable to those reported for human serum, 0.3– 4.0 ng/ml. Within 12 h after birth, all pups from control and BPA-consuming F0 dams were cross-fostered, i.e. to dams on control diet that had given birth within the past 24 hours.

F1 juveniles of both sexes (about 10 animals per group) underwent evaluation for Social interaction on PND 20-21 (10 minutes), Social preference on PND 24 (10 minutes) and tested in an Elevated plus maze tests (EPM) on PND 22. Juveniles of the F2 and F4 generation (obtained by breeding brother-sister pairs) were assessed in the free social interaction test. Expression of several genes (Er-alfa, Er-beta, membrane bound oestrogen receptor, oestrogen-related receptor gamma, oxytocin, vasopressin and their respective receptors) were measured in the brain of 18-day embryos of the F1 and F4 generation by microarray analysis and quantitative real time Polymerase Chain Reaction (QPCR). Two-ways ANOVA (sex, diet) followed by Fisher's exact post hoc test that adjusts significance levels and takes multiple comparisons into account, was used for data analyses.

Juvenile F1 mice exposed to BPA displayed significantly fewer social interactions as compared with control mice (side-by-side interactions and frequency of anogenital investigation), whereas the frequency of play solicitation was higher. In juvenile BPA-male, but not female, the social preference for an adult male was decreased. In later generations (F2 and F4), the effect of BPA was an increase of these social interactions. None of these behaviours appeared to be sexually dimorphic, nor was there any interaction between sex and diet. In the EPM no effect of BPA was revealed as the time spent in the open arms and closed arms and the numbers of crosses between arms were similar in both groups. In the BPA exposed-line, brains from embryos of F1 mice had lower gene transcript levels for several oestrogen receptors, oxytocin, and vasopressin as compared with controls; decreased vasopressin mRNA persisted into the F(4) generation, at which time oxytocin was also reduced but only in males. The authors conclude that exposure to a low dose of BPA during gestation has long-lasting, transgenerational effects on mRNA in brain and social behaviours.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:

Strengths:
- BPA measurement in animal samples (serum)
- Phytoestrogen-free diet (F0-generation only)

Weaknesses:
- Single dose level study
- Study design not appropriate to the scope (the number of dams in F0 generation was limited)
- Insufficient study reporting (animal age and body weight not given; no normalization of food consumption on body weight, potential variability of exposure; lack of information of control of environmental contamination of BPA, i.e. cages, bedding and water bottles; lack of

information of mixed litters e.g. number of pups and sex ratio; reproductive outcome of the breeding brother-sister pairs done to obtain F2 to F4 generation is not given)
- Inappropriate statistics (litter effect not properly considered)

The CEF Panel noted the the study addressed association of BPA behavioural effects with expression of genes implicated in regulation of social behaviour and related sex dimorphism (ERs, oxytocin and vasopressin) in embryos across generations. The CEF Panel however considered the use of foster dams to ensure gestational BPA exposure challenging as it resulted in mixed litters and in some cases tail clipping of the pups, which may have influenced on their social development and biased the subsequent testing of social interactions. Exposure was estimated (20 µg daily) on the basis of the quantity of food ingested daily, but it is unclear if the authors calculated the amount of food consumed daily by each subject. The study thus has methodological limitations. The results on social behaviour throughout the generations were inconsistent (social interaction decreased in the F0 generation but increased in the F2 and F4 generations), whereas the effects on gene expression from F1 to F4 appeared persistent. The CEF Panel acknowledges that variance in appearance of epigenetic expression may have influence on the inconsistency.

This paper is included in the WOE table because of its relevance to one or more questions addressed there.

Wolstenholme JT, Goldby JA and Rissman EF, 2013. Transgenerational effects of prenatal bisphenol A on social recognition. Hormones and Behavior, 64, 833–839

Female C57BL/6 mice (F0) received 7-10 days prior to mating and throughout gestation phytoestrogen-free chow with (n=22) or without (n=74) BPA at 5 mg BPA per kg chow, which was calculated by the author to represent a daily intake of 20 µg BPA per dam. The subsequent F1 to F3 generations received standard rodent chow containing phytoestrogens and no BPA exposure. Within 12 h after birth, pups (F1) were cross fostered to control dams to limit BPA exposure to gestation in the first generation only. F0 foster dams (n = 48) retained two biological pups not included in the study, and received four F1-foster pups from the same litter (control litters n=26, BPA litters n = 22). Sibling pairs of F1 and F2 were bred to obtain the F2 and F3 generations (F1, BPA n=9, control n=9, F2, BPA n=15, control n=15).

First (F1) and third (F3) generation juveniles were tested for Social recognition at postnatal day (PND) 21 and in the open field at PND 23–24. Adult F3 mice of both sexes were tested for olfactory discrimination. Each mouse was tested in only one behaviour task and litter was taken into consideration. Two-way ANOVA with diet and sex as main factors was used for all behavioural data collected, in addition with trials as the repeated measure for social recognition and odour discrimination. Fisher's Exact post-hoc test was used for following up analyses.

BPA exposed juvenile F1 and F3 mice displayed higher levels of investigation than controls during the eight first trials in the Social recognition task. In the last trial, F3 male of the BPA exposed line, spent less time with the novel stimulus ovarioectomized mouse compared to other groups. In the open field no differences were noted for F1 mice, but increased activity in F3 BPA line age mice compared to controls. No group differences appeared for olfactory discrimination (assessed in F3 mice only). The authors conclude that these results show that BPA exposure during gestation has long lasting, transgenerational effects on social recognition and activity in mice.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:
- Phytoestrogen-free diet (F0 dams)

Weaknesses:
- Single dose level study

- Insufficient study reporting (information on sex ratio in the F1 litters is missing, as well as reproductive outcome of the sibling mating performed to obtain F2 and F3 generations, unclear whether the authors actually measured the daily amount of feed consumed for each dam or averaged the amount based on previous knowledge; lack of information of control of environmental contamination of BPA, i.e. cages, bedding and water bottles)
- F1-F3 fed chow containing phytoestrogens

Overall, the CEF Panel noted that the use of F0 foster dams to ensure gestational BPA exposure only is challenging as it resulted in mixed litters. Compared to the two previous studies of this author (see above) it is considered an improvement that the mixed litters were standardized to six pups, and that tail clipping for identification purposes were limited to the biological pups which was not included in this study. One dose level of 5 mg BPA per kg chow was used, which is equivalent to about 1 mg BPA/kg bw per day based on a conversion factor of 0.2 (EFSA, 2012). However, exposure was estimated by the authors to be 20 µg daily per dam on the basis of the quantity of daily feed consumption, but it is unclear whether the authors actually measured the daily amount of feed consumed for each dam or averaged the amount based on previous knowledge. The study thus has methodological limitations.

This paper will be included in the WOE table because of its relevance to one or more questions addressed there.

(4) Studies examining the effects of BPA on sensory-motor function

Ferguson SA, Law CD and Abshire JS, 2012. Developmental treatment with bisphenol A causes few alterations on measures of postweaning activity and learning. Neurotoxicology and Teratology, 34, 598-606.
For study details see (2) Studies examining effects of BPA on learning and memory

Wolstenholme JT, Goldby JA and Rissman EF, 2013. Transgenerational effects of prenatal bisphenol A on social recognition. Hormones and Behavior, 64, 833–839
For study details see (3) Studies examining effects of BPA on social behaviour

(5) Other studies

No WoE was performed for the endpoint addressed by these studies.

Jones BA, Shimell JJ, Watson NV, 2011. Pre- and postnatal bisphenol A treatment results in persistent deficits in the sexual behavior of male rats, but not female rats, in adulthood. Hormones and Behavior 59, 246–251

Fifteen Long-Evans pregnant dams were orally exposed to corn oil (controls), 5 µg/kg, 50 µg/kg, 500 µg/kg or 5 mg/kg body weight (bw) PBA by daily drinking from a syringe from gestation day 7 until lactation day 14 (3 dams/group). Adult offspring (postnatal day 90 and 120, n=12/group/sex) were evaluated for sexual behaviour by use of stimulus animals that were sexually experienced but gonadectomized and treated with male/female hormones, respectively.

Male offspring were tested on three consecutive days to gain sexual experience and then they were finally tested seven days after the initial test day, i.e. totally four test days. Data were collected at the initial and final test days, representing sexually naïve and sexually experienced males, respectively. Test duration was until occurrence of the first mount following ejaculation and maximum 30 min if ejaculation failed. For each male the number of anogenital investigations (AGI), mounts and intromissions of the stimulus female were noted, as well as the latencies to mount, intromit and ejaculate, and the post-ejaculatory interval (PEI; latency to first mount of the next bout of copulation). An index of copulatory efficiency (CE: # intromissions/# of mounts) was created by the authors.

Consummatory sexual behaviour is referred to as CE and intromissions, and appetitive sexual behaviour as PEI and latencies to mount, intromit and ejaculate.

Females were tested at the day of behavioural ooestrous on two occasions which were separated by the duration of one ooestrous cycle (i.e. 4 days). On each test occasion the total number of proceptive behaviours as well as the lordosis quotient (i.e. number of lordosis shown per 10 mounts×100) was tabulated for each female. The proceptive behaviours (i.e. ear wiggling and hopdarting behaviour) were observed until the female had received 10 mounts with pelvic thrusting by the stimulus male.

Data were analysed by one-way ANOVA with post-hoc Tukey's analyses for testing of specific group differences (i.e. comparing all possible pairs of means). The observed non-linear dose response in males was sought by the authors to be determined, as a post hoc analysis with the one-way ANOVA, whether it exhibited a significant quadratic polynomial component (i.e. displays concavity, a single bend either upward or downward). Additionally, the authors transformed different parameters of the behavioural endpoints measured in the same animal into a z-score for the evaluation of its performance averaged over all parameters (i.e. for each animal and test day, a composite score of the recorded data relative to that of the other animals on that test day). Further, in order to ensure that the individual characteristics of dams were not significantly affecting the parameters, ANOVA and subsequent Tukey's post-hoc tests was used to determine if litter membership had any significant effects on the observed behaviours within each dose group.

No statistically significant differences between groups were found at the initial day of testing, which according to the authors might be as expected due to the large variances associated with sexual inexperience at the initial days.

In females, perinatal BPA-exposure did not statistically significantly influence adult sexual behaviour at any dose or time tested, and according to the authors a study limitation could be that no male-typical behaviours were examined (e.g. partner preference).

For males, neither AGI nor number of mounts showed any group differences. On day 1, only high dose BPA-males differed statistically significantly from other groups with increased consummatory behaviour compared to other BPA-groups, and increased appetitive behaviour compared to controls and BPA 5 µg/kg bw (latencies) and 50 µg/kg bw (latencies and PEI). On day 4, the statistical picture was different. In short, both controls and high dose BPA-males showed increased consummatory behaviour compared to 50 µg/kg-group. High dose BPA-males had shorter PEI than 50 µg/kg-males. The 50 µg/kg-group showed longer latencies to intromit and ejaculate than controls, 500µg/kg and high dose groups. The authors' conclude that "*Males receiving low dose perinatal BPA (50 µg/kg bw per day) showed persistent deficits in sexual behavior in adulthood. Males receiving the highest dose (5 mg/kg bw per day), however, were indistinguishable from controls with respect to consummatory sexual behaviors but showed decreased latencies to engage in those behaviors when sexually naïve, with significant non-linear, or U-shaped, dose–response relationships observed on the first and last day of testing*."

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:

Strengths:

- Number of BPA doses (4)
- Blind evaluation of the observed sexual behaviour
- Non-PC cages used for dams and offspring, and BPA-free water sacks for dams

Weaknesses:

- Study design not appropriate to the scope (small number of dams per group (2-3), littermates treated as independent observations)

- Study reporting (limited information of dams, e.g. body weight, signs of toxicity; number of stimulus animals not given, e.g. same number as test animals; test animal body weight and developmental landmarks of sexual maturation not reported)
- Phytoestrogen content in the standard rat chow not reported
- Inappropriate statistics (litter effect not appropriately addressed; uncertainty whether the statistically significant differences were due to dose or other study factors, in particular the composite z-score analysis will be sensitive to small differences among dose groups other than the dose
- In the figures it is not reported what the whiskers represent, e.g. SD or SEM.
- There was no mentioning of the order of the various treatments in the individual animals, i.e. when treatments were completed for all animals in a given dose group, and then for the next dose group, this could easily give rise to different responses among groups which are unrelated to the dose, in particular for behavioural parameters.

There was no mentioning of the order of the various treatments in the individual animals, i.e. when treatments were completed for all animals in a given dose group, and then for the next dose group, this could easily give rise to different responses among groups which are unrelated to the dose, in particular for behavioural parameters. The CEF Panel noted that control of environmental BPA contamination was done by housing rats in polysulfone cages and by use of BPA-free water sacks for dams. However, both dams and offspring were fed standard Purina rat chow and no information about the bedding used is given. All behaviours were scored by an investigator blind to experimental condition. Sexual maturation usually occurs in rats at the age of 2-3 months (60-90 days). In the study, rats were tested at the age of 90-120 days and no information about developmental landmarks for sexual maturation was reported. Age has been found to be crucial for the sexual maturation of female rats, whereas body weight has been found to be more important for the sexual maturation of male rats. Differences in sexual maturation might influence the performances. LE-males are recommended to be housed individually and not group housed as done in the study. Sexual experience in test males is presumed to be present after being presented for stimulus females on three occasions, but no information to ensure this is given, e.g. that all males actually had copulated. Studies have shown that male rats with no sexual experience exhibit increases in the latencies for intermission and ejaculation and PEI period. Since stimulus females were injected 4 h prior to onset of testing for inducing sexual receptivity, number of stimulus females is presumably the same as number of test males, but no information is given. The underlying cause of the reported statistically significant behavioural differences appears unclear, and may be caused e.g. by chance findings of multiple testing.

The CEF Panel considers the main weakness in this study to be that both male and female test animals were littermates representing only 2-3 dams per exposure group. OECD guidance for developmental studies states that the statistical unit of measure should be the litter and not the pup (OECD, 2007, Test No. 426). The use of littermates leads to p-values that are higher than justified by the data. Additionally, the z-score approach implies that statistically significant differences due to other study factors than dose are amplified.

This paper is not included in the WoE table because it is not relevant to any of the questions addressed there, but it is included in the section 1.2 on NMDR.

Ishido M, Masuo Y, Terasaki M and Morita M. 2011. Rat hyperactivity by bisphenol A, but not by its derivatives, 3-hydroxybisphenol A or bisphenol A 3,4-quinone. Toxicology Letters, 206, 300-305.

Ishido et al. intended to assess the effects of acute neonatal exposure to BPA on spontaneous motor activity at young adulthood. Approximately 50 male pups were born from 10 pregnant Wistar rat females, 5–7 of which were randomly housed. Standard feed and distilled water were available ad libitum. Five-day old pups (n = 6 per group) received an intracisternal injection of 10 µl vehicle (control) or 20 µg bisphenol A or its derivatives, equivalent to 87 nmol chemical/10 µl vehicle/pup. In

4-5 weeks old males, activity was measured at 15-min intervals for 22–24 h under a 12-h light–dark cycle by use of a Supermex sensor head which detects body heat and an array of Fresnel lenses placed above the cage which monitors motion.

Administration of BPA induced statistically significant nocturnal hyperactivity in rats at 4-5 weeks of age ($p<0.05$ analysed by Student's *t*-test) compared to controls, whereas administration of BPA derivates (3-hydroxybisphenol A and bisphenol A 3,4-quinone) did not affect this same endpoint.

The authors found significant accumulation of BPA (1.385 ng/tissue) in the brain at 8 weeks of age, but not of the other compounds. These findings led the authors to suggest that the parent compound BPA crosses the immature blood-brain barrier (BBB) and exert long-lasting effects on behaviour with mechanisms others than its suggested anti-estrogenic action (i.e. refers to previous paper of the same group indicating reduction of brain tyrosine hydroxylase activity after BPA exposure).

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:
- None

Weaknesses
- Single dose level study
- Single acute dose administration
- Small sample size
- One sex only (males)
- Insufficient study reporting (unclear whether one dose level or several dose levels were used; animal age and body weight not given; animal diet phytoestrogen content not reported)
- Inappropriate statistics(litter effect not considered)

Overall, the CEF Panel noted that the main limitations of this study are the limited sample size (n = 6), the intracisternal exposure route, the lack of consideration of the litter effect, the small behavioural changes found, only 1.3 times higher than controls and only nocturnal and not diurnal activity affected by BPA, and the use of a single BPA administration. It is unclear whether one dose level or several dose levels were used as it under "Results" refers to other dose levels than described in Materials and methods.

This paper is not included in the WoE table because lack of relevance to one or more questions addressed there.

(6) Studies examining the morphological, molecular and biochemical changes in the brain

No WoE was performed for the endpoint addressed by these studies.

Cao J, Mickens JA, McCaffrey KA, Leyrer SM, Patisaul HB. 2012. Neonatal Bisphenol A exposure alters sexually dimorphic gene expression in the postnatal rat hypothalamus. Neurotoxicology, 33, 23--36.

The goal of this study was to determine if neonatal BPA exposure interferes with sex specific gene expression of oestrogen receptor alpha (ERα), ER beta (ERβ) and kisspeptin (Kiss1) in the anterior and mediobasal hypothalamus. Time mated Long Evans rats (n=13) delivered pups at postnatal day (PND) 0 and the pups were daily exposed to vehicle (10% ethanol in sesame oil), 10µg oestradiol benzoate (EB), 50 mg/kg BPA or 50 µg/kg BPA by subcutaneous injection from the day of delivery (PND 0) to PND 2 (n=6-9 per sex and group representing a minimum of 3 litters). Litter sizes (9 to 17 pups) were not standardized for size or sex ratio. All pups within the litter were administered the same compound to prevent cross-contamination (3 litters each for vehicle, EB, and low dose BPA, 4 litters for high dose BPA). Breeding and rearing procedures included control for potential environmental contamination (phytoestrogen free diet, polysulfone cages). At PNDs 4 and 10, pups were sacrificed

and their heads subjected for cryosectioning. In situ hybridization histochemistry (ISHH) was used to investigate gene expressions. Data was analysed by two-ways ANOVA (sex and exposure). Significant interactions were followed by one-way ANOVA to find effect of exposure within each sex. Then the Dunnett's Multiple Comparison post hoc test was used to compare same sex exposure and control groups.

There were no significant impacts of BPA in the mediobasal hypothalamus. Within the anterior hypothalamus ERα expression was augmented by BPA in PND 4 females, and then fell to male-typical levels by PND 10. ERβ expression was not altered by BPA on PND 4, but significantly decreased or eliminated in both sexes by PND 10. Kiss1 expression was diminished by BPA in the anterior hypothalamus, especially in females. The BPA effects did not mirror those of oestradiol benzoate, supporting the view that the interference of BPA with early hypothalamic organization involves mechanisms different from its estrogenic action.

Comments from the CEF Panel:
The control of environmental BPA contamination includes cages (polysulfone), feed (phytoestrogen free), and bedding (woodchip), but the type of water bottles is not mentioned. A positive control, oestradiol benzoate, was used. In the study, 13 dams and three litters per dose groups were used. Thus, littermates were investigated. Direct exposure to neonatal pups was used and it cannot be excluded that handling at delivery to PND 2 may influence gene expression. Considering the BPA dose span, 0.05 mg/kg bw and 50 mg/kg bw, a dose-response would have been expected, but did not clearly appear. In the statistics, the litter effect was not properly considered.

Cao J, Rebuli ME, Rogers J, Todd KL, Leyrer SM, Ferguson SA and Patisaul HB, 2013. Prenatal bisphenol A exposure alters sex-specific estrogen receptor expression in the neonatal rat hypothalamus and amygdala. Toxicological Sciences, 133, 157-173.

This study was specifically aimed at assessing whether prenatal BPA exposure altered sex-specific ESR1 (ERα) and ESR2 (ERβ) expression in postnatal limbic nuclei. Sprague Dawley rats were mated and gavaged on gestational days 6-21 with vehicle, 2.5 or 25 µg/kg bw per day BPA, or 5 or 10 µg/kg bw per day ethinyl oestradiol. An additional group was restrained but not gavaged (naïve control). Rearing conditions were controlled to avoid any potential source of environmental contamination. Offspring (n = 5-8 per sex/group) were sacrificed the day after birth to quantify ESR gene expression throughout the hypothalamus and amygdala by in situ hybridization. Data were analysed by two-way ANOVA (sex, exposure) followed by and Holm-Sidak multiple comparison tests. Naïve controls were not included in the overall analysis, since a separate analysis showed difference between controls and naïve controls.

Relative to the vehicle group, significant effects of BPA, mostly in the direction of the effects attributable to ethinyl oestradiol, were observed on ESR1 and ESR2 expressions throughout the mediobasal hypothalamus and amygdala in both sexes: the regions more sensitive to BPA effects include specific subregions of the amygdala. Significant differences in ESR expression were also observed in the mediobasal hypothalamus and amygdala of the naïve control group compared with the vehicle group, highlighting the potential for gavage to influence gene expression in the developing brain.

Comments from the CEF Panel:
Control of environmental BPA contamination was performed including a low phytoestrogen diet and glass bottles. This study presents a detailed analysis of ESRs expression in different sub regions of hypothalamus and amygdala following prenatal treatment with BPA or ethinyloestradiol (EE). Previous studies have consistently reported sex-dimorphic effects of developmental BPA exposure on ESR expression. Treatment schedule, route and doses used are relevant, both a positive control (EE) and naïve control were included and it was properly controlled for litter effect. The study is large and pup samples were from dams bred at different time points. The same litter samples were averaged. Advanced statistics comparisons have been used, but it is unclear whether multiple endpoint has been

considered. ESR expression is sexually dimorphic but neither BPA nor EE treatments interfere with such sex dimorphism. Furthermore is difficult to evidence an effect of BPA per se: both BPA and EE appear to enhance ESR expression in most of the sub-regions considered in comparison to vehicle-treated control. The enhanced ESR expression in naive controls makes the interpretation of the findings difficult: gavage per se seems to reduce ESR expression (stress effect) and the estrogenic stimulation (either BPA or EE) could somewhat reduce the "gavage" effects with mechanisms which are far to be understood. In conclusion the study suggests the estrogenic activity of BPA at very low doses shortly after termination of the prenatal exposure.

He Z, Paule MG and Ferguson SA, 2012. Low oral doses of Bisphenol A increase volume of the sexually dimorphic nucleus of the preoptic area in male, but not female, rats at postnatal day 21. Neurotoxicology and Teratology, 34, 331-337.

He et al. aimed at evaluating the effects of pre- and postnatal treatment with low BPA doses on the volume of the sexually dimorphic nucleus of the preoptic area (SDN-POA) of weanling rats. Pregnant Sprague-Dawley rats were orally gavaged with vehicle, 2.5 or 25.0 µg BPA/kg bw per day, or 5.0 or 10.0 µg ethinyl oestradiol (EE)/kg bw per day, on gestational days 6-21. Beginning on the day after birth, offspring of both sexes were orally treated with the same dose their dam had received. An additional group was restrained but not gavaged (naïve control). On PND 21, offspring (n=10-15/sex/group; 1/sex/litter) were perfused and volume evaluation was conducted blind to treatment. SDN-POA outline was delineated using calbindin D28K immunoreactivity. Low-phytoestrogen diet was given. Average SDN-POA volumes were analysed by two-way ANOVA (exposure, sex) and the Holm–Sidak method was used for interaction comparisons to the vehicle controls. Correction for anogenital distance (AGD) was not included in the statistical analyses.

Pairwise comparisons of the significant treatment by sex interaction indicated that neither BPA doses affected female SDN-POA volume. However, females treated with 5.0 or 10.0µg/kg EE exhibited volumes that were larger than same-sex controls, respectively ($p<0.001$). Males treated with either BPA dose or 10.0µg/kg/day EE had larger volumes than same-sex controls ($p<0.006$). These data indicate that BPA can have sex-specific effects on SDN-POA volume and that these effects manifest as larger volumes in males.

Comments from the CEF Panel:
Control of environmental BPA contamination was performed including a low phytoestrogen diet and glass bottles. A positive control was used (EE), but it was not properly positive for males. The effect of the highest dose used of EE was very similar to that of BPA, suggesting the use of other positive controls. The authors explained this as being due to the existence of an upper limit in males (e.g. a saturation value). Litter effect, statistical power (at least n=10 rats per group, 1/sex/litter) and vehicle controls were properly considered. The presence of a higher dose of BPA would have been important in assessing whether the effect is U-shaped. A weakness of this study is the consideration of a single histological endpoint at PND 21 and the lack of testing for functional effects of reproductive and sex dimorphic behaviours.

Komada M, Asai Y, Morii M, Matsuki M, Sato M, Nagao T. 2012. Maternal bisphenol A oral dosing relates to the acceleration of neurogenesis in the developing neocortex of mouse fetuses. Toxicology, 295, 31-38.

The study by Komada et al. was aimed at describing the effects of gestational exposure to BPA on the hippocampal neurogenesis of the fetal mouse. Young C57BL/6J mice were bought and then mated in house. The presence of a seminal plug was denoted embryonic days (E) 0. The mice had free access to standardised pellets and drinking water in glass bottles prior to and after mating. The pregnant mice were given corn oil or 200 µg/kg BPA by oral gavage from E 8.5 to E 13.5. At E 14.5, dams were killed and fetuses were subjected to histological evaluation (pups representing 6 litters per group).

Prior to killing of the dams, CldU and IdU were injected 24 h and 1 h, respectively, in order to elucidate the histological fetal changes.

Data presented by the authors indicated that maternal exposure to 200 µg/kg BPA was associated with accelerated neurogenesis and hyperplasia of cortical plate during telencephalon development, as demonstrated by increases of the thickness of the plate. Moreover, BPA-fetuses showed a reduction of neural stem/progenitor cells as a result of the promotion of neurogenesis in the dorsal telencephalon. Through immunostaining, the authors demonstrated that BPA exposure is specifically associated with the acceleration of neurogenesis of neural progenitor cells (IPCs) in the sub-ventricular zone (SVZ) and the differentiation of radial glial cells (RGCs) of dorsal telencephalon. In the BPA-treated group, the cell cycle was prolonged compared to controls.

Comments from the CEF Panel:
Environmental contamination of BPA is addressed as information about water bottles of glass, use of polypropylene cages and corncob bedding is given. The authors claim that BPA affects neuronal proliferation at critical developmental phases, likely by interfering with the important neurotrophic role of steroids and related receptors in brain development. There is however no specific statistical analysis Section, making it difficult to understand what is reported (SEM or SD) in figures. In addition, both male and female fetuses were considered, but no discussion of a sex effect (either significant or insignificant) is reported. Finally, it is impossible to evaluate whether the maternal factor has been considered, although on the basis of the number of treated pregnant females and of offspring of both sexes examined, it appears that there are at least four fetuses for each treated dams in the final experimental groups.

Viberg H and Lee I, 2012. A single exposure to Bisphenol A alters the levels of important neuroproteins in adult male and female mice. NeuroToxicology, 33, 1390-1395.

The aim of the study was to evaluate whether a single exposure to BPA during the brain growth spurt can alter the adult levels of proteins important for normal brain development (CaMKII and synaptophysin). Pregnant NMRI mice were fed standardised pellets and tap water ad libitum prior to and after delivery. Litters were culled to 10-14 pups within the first 48 hours. On PND10, male pups were given 0.32, 3.2 or 4.8 mg/kg bw and female mice were given 4.8 mg/kg bw in a single dose via metal gastric tube. Controls were gavaged vehicle (10ml 20% fat solution/kg bw). Males were sacrificed 24 h or 5 months after the BPA exposure (n=6 males), but females only after 5 months (n=8-9). The cerebral cortex and hippocampus brain regions were dissected out and frozen in liquid nitrogen and stored until protein analysis for CaMKII, GAP-43, synaptophysin and tau. Male data were analysed by one-way ANOVA and Newman–Keul's post hoc test (GraphPad Prism 5.01). The collected data from the adult females was analysed by Student's t-test. For neonatally mice, no statistically significant group differences for synaptic proteins in the cerebral cortex or the hippocampus appeared. For adult males, no group differences for synaptic proteins were shown in hippocampus or for CaMKII, GAP-43 and tau protein levels in the cerebral cortex, but for increased cerebral synaptophysin at the two higher BPA doses (3.2 or 4.8 mg/kg bw). For adult females, BPA caused increased synaptophysin in cerebral cortex and decreased CaMKII in both cerebral cortex and hippocampus. No other treatment effects were seen in females. According to the authors, the findings of this study indicate that a single neonatal exposure to BPA, on postnatal day 10, during the peak of the brain growth spurt, can alter the adult levels of proteins important for normal brain development (CaMKII and synaptophysin). These alterations are induced in both male and female mice and effects are seen in both hippocampus and cerebral cortex.

Comments from the CEF Panel:
Information on control of environmental contamination of BPA is lacking (i.e. cages, feed, water bottles, or bedding). The number of dams and their general reproductive outcome including pup sex ratio is not given. The litters were standardised with regard to size (12-14 pups) but sex ratio is not given. Chosen doses were relevant, although the exposure was single dose. Sample size was limited

for males (n=6), and acceptable for females (n=8-9) but it is unclear whether each sample represented one litter. In the statistics, the litter effect is not taken into consideration, thus the results presented may be questioned.

Xu X, Xie L, Hong X, Ruan Q, Lu H, Zhang Q, Zhang G, Liu X, 2013b. Chemosphere. Perinatal exposure to bisphenol-A inhibits synaptogenesis and affects the synaptic morphological development in offspring male mice. Chemosphere, 91, 1073-1081.

This study was aimed at evaluating whether perinatal exposure to BPA has effects on hippocampal synaptogenesis in exposed offspring. Pregnant ICR mice were orally exposed by injection from gestational day 7 through PND 21 to BPA at doses of 4, 0.4 or 0.04 mg/kg/bw day. Only male pups coming from 6 different litters (n= 6 male/group) were used in the study. Offspring were sacrificed on PND 14, 21 or 56 and brain processed for measurement of synaptic density and synaptic interface structure by the electron microscopy. The expression of Synapsin 1, PSD 95 and the levels of NMDA receptor subunit NR1, AMPA receptor subunit GluR1 were measured by immunoblotting in the synaptic fraction (n= 4 male/group). Synaptic density and the synaptic interface structure parameters were evaluated by two-way (age, exposure) repeated measure analysis of variance (ANOVA). One-way analysis of variance was applied to the Western blot data. Difference between groups was tested by use of Tukey's test.

Results showed that the numeric synaptic density of pyramidal cells of hippocampal CA1 area was significantly reduced by BPA (main effect of the treatment $p < 0.001$). The higher dose of BPA reduced synaptic density at all three ages considered, while the lower dose reduced this same measure at PND 14 and 56. No effects of the intermediate dose were found. The reduced numeric density was paralleled by alteration of synaptic ultrastructural parameters, i.e. curvature of synaptic interface, width of synaptic cleft and thickness of Post Synaptic Density (PSD), affected by BPA exposure in a dose-dependent fashion. PSD was reduced by BPA exposure while the synaptic cleft was enlarged and the length of the active synaptic zone reduced: the authors hypothesize that BPA affects not only synaptogenesis but possibly the efficacy of CA1 neurotransmission. The expression of synapsin-1, PSD 95 (both markers of synaptic functionality) as well as the expression of glutamate receptor subunits appeared significantly down-regulated on PND 14, 21 and 56 (AMPA receptors) and PND 21 and 56 (NMDA receptors). These findings replicate those already reported in a previous study of the same group (Xu et al., 2010) where a reduced expression of NMDA receptor subunits in the hippocampus on PND 21 and 56 was associated with impaired memory capabilities in BPA exposed mouse offspring.

Comments from the CEF Panel:
Information on control of potential sources of environmental BPA contamination is lacking (i.e. cages, feed, water bottles, or bedding). Route of exposure is oral but it is unclear whether "oral injection" refers to the use of gavage or not. The litter effect is considered, the sample size is acceptable for the electronic measurements (n=6) but rather small for the immunoblotting studies (n=4). Only male offspring are examined which leaves possible sex differences behind. This study follows previous studies of the same group and was focused on the effects of perinatal BPA exposure on hippocampal synaptogenesis, synaptic protein and glutamate neurotransmission.

(iii) In vitro studies

Appraisal of strengths and weaknesses and WoE analysis were not carried out for in vitro studies.

Seki S, Aoki M, Hosokawa T, Saito T, Masuma R, Komori M and Kurasaki M, 2011. Bisphenol-A suppresses neurite extension due to inhibition of phosphorylation of mitogen-activated protein kinase in PC12 cells. Chemico-Biological Interactions, 194, 23-30.

Seki and co-workers assessed the morphological changes in nerve growth factor (NFG)-induced differentiation caused by bisphenol-A in a PC12 cell system. In addition, to clarify whether BPA

affects the early and late stages of the NGF-signalling pathway in cell differentiation, changes of phosphorylation of MAP kinases and cAMP-response element binding protein (CREB) in PC12 cells treated with and without BPA in medium containing NGF were investigated using Western blot analysis. The results indicate that BPA significantly inhibited phosphorylation of CREB and ERK1/2 MAPK. When a BPA concentration of 10 ng/ml (corresponding to 50 nM) was added to medium containing NGF, it inhibited neurite extension.

Tanabe N, Yoshino H, Kimoto T, Hojo Y, Ogiue-Ikeda M, Shimohigashi Y and Kawato S, 2012. Nanomolar dose of bisphenol A rapidly modulates spinogenesis in adult hippocampal neurons. Molecular and Cellular Endocrinology, 351, 317-325.

The study by Tanabe et al. aimed at assessing the effects of BPA on spinogenesis in rat derived hippocampal slices. In addition to BPA concentrations ranging from 1 nM to 10 µM, the authors also assessed the effect of different compounds (hydroxytamoxifen, an antagonist of both estrogen-related receptor gamma (ERRγ) and estrogen receptors (ERα/ERβ), ICI, an antagonist of ERα/ERβ, the MAP kinase inhibitor PD98059, and the blocker of NMDA receptors, MK-801) to obtain insight into the mechanisms implicated in BPA modulation of spinogenesis. Spinogenesis was significantly enhanced by BPA added to isolated hippocampal slices obtained from untreated adult Wistar male rats. The results suggest that the action of BPA on ERRγ may contribute to the observed rapid effect on spinogenesis. Finally, the authors also measured BPA concentration in hippocampus through mass-spectrometry, finding an average concentration of 14.6 ± 1.8 ng/g wet weight (64 ± 8 nM) from 4 animals.

Although the findings presented by the authors are preliminary and should be confirmed in vivo, the study shows that internal BPA concentration in the nanomolar range may change density and morphology of spinogenesis of hippocampal neurons from adult rats. The reported BPA concentration in the hippocampus is surprisingly high considering that rats after oral treatment with 100 µg/kg bw have unconjugated BPA serum concentrations of 0.1 mg/ml (0.5 nM; Doerge et al., 2011b).

Warita K, Mitsuhashi T, Ohta KI, Suzuki S, Hoshi N, Miki T and Takeuchi Y, 2013. In vitro evaluation of gene expression changes for gonadotropin-releasing hormone 1, brain-derived neurotrophic factor, and neurotrophic tyrosine kinase, receptor, type 2, in response to bisphenol A treatment. Congenital Anomalies (Kyoto), 53, 42-45.

The present short communication reports effects of bisphenol A (BPA) on gene expressions of embryonic mouse hypothalamic cells by using the cell line mHypoE-N44. Normal maturation of the hypothalamic-pituitary-gonadal axis in fetuses is, among other thing, dependent on proper development of the hypothalamic neurons that release gonadotropin-releasing hormone 1 (GNRH1). Additionally, most embryonic GNRH1 neurons express neurotrophic tyrosine kinase, receptor, type 2 (NTRK2), which is the high-affinity receptor of brain-derived neurotrophic factor (BDNF) that supports the survival of a variety of embryonic neural types.

BPA was dissolved in dimethyl sulfoxide (DMSO; final concentration, 0.1%). The vehicle DMSO (0.1%) showed no cell toxicity or effects on cell survival or ability to divide. Cells exposed to DMSO 0.1% served as controls, and each exposure experiment was performed in three replicate wells.

mHypoE-N44 cells were incubated with 0.02, 0.2, 2, 20, and 200 µM BPA for 3 hours and evaluated for *Gnrh1* gene expression by use of reverse transcription polymerase chain reaction (RT-PCR) with glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) mRNA as internal positive control. Equivalently, mHypoE-N44 cells were examined for gene expressions of *Bdnf* and its high-affinity receptor *Ntrk2* after incubation with 200 µM BPA for 3, 6 and 12 hours. Statistical analyses were performed using one-way analysis of variance (ANOVA) and Bonferroni–Dunn post-hoc tests.

Results showed that cells treated for 3 hours with the highest concentration of BPA, 200µM, expressed statistically significant decreased levels of *Gnrh1* compared to control cells. In cells treated with 200

µM BPA for 6 and 12 hours, the mRNA level for brain-derived neurotrophic factor (*Bdnf*) was significantly decreased, whereas that for neurotrophic tyrosine kinase, receptor, type 2 (*Ntrk2*) did not change.

The authors' conclude that these results indicate that *Gnrh1* gene expression in mice fetuses is not affected by exposure to less than 20 µM BPA and that the adverse effects of BPA on the BDNF-NTRK2 neurotrophin system are induced by decreased mRNA level of the ligand BDNF, not of its receptor.

Immune effects

(i) Human studies

Clayton EMR, Todd M and Aiello AE, 2011. The impact of Bisphenol A and Triclosan on Immune Parameters in the US population, NHANES 2003-2006. Environmental Health Perspectives, 119, 390-396.

Clayton et al. (2011) examined possible associations of urinary BPA levels with serum cytomegalovirus (CMV) antibody levels and diagnosis of allergies or hay fever in US adults and children >6 years of age (n=3 728), for which the survey and laboratory data from the 2003–2006 US NHANES were used. The exposure was assessed by measuring BPA in spot urines. Total (free and conjugated) urinary BPA was measured by solid phase extraction (SPE) coupled with liquid chromatography–tandem mass spectrometry (LC–MS–MS, LOD 0.36 ng/ml) at the Center for Disease Control and Prevention (CDC). CMV-specific IgG was measured using an enzyme-linked immunosorbent assay (ELISA) and CMV-specific IgG optical density was reported [measured in arbitrary units (AU) per milliliter] and used as a continuous outcome variable. CMV seropositivity was defined by NHANES based on this optical density measure. A diagnosis of allergy or hay fever was determined by two questions in the NHANES interview. Respondents were asked: "Has a doctor or other health professional ever told you that you have allergies/hay fever?" A respondent was coded as having allergies or hay fever if the response to either question was yes. Multivariate ordinary least squares linear regression models were used to examine the association of BPA with CMV antibody titers, and multivariate logistic regression models to investigate the association of these chemicals with allergy or hay fever diagnosis. Statistical models were stratified by age (<18 years and ≥18 years). In analyses adjusted for age, sex, race, body mass index, creatinine levels, family income, and educational attainment, in the ≥18-year age group, higher urinary BPA levels were associated with higher CMV antibody titers ($p<0.001$). In the <18-year age group, lower levels of BPA were associated with higher CMV antibody titers ($p<0.05$). BPA showed no association with allergy or hay fever diagnosis.

Comments from the CEF Panel:
The CEF Panel identified the following strengths and/or weaknesses in this study:
Strengths:

- Large sample size
- Analytical method (SPE LC–MS–MS)
- Quality control, including blanks and quality assurance procedures

Weaknesses:

- Cross–sectional study design
- Single exposure measurements
- Single spot urine BPA measurement
- Confounding by diet or by concurring exposure factors not considered or not reported
- Unclear clinical relevance (inconsistent results between groups stratified by age)

The authors do not offer an explanation for these contrasting observations, and conclude that additional studies should be done to further investigate these findings.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Donohue KM, Miller RL, Perzanowsky MS, Just AC, Hoepner MS, Arunajadai S, Canfield S, Resnick D, Calafat AM, Perera FP and Whyatt RM, 2013. Prenatal and postnatal bisphenol A exposure and asthma developoment among innet-city children. Journal of Allergy and Clinical Immunology, 131, 736-742.

Donohue et al. (2013) examined possible associations of urinary BPA levels with wheeze and asthma in a prospective cohort of 568 Dominican (65%) and African American (35%) mothers and children in New York. Total (free plus conjugated) was determined by solid phase extraction (SPE) coupled with liquid chromatography tandem mass spectrometry (LC-MS-MS, LOD 0.4 ng/ml) at the Center for Disease Control and Prevention (CDC). BPA concentration in spot urine samples was measured in the pregnant women in the third trimester of pregnancy (n=375), and in the children at 3 (n=408), 5 (n=401), and 7 (n=318) years of age. Women were screened to avoid enrollment of active smokers. Wheeze questionnaire were administered in the last 12 months at the ages of 5, 6, and 7 years, and asthma was determined by a physician at ages between 5 and 12 years, using the following criteria: respiratory symptoms, FEV1 measurements, IgE measurements, asthma medication. FENO (fraction of exhaled nitric oxide) measurements were done at ages between 5 and 11 years. Maternal prenatal urinary BPA levels were lower than all BPA levels measured in children and did not correlate with child urinary BPA.

Results for *prenatal* BPA: Contrary to what was hypothesized, the prenatal urinary BPA concentration was associated inversely with wheeze at age 5 years (odds ratio [OR], 0.7; 95% CI, 0.5–0.9; p=0.02), but no associations were found for prenatal BPA values with wheeze at age 6 or 7 years or with asthma at ages between 5 and 12 years or with serotopy at age 7 years. Results for *postnatal* BPA: urinary BPA concentrations at age 3 years were associated positively with wheeze at ages 5 years (OR, 1.4; 95% CI, 1.1–1.8; p=0.02) and 6 years (OR, 1.4; 95% CI, 1.0–1.9; p=0.03). BPA concentrations at age 7 years were associated with wheeze at age 7 years (OR, 1.4; 95% CI, 1.0–1.9; p=0.04) and FENO values (β=0.1; 95% CI, 0.02–0.2; p=0.02). BPA concentrations at ages 3, 5, and 7 years were associated with asthma (OR, 1.5 [95% CI, 1.1–2.0], p=0.005; OR, 1.4 [95% CI, 1.0–1.9], p=0.03; and OR, 1.5 [95% CI, 1.0–2.1], p=0.04, respectively). No associations with seratopy at 7 years were noted. Although the strength of the associations is modest, this is the first cohort study to report an association between childhood urinary BPA concentrations and asthma in children. There negative correlation between maternal BPA and wheeze at 5 years was unexpected. Only few children had all the repeated measures (n=82).

CEF Panel:
The CEF Panel identified the following strengths and/or weaknesses in this study:
Strengths:
- Longitudinal follow up
- Large sample size
- Repeated measurements
- Analytical method (LC-MS-MS)
- Quality control, including blanks and quality assurance procedures
- Multiple outcome assessment for asthma

Weaknesses:
- Single spot urine BPA measurements
- Confounding by diet or by concurring exposure factors not considered
- Inconsistency of results between groups
- Inconsistent results amongst different studies

Overall the CEF Panel notes that the odd ratio's observed were modest, and as for all observational studies, unmeasured confounding might have biased the results. Potential confounding for diet was not considered. Yet, the findings are in agreement with other evidence that BPA may be associated with adverse respiratory outcomes.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Savage JH, Matsui EC, Wood RA and Keet CA, 2012. Urinary levels of triclosan and parabens are associated with aeroallergen and food sensitization. Journal of Allergy and Clinical Immunology, 130, 453-460.

The aim of this study was to determine the association between urinary endocrine-disrupting compounds (EDCs), namely BPA, triclosan, benzophenone-3, propyl, methyl, butyl, and ethyl parabens, with specific aeroallergen and food allergen sensitization. Data were obtained from the 2005–2006 National Health and Nutrition Examination Survey (NHANES) and urinary BPA, triclosan, benzophenone-3, propyl, methyl, butyl, and ethyl parabens and specific IgE levels were available for 860 children aged 6–18 years. Urinary BPA and other EDCs were measured by liquid chromatography tandem mass spectrometry (LC-MS-MS, LODs 0.1–2.0 ng/ml). Aeroallergen and food sensitizations were defined as having at least one positive (≥0.35 kU/L) specific IgE level to an aeroallergen or a food. Analyses were adjusted for urinary creatinine level, age, sex, ethnicity, and poverty index ratio. In contrast to triclosan and propyl and butyl parabens, no associations between urinary BPA levels and sensitization were observed.

Comments from the CEF Panel:
The CEF Panel identified the following strengths and weaknesses in this study:
Strengths:
- Large sample size
- Analytical method (LC-MS-MS)
- Quality control, including blanks and quality assurance procedures
- Multiple outcome assessment for allergen sensitisation

Weaknesses:
- Cross-sectional study design
- Single spot urine BPA measurement
- Confounding by diet or by concurring exposure factors not considered
- Inconsistent results amongst different studies

Overall the CEF Panel notes that the manuscript is interesting and the scientific soundness is acceptable in light of the limitations related to the cross-sectional design and single spot urine samples. The fact that urinary BPA was not significantly related to aeroallergen and food sensitization is a negative but quite relevant result.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Spanier AJ, Kahn RS, Kunselman AR, Hornung R, Xu Y, Calafat AM and Lanphear BP, 2012. Prenatal Exposure to Bisphenol A and Child Wheeze from Birth to Three Years. Environmental Health Perspectives, 120, 916-920.

Spanier et al. (2012) examined prenatal BPA exposure and childhood wheeze from birth to 3 years of age in 365 mother–child pairs. The exposure was assessed by measuring BPA in spot urines from mothers at 16 and 26 weeks of gestation and at birth. Total (free plus conjugated) was determined by solid phase extraction (SPE) coupled with liquid chromatography tandem mass spectrometry (LC-MS-

MS, LOD 0.4 ng/ml) at the Center for Disease Control and Prevention (CDC). Tobacco exposure was measured by means of serum concentration of a metabolite of nicotine. Childhood wheeze was surveyed every 6 months from birth to 3 years by trained research assistants at home visits or by phone, using questions parallel to the NHANES wheeze question. Questions included the number of wheeze attacks, and the outcome was dichotomized to no wheeze versus any wheeze at each time point. The results were mainly negative. When prenatal BPA exposure was modelled as a continuous variable (mean of three values), BPA was not related with childhood wheeze. However, when urinary BPA was categorized above or below the median value, a significant positive relationship with wheeze was found at six months of age, but there was no evidence of a persistent positive association by three years of age.

Comments from the CEF Panel:
The CEF Panel identified the following strengths and weaknesses in this study:
Strengths:
- Longitudinal follow up
- Large sample size
- Repeated measurements
- Analytical method (LC-MS-MS)
- Quality control, including blanks and quality assurance procedures
- Multiple outcome assessment for wheeze

Weaknesses:
- Single spot urine BPA measurements
- Confounding by diet or by concurring factors (other than active smoking during pregnancy) not reported
- Unclear clinical relevance (relevance of wheeze difficult to interpret in the absence of effects on sensitisation)
- Inconsistent results amongst different studies

Overall the CEF Panel notes that the study is generally sound, but the CEF Panel considers that this categorization of BPA exposure to be questionable and notes that exposure after birth was not considered. The relevance of wheeze is of difficult interpretation in the absence of effects on sensitisation.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Vaidya SV and Kulkarni H, 2012. Association of Urinary Bisphenol A Concentration with Allergic Asthma: Results from the National Health and Nutrition Examination Survey 2005-2006. The Journal of Asthma, 49, 800-806.

Vaidya et al.(2012) examined whether urinary BPA concentration was associated with allergic asthma using data from the National Health and Nutrition Examination Survey 2005–2006 survey. The sample size was large (n=2548). Total (free plus conjugated) was determined by solid phase extraction (SPE) coupled with liquid chromatography tandem mass spectrometry (LC-MS-MS, LOD 0.4 ng/ml). The exposure was spot urine BPA measurement, allergic asthma was defined as a history of asthma ever, high eosinophil count, and high total IgE or atopy. The results showed that 10-fold increase in BPA was independently associated with a higher likelihood of allergic asthma in females [odds ratio (OR)=2.21, p=0.032] but not in males (OR=0.83, p=0.474). These findings were reaffirmed when allergic asthma was defined based on atopy rather than total IgE (OR=2.45, p=0.001 in females and OR=0.83, p=0.605 in males). Urinary BPA was significantly associated with sensitization to various specific allergens in a dose-response manner.

Comments from the CEF Panel:
The CEF Panel identified the following strengths and weaknesses in this study:

Strengths:

- Large sample size
- Analytical method (LC-MS-MS)
- Quality control, including blanks and quality assurance procedures
- Multiple outcome assessment for asthma

Weaknesses:

- Cross-sectional study design
- Single spot urine BPA measurement
- Not adjusted urine samples
- Confounding by diet or by concurring factors not reported
- Unclear clinical relevance (gender-related differences)
- Inconsistent results amongst different studies

Overall the CEF Panel notes that the relevance of this study is limited by several methodological issues (cross-sectional study and single spot urines) and statistical handling. Urinary BPA measurements were not adjusted for creatinine or specific gravity data and log of BPA was used as dependent variable in spite of several samples <LOD. Differences were found between included and excluded subjects, but the differences were not considered clinically meaningful. The authors acknowledged the main limitations of the study.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

(ii) Animal studies

Bauer S, Roy A, Jason E, Chapman T, Georas S. Lawrence BP. The effects of maternal exposure to bisphenol A on allergic lung inflammation into adulthood. Toxicological Sciences, 130, 82-93.

Bauer et al. (2012) administered BPA by gavage to pregnant C57Bl/6 damns from gestation day 6 until post-natal day 21 to 0, 0.5, 50, or 500 µg BPA/kg/day. To induce allergic inflammation, adult offspring were mucosally sensitized with inhaled ovalbumin containing low dose lipopolysaccharide or intraperitoneally sensitized using ovalbumin with "alum" (not further specified by the authors), followed by ovalbumin aerosol challenge. In the mucosal sensitization model, female offspring maternally exposed to ≥ 50 µg BPA/kg/day displayed enhanced lymphocytic and lung inflammation, assessed by histopathology, compared to controls. This effect was evident in females but not males. There were no clear dose-related effects on cell numbers in broncholveolar lavages of the animals. Peritoneally sensitized female but not male mice maternally exposed to ≤ 50 but not 500 µg/kg showed a dampened effect on numbers of eosinophils in lung lavage, but there was no clear dose-response relationship. In the peritoneal model, there was no effect on airway hyperresponsiveness in females noted. Unfortunately, this clinical outcome was not measured in the more relevant mucosal model.

Comments from the CEF Panel:
The CEF Panel identified the following strengths and weaknesses in this study:
Strengths:

- Number of doses (≥3)
- Phytoestrogen –free diet
- Oral administration via gavage
- BPA measurement in biological samples

Weaknesses:

- Study design not appropriate to the scope (Lower end of the dose-response was performed in a separate experiment)

Overall, the CEF Panel considered that the inflammation seen in the mucosal model in females is an adverse effect, indicative of stimulation of allergic asthma. It is not known why females but not males show these effects. The effect seems subtle, as effects on eosinophils in the lavage could not be detected, but airway hyperreactivity was not assessed in that model. In the peritoneal sensitization model, the females showed dampened eosinophil numbers after inhalation challenge to ovalbumin in the lung lavage, but only below 50 µg/kg bw per day, and not at the highest concentration. Hence, there is no clear dose-response relationship, while the CEF Panel considers this intraperitoneal model less relevant for allergic asthma.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Kendziorski JA, Kendig EL, Gear RB, Belcher SM, 2012. Strain specific induction of pyometra and differences in immune responsiveness in mice exposed to 17α-ethinyl estradiol or the endocrine disrupting chemical bisphenol A. Reproductive Toxicology, 34, 22-30.

Kendziorsky et al. (2012) administered BPA in the diet of CD1 and C57Bl mice (n= 5 per group) at levels of 0, 0.03, 0.3 or 30 mg/kg diet, (estimated to be approximately equivalent to 0, 4, 30-40 or 4000 µg BPA/kg bw per day) from before mating, through gestation, parturition and weaning (in F0 females) and until weeks 19-23 (F0 females). 17α-ethinyl oestradiol (EE; 0.01, 0.1 or 1.3 mg/kg diet) was also administered to separate groups of mice (n=4), equivalent to approximate intakes of 0, 1.2-1.4, 12-15 or 1160 µg DES/kg bw/ day. Reproductive performance was assessed and uterine pathology of the F0 females was investigated following sacrifice at weeks 19-23.
The authors observed pyometra, i.e. inflammation in the uterus in in a small minority of C57Bl mice receiving 0.3 mg BPA/kg diet, accompanied by changes in uterine morphology. A 5-fold, statistically significantly more pronounced presence of macrophages was observed in the uteri of all C57B1 females at this dose. Pyometra was also observed in the 15µg/kg-d EE treatment group, but no such changes were seen in CD1 mice. The authors concluded that BPA enhances immune responsiveness of the uterus and that heightened responsiveness in C57BL/6 females is related to increased susceptibility to pyometra.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:
- Positive control included
- Number of doses (≥3)
- Phytoestrogen –free diet

Weaknesses:
- Small sample size

Overall, the CEF Panel noted that the relevance of the macrophage infiltration in terms of pyometra is not clear, and the conclusion of the authors that BPA enhances immune responsiveness is speculative. The pyometra was observed only in a minority of animals at highest dose. These notions do not take away from the fact that infiltration of macrophages may be adverse. A clear deficiency in the study is that a dose-response relationship has not been established, although it is not totally clear from the paper whether uterine changes were investigated in the animals receiving 30 mg BPA/kg diet.
This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Lee J, Lee SJ, Lim KT and 2012a. CTB glycoprotein (75kDa) inhibits IgE releasing, TNF-α and IL-6 expressed by bisphenol A *in vivo* and in vitro. Food and Chemical Toxicology, 50, 2109-2117.

Lee et al. (2012a) described studies in which adult female BALB/c mice (n=6) were injected intraperitoneally with 5 mg BPA/kg body weight per day for 4 weeks. The treatment resulted in increases in several non-specific inflammatory mediators and total levels of IgE. These effects were diminished or blocked in the presence of a glycoprotein derived from Cudrania tricuspidata Bureau (CTB), investigation of such an inhibitory effect being the main purpose of the study.

In the in vitro part of the study, the authors examined the effects of BPA (50 µM) on extracellular signal-regulated kinases (ERK) and p38 mitogen-activated protein kinase (MAPK), activator protein (AP)-1, expressions of pro-inflammatory cytokines, nitric oxide (NO) production and cyclooxygenase (COX)-2 in pre-mast cells (RBL-2H3 cells) BPA was found to stimulate expression of these various markers, indicative of an immunomodulatory effect. CTB glycoprotein blocked or partially inhibited the stimulatory effect of BPA.

Comments from the CEF Panel:
The CEF Panel identified the following weaknesses in the study:

Strengths:
- None

Weaknesses
- Small sample size
- Test performed in one sex only
- Single dose level study (to show effects on total IgE non-specific inflammatory mediators).
- Study design not appropriate to the scope (no functional endpoints assessed)

Overall the CEF Panel considers that while BPA had a number of effects that may be seen as immunomodulatory, only one concentration of BPA was used, no functional endpoints were investigated and the number of animals was relatively small.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Nakajima Y, Goldblum RM, Midoro-Horiuti T, 2012. Fetal exposure to bisphenol A as a risk factor for the development of childhood asthma: an animal model study. Environmental Health, 11, 8.
Nakajima et al. (2012) exposed female Balb/c mice to 10 µg/ml BPA in their drinking water from one week prior to gestation until the end of the study on day 25 post partum. Some pups were left with their mothers, but other pups were switched to unexposed mothers within the first 48 hours of life, so that exposure took place during the entire study period, only prenatally, or only postnatally. Pups were sensitized to ovalbumin at day 4 after birth and challenged at days 18, 19 and 20 after birth. Airway hyperreactivity to methycholine as well as inflammation by evaluating eosinophils in bronchoalveolar lavage were assessed in 22 day old pups. Pups exposed in utero or through mother's milk in addition to in utero exposure showed increased airway hyperreactivity and increased eosinophil numbers in bronchoalveolar lavage fluid. Pups exposed only post-natally did not show such effects. The authors concluded that prenatal exposure to BPA, followed by postnatal allergic sensitisation and challenge, promoted the development of experimental allergic asthma. They suggested that delayed expression of BPA-metabolising enzymes may explain, at least in part, the enhanced fetal susceptibility.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:

Strengths:
- Phytoestrogen-free diet
- Use of non-PC cages and of non plastic bottles

Weaknesses:
- Animal age and body weight not given
- Small sample size
- Single dose level study
- Study design not appropriate to the scope (administration via drinking water, but water consumption not measured)

Overall the CEF Panel considers that this study shows enhancement of ovalbumin-induced airway hyper-reactivity in mice and increased numbers of eosinophils in bronchoalveolar lavage after pre- and postnatal exposure to BPA, but no dose-response relationship was assessed.
This study is included in the WoE table because of its relevance to one or more review questions addressed there.

(iii) In vitro studies

Appraisal of strengths and weaknesses and WoE analysis were not carried out for in vitro studies.

Pisapia L, Del Pozzo G, Barba P, Caputo L, Mita L, Viggiano E, Russo GL, Nicolucci C, Rossi S, Bencivenga U, Mita DG and Diano N, 2012. Effects of some endocrine disruptors on cell cycle progression and murine dendritic cell differentiation. General and Comparative Endocrinology, 178, 54-63.

Pisapia and coll. investigated the effect of several substances including BPA on the differentiation of bone marrow dendritic cells isolated from female mice and cultured in hormone-deficient medium. BPA at 10^{-7} M, 10^{-6} M and 10^{-5} M induced the differentiation of 62%, 70% and 91% of the cells to the CD11c+ phenotype, respectively. The CEF Panel noted that due to high BPA concentrations and the specific culture conditions the relevance of this finding for the in vivo situation is not clear.

Cardiovascular effects

(i) Human studies

BPA effects on coronary artery disease/heart attack

Lakind JS, Goodman M and Naiman DQ, 2012. Use of NHANES Data to Link Chemical Exposures to Chronic Diseases: A Cautionary Tale. PLoS One. 2012;7(12):e51086.

The authors reanalysed data from four datasets in the National Health and Nutrition Examination Survey (NHANES). Data on urinary BPA and health outcomes from 2003-2004, 2005-2006, 2007-2008, and 2009–2010 were available. The aim was to examine the consistency of the association between urinary BPA measures and diabetes, coronary heart disease (CHD), and/or heart attack across datasets when consistent scientifically and clinically defined criteria were applied. The study sample included n=4811 for CVD, n=4811 for heart attack and n=4823 for diabetes. Samples were analysed by on line solid-phase extraction (SPE) coupled with liquid chromatography tandem mass spectrometry (LC-MS-MS, LOD 0.4 ng/mL). All multivariable analyses were controlled for a priori selected potential confounders including, but not limited to, those used in the previous studies. The models included the following covariates: creatinine, age, gender, race/ethnicity, education, income, smoking, body mass index, waist circumference, heavy drinking, family history of diabetes (in the analyses of diabetes) or heart attack/angina (in the analyses of CHD and heart attack), hypertension, sedentary activity, blood cholesterol, and daily energy intake. Urinary BPA was not significantly associated with adverse health outcomes for any of the NHANES surveys, with ORs (95% CIs) ranging from 0.996 (0.951–1.04) to 1.03 (0.978–1.09) for CHD, 0.987 (0.941–1.04) to 1.04 (0.996–1.09) for heart attack, and 0.957 (0.899–1.02) to 1.01 (0.980–1.05) for diabetes. When the data from

four surveys were pooled, the ORs (95% CIs) for the full model that included all covariates were 1.004 (0.998–1.009) for CHD, 1.002 (0.998–1.007) for heart attack, and 0.995 (0.982–1.007) for diabetes. The choice of covariates had only minor effect on point estimates. The authors concluded that the discrepancy between their findings on diabetes and those reported previously was largely explained by the choice of case definition. For discrepancy between results of this study and previous findings for CHD, the authors concluded that this was in part attributable to differences in inclusion criteria. In the current study, no subjects were excluded based on very high BPA concentrations.

The authors provided an example of reverse causality obscuring possible conclusions from cross-sectional studies: "In all analyses, cholesterol levels were statistically significantly inversely associated with heart attack and CHD. Given the well-documented positive association between cholesterol and heart disease from prospective studies, the most logical explanation for the observed result is reverse causation, i.e. it is likely that diagnoses of heart attack or CHD, which preceded the cholesterol measurements in NHANES, likely triggered changes in lifestyle or use of medications that resulted in lower cholesterol levels."

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- Large sample size
- Urine, container specified
- Standardised samples (urinary creatinine included in the model as independent variable)
- Analytical method (SPE LC-MS-MS)
- Quality control, including blanks and quality assurance procedures

Weaknesses:

- Cross-sectional study design
- Single spot urine BPA measurement
- No distinction between unconjugated and conjugated BPA
- Confounding by diet or by concurring exposure factors not considered
- Inconsistency in results among different studies

Overall, the CEF Panel considers that this study shows how relatively minor decisions made a priori (clinical definition of diabetes and inclusion of participants with higher levels of BPA) affected the previously reported results and conclusions of associations between urinary BPA exposure and chronic disease. This study does not add to the evidence as to whether or not BPA is a risk factor for chronic disease, but highlights that using data from cross-sectional studies like NHANES surveys to draw conclusions about relations between short-lived environmental chemicals and chronic diseases is inappropriate.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Lind PM and Lind L, 2011. Circulating levels of Bisphenol A and Phthalates are related to Carotid Atherosclerosis in the Elderly. Atherosclerosis, 218, 207-213.

The authors investigated whether circulating levels of BPA and phthalate metabolites were related to atherosclerosis. The study was carried out as a cross-sectional analysis of 1 016 subjects all aged 70, within a population-based cohort in Sweden. BPA and 10 phthalate metabolites were measured in serum by solid phase extraction (SPE) coupled with liquid chromatography tandem mass spectrometry (LC-MS-MS, LOD 0.2 ng/mL). Atherosclerosis was defined by the prevalence of overt plaques and echogenectity (grey scale median, GSM) of carotid artery plaques recorded by ultrasound in both of the carotid arteries. Additionally, the thickness (IMT) and echogenicity (IM-GSM) of the intima-

media complex were measured. BPA was not significantly associated with the number of arteries with plaque or intima-media thickness, while these associations were significant for mono-methyl phthalate (MMP). High levels of BPA as well as some phthalates were associated with an echogenic IM-GSM and plaque GSM, suggesting a role for plaque-associated chemicals in atherosclerosis. The models were adjusted for multiple CV risk factors.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- Large sample size
- Analytical method (SPE LC-MS-MS)
- Quality control, including blanks and quality assurance procedures

Weaknesses:

- Cross-sectional design
- Selection bias (Caucasians aged > 70, better health conditions)
- Serum BPA measurement (invalid exposure measurement)
- Single BPA measurements
- No distinction between unconjugated and conjugated BPA
- Handling of values below LOQ not reported
- Confounding by diet or by concurring exposure factors not considered
- Generalisability to the total population
- Inconsistency in results among different studies

Overall, the CEF Panel considers that the significant relationship between serum level of BPA and echogenicity of the intima-media complex and plaque echogenicity in 1 016 subjects aged 70 is potentially interesting, but the study has main limitations (e.g. cross-sectional design and invalid exposure assessment using serum BPA without distinction between unconjugated and conjugated BPA). The four phthalates and BPA were analyzed separately, and the relationship between the measured substances would be interesting.

The CEF Panel notes that no formal adjustment for multiple testing was made and the explanation for not performing it provided by the authors is not convincing. Further covariates for which information was available were not adjusted for in the models (e.g. myocardial infarction, drugs other than statins); the authors neither describe nor discuss this choice. Some of the CV risk factors included in the analysis for adjustment were collected via administration of a questionnaire (e.g. smoking, statin use); there is a possibility for information bias due to self-reporting.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Melzer D, Osborne NJ, Henley WE, Cipelli R, Young A, Money C, McCormack P, Luben R, Khaw KT, Wareham NJ and Galloway TS, 2012a. Urinary Bisphenol: A Concentration and Risk of Future Coronary Artery Disease in Apparently Healthy Men and Women. Circulation, 125, 1482-1490.

This is an observational nested case-control prospective study using data from a 10 year follow up of the EPIC-Norfolk cohort in the UK to examine associations between urinary BPA concentration and later coronary artery disease (CAD). Total BPA (unconjugated and conjugated) was determined in spot urine, by solid phase extraction (SPE) coupled with liquid chromatography tandem mass spectrometry (LC-MS-MS, LOQ 0.50 ng/mL). The results showed that higher urinary concentrations of total BPA were associated with increased risk of developing coronary artery disease defined as hospital admittance or death because of myocardial infarction). One SD (4.45 ng/mL) increase in urinary total BPA in a partly adjusted model resulted in and increased risk of OR: 1.13 (95%CI: 1.02–

1.24) and in a fully adjusted model of OR: 1.11 (95% CI: 1.00–1.23, p=0.058). Blood and urine samples were taken between March 1993 and April 1998. Follow-up was until first endpoint event or December 2003. The longest observation interval between urine sampling (and estimation of BPA concentrations in urine) and observation of the endpoint (if not the endpoint occurred in between) is 10 years 9 months; the shortest is 5 years 8 months.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- Longitudinal follow up
- Analytical method (SPE LC-MS-MS)
- Quality control, including blanks and quality assurance procedures

Weaknesses:

- Single spot urine BPA measurement
- Confounding by diet or by concurring exposure factors not considered
- Unclear clinical relevance (small effect size)
- Generalisability to the overall population

Overall the CEF Panel notes that the longitudinal design in a European population can be seen as a major strength in comparison to previously reported cross-sectional associations in more highly exposed US study populations. BPA exposure was measured in spot urine samples with limited information on the conditions of sampling, except that the BPA measures were from single-spot urine specimens and that urine samples were taken at the same time of day for each respondent. However, the authors reported that they followed WHO guidelines as regards biomonitoring of BPA exposure as well as GLP and inclusion of reagent blanks**.**

The endpoint in this study was CAD, and the authors only identified cases that were admitted to the hospital or died because of myocardial infarction. Validation of diagnoses was only performed in the cases of myocardial infarction. CAD is a disease where patients do not necessarily need to be admitted to a hospital. Hence, the possibility is given that subjects in the control group have a CAD which was not leading to a hospital admission. The authors excluded subjects with diabetes. Several statistical models were reported, of which only the fully adjusted model D is acceptable because in partly adjusted models the known risk factors were not taken into consideration. If adjusted for BMI, cigarette smoking, average of the 2 systolic blood pressure readings (in mm Hg), total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, triglycerides, and level of physical activity the odds ratio decreased to 1.11 (CI 1.00–1.23), p=0.058. It is also of interest to note that removal of the cases of the first three years in the post hoc sensitivity analysis changed the odds ratio to 1.12 (CI 1.00–1.26) p=0.05. The authors indicate that they measured C-reactive protein (also associated with CAD) but they did not explain why the measurements were not included into the model. However, this seems not to be of influence in the post hoc analysis.

The discussion part infers from kinetic data in rodents to the human situation which is not scientifically correct given the fact that in rodents enterohepatic recirculation influences the terminal half-life whereas in humans BPA is not undergoing enterohepatic recirculation. Hence, their conclusion that under normal condition of food intake single spot urinary concentration of total BPA is a valuable estimate for intake is not substantiated and is in contrast to experimental findings mimicking normal conditions of food intake (Teeguarden et al., 2011). The study may be confounded by the fact that CAD cases were only cases with hospital admission thus not including cases in outpatient care (whether this omission is distributed equally between cases and controls is unclear and here the fact that they did not match the controls may play a role). The association observed in the study was weak.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Melzer D, Gates P, Osborn NJ, Henley WE, Cipelli R, Young A, Money C, McCormack P, Schofield P, Mosedale D, Grainger D and Galloway TS, 2012b. Urinary Bisphenol A concentration and angiography-defined coronary artery stenosis. PLoS One. 2012 ;7(8):e43378. doi: 10.1371/journal.pone.0043378. Epub 2012b Aug 15.

The study aim was to estimate associations between BPA exposure assessed in spot urine and angiographically graded coronary atherosclerosis in 591 patients participating in The Metabonomics and Genomics in Coronary Artery Disease (MaGiCAD) study in Cambridgeshire UK. Total BPA (unconjugated and conjugated) was determined in spot urine, by solid phase extraction (SPE) coupled with liquid chromatography tandem mass spectrometry (LC-MS-MS, LOQ 0.50 ng/ml). Total BPA was compared with grades of severity of coronary artery disease (CAD) on angiography. Linear models were adjusted for BMI, occupational social class and diabetes status. Severe (one to three vessel) CAD was present in 385 patients, 86 had intermediate disease (n=86) and 120 had normal coronary arteries. The (unadjusted) median urinary BPA concentration was 1.28 ng/mL in patients with normal coronary arteries and 1.53 ng/mL in patients with severe CAD. Compared to those with normal coronary arteries (n=120), urinary BPA concentration was significantly higher in those with severe CAD (n=385) (OR per uBPA SD=5.96 ng/mL OR=1.43, CI 1.03 to 1.98, p=0.033), and near significant for those with intermediate disease (n=86) (OR=1.69, CI 0.98 to 2.94, p=0.061). Patients with severe CAD were further divided into three groups with scores denoting disease in 1, 2 or three vessels (148 with 1 VD, 123 with 2VD, 114 with 3VD). Significant associations with BPA were seen only for patients with 1VD and 3VD.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- Longitudinal follow up
- Analytical method (SPE LC-MS-MS)
- Quality control, including blanks and quality assurance procedures

Weaknesses:

- Single spot urine BPA measurement
- Confounding by diet or by concurring exposure factors not considered
- Generalisability to the overall population
- Inconsistent results amongst different studies

Overall, the CEF Panel considers that the finding of higher BPA exposure in those with severe coronary artery stenosis compared to those with no vessel disease is potentially interesting, despite the relative weak associations and the limitations concerning the clinical usefulness of the criteria used to classify severe and intermediate patients. Whereas nearly the same number of patients were in one the three groups (1 VD, 2VD, 3 VD), there was no association between the group of patients with 2 VD and uBPA (OR per SD uBPA 1.2 (0.7–2.04)), indicating an unknown influence in the statistical model used. In addition, it should be critically noted that the scoring category used has not been identical with the one used in the original paper and that the cumulative assessment of one to three vessel disease might be made from a statistical point of view but is without clear clinical relevance. Classification of all other coronary lesions as intermediate if the criteria were not met is also clinically not justified. BPA was used as a continuous variable, but analytical aspects were sufficiently justified. Urinary BPA varied from below levels of detection to 69.4 ng/mL, with a median of 1.28 ng/mL (unadjusted) in patient with normal coronary arteries, and 1.53 ng/mL in those severe disease, while median concentration in the intermediate group was higher (1.77 ng/mL). Urinary BPA was associated with severe disease in a model adjusted for age, sex, BMI category, occupational social class and diabetes status, but potential confounding by diet was not considered. The authors modelled urinary BPA as a continuous variable, but the standard deviation was very high (5.96 ng/mL) and it could be questioned whether it was appropriate to use the SD increase as the independent value in OR calculation. Such an increase is higher than the most part of the performed measures. Urinary BPA

values were not normalized to urinary creatinine level, but for blood urea creatinine ratio, thus they are not comparable to values of other studies available in the peer-reviewed literature. This is a very important limitation. The authors discuss the limitation related to use of spot urine samples, and say it is "likely that the use of single spot samples would, if anything, result in a smaller (diluted) estimate of the strength of the association between BPA and CAD".

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Olsén L, Lind L and Lind PM, 2012. Associations between circulating levels of bisphenol A and phthalate metabolites and coronary risk in the elderly. Ecotoxicology and Environmental Safety, 80, 179-183.

This is a cross sectional study (n=1 016 with an age >70) that examined associations between serum concentrations of total BPA and coronary risk. The study dates were not reported. Blood samples were taken to measure lipids including LDL-, HDL-cholesterol and triglycerides as well as glucose (fasting state). Blood pressure was measured and a self-reported questionnaire was used to assess smoking habits, history of cardiovascular diseases and drug use. From these data the Framingham risk score was determined. The blood sample was also used to measure phthalate and its metabolites as well as total BPA by solid phase extraction (SPE) coupled with liquid chromatography tandem mass spectrometry (LC-MS-MS, LOD 0.2 ng/ml) after enzymatic hydrolysis. The authors evaluated whether the circulating levels of the chemicals or their metabolites were related to one of the risk factors for coronary heart disease, and found no associations.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- Large sample size
- Analytical method (SPE LC-MS-MS)
- Quality control, including blanks

Weaknesses:

- Cross-sectional design
- Selection bias (age >70)
- Serum BPA measurement (invalid exposure measurement)
- Single BPA measurements
- No distinction between unconjugated and conjugated BPA
- Handling of values below LOQ not reported
- Confounding by diet or by concurring exposure factors not considered
- Generalisability to the total population
- Inconsistency in results among different studies

Overall, the CEF Panel notes that the study sample is the same as in the study by Lind and Lind (2011), and many of the limitations noted above relate to the Olsén study. The study, which did not find a correlation between the serum concentration of BPA and the risk factors, cannot be taken as dismissing a correlation.

This paper is included in the WoE table because of its relevance to one or more review questions addressed there.

BPA effects on metabolic syndrome, hypertension, and peripheral artery disease

Bae S, Kim JH, Lim YH, Park HY and Hong YC, 2012. Associations of Bisphenol A Exposure With Heart Rate Variability and Blood Pressure. Hypertension, 60, 786-793.

The aim of the study was to investigate the associations of urinary BPA with heart rate variability and blood pressure. The study comprised 560 non-institutionalized elderly citizens recruited in Seoul during the years 2008–2010. All of the participants were ≥60 years old. The participants took medical examinations ≤5 times. Heart rate variability, blood pressure and spot urine BPA concentration, were measured at each time. Hypertension was defined as systolic blood pressure (SBP) ≥140 mm Hg or diastolic blood pressure (DBP) ≥90 mm Hg A total of 1 511 observations from 521 participants were included in the analyses. Total BPA was measured by solid phase extraction (SPE) coupled with liquid chromatography tandem mass spectrometry (LC-MS-MS, LOD 0.01 µg/l) after enzymatic hydrolysis. When urinary BPA was modelled on the continuous scale, urinary BPA was negatively associated with the heart rate ($p<0.001$) and positively associated with blood pressure (SBP: $p=0.073$, DBP: $p=0.038$). When urinary BPA was divided into quartiles, no significant association was found for comparison of the fourth quartile compared with the first quartile of urinary BPA concentration. However, the association was statistically significant when the analyses were restricted to participants who did not report previous history of hypertension (n=258), with adjusted OR: 2.35 (95% CI, 1.33–4.17). The association was stronger in women than in men.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- Urine, container specified
- Standardized samples (urinary creatinine)
- Analytical method (SPE LC-MS-MS)

Weaknesses:

- Cross-sectional study design
- Selection bias (age ≥60)
- Single spot urine BPA measurement
- No quality control (e.g., blanks) and quality assurance procedures
- No distinction between unconjugated and conjugated BPA
- Confounding by diet or by concurring exposure factors not considered
- Generalisability to the total population

Overall, the CEF Panel notes that, although some significant associations between urinary BPA levels and hearth rate variability and hypertension were described, the clinical/pathological significance remains doubtful. Fasting blood glucose and alcohol consumption was included among adjusting variables, but other dietary information was not considered. The study has a fair sample size. However, according to the authors the sample size became small after stratification by the previous history of hypertension and sex. The study is limited by use of single spot urine samples and cross-sectional design. The generalisability of the study population is also questionable. The authors sometime overestimated the results, in particular those of clinical relevance.

This paper is included in the WoE table because of its relevance to one or more review questions addressed there.

Shankar A and Teppala S, 2012. Urinary Bisphenol A and Hypertension in a Multiethnic Sample of US Adults. Journal of Environmental and Public Health, 2012, Article ID 481641, 5 pages.

This is a cross-sectional study that examined the association between urinary BPA levels in 1 380 subjects from the National Health and Nutritional Examination Survey 2003–2004 and hypertension, defined as blood pressure-reducing medication use and/or blood pressures >140/90 mm Hg (n=580). Complete data was available for 1 380 participants, of whom 580 had hypertension. Total BPA was measured in spot urine samples by solid phase extraction (SPE) coupled with liquid chromatography

tandem mass spectrometry (LC-MS-MS, LOD 0.36 ng/mL). The results showed a positive association between increasing levels of urinary BPA and hypertension independent of confounding factors such as age, gender, race/ethnicity, smoking, body mass index (BMI), diabetes mellitus and total serum cholesterol levels. Compared to tertile 1 (referent), the multivariate-adjusted odds ratio (95% confidence interval) of hypertension associated with tertile 3 was 1.50 (1.12–2.00); p-trend=0.007. The association was consistently present in subgroup analyses by race/ethnicity, smoking status, BMI, and diabetes mellitus.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- Analytical method (SPE LC-MS-MS or GC-MS)
- Quality control, including blanks and quality assurance procedures

Weaknesess:

- Cross-sectional study design
- Single spot urine BPA measurement
- No distinction between unconjugated and conjugated BPA
- Confounding by diet or by concurring exposure factors not considered
- Generalisability to the total population

Overall, the CEF Panel notes that the hypertension endpoint was measured (method adequate) and the diagnostic criteria used were appropriate. Age, gender, race/ethnicity, smoking status, alcohol intake (g/day), level of education, history of diabetes and oral hypoglycemic intake or insulin administration were assessed using a questionnaire. The authors adjusted for the following confounding factors; age, gender, race/ethnicity, smoking, body mass index (BMI), diabetes mellitus and total serum cholesterol levels, but no dietary data were included. The CEF Panel also notes that the study has main limitations, e.g. the use of single spot urine samples to assess total BPA exposure and the cross-sectional design, which makes the study unsuitable for causal inference.

This paper is included in the WoE table because of its relevance to one or more review questions addressed there.

Shankar A, Teppala S and Sabanayagam C, 2012a. Bisphenol A and Peripheral Arterial Disease: Results from the NHANES. Environmental Health Perspectives, 120, 1297-1300.

The authors analysed data from the US NHANES 2003/04 with a sample size of 745 subjects. The aim of the study was to investigate the potential association between single spot urine BPA concentrations and peripheral arterial disease (PAD) defined as ankle-brachial index <0.9 (n=63). Total BPA was measured in spot urine samples by solid phase extraction (SPE) coupled with liquid chromatography tandem mass spectrometry (LC-MS-MS, LOD 0.36 ng/ml). Urinary total BPA was categorized into tertiles (<1.4 ng/ml, 1.4-3.5 ng/ml, >3.5 ng/ml). The results showed a significant, positive association between increasing levels of urinary total BPA and PAD before and after adjustment for confounders (age, gender, race/ethnicity, education, smoking, body mass index (BMI), diabetes mellitus, hypertension, urinary creatinine, estimated glomerular filtration rate, and serum cholesterol levels). The multivariable-adjusted odds ratio (95% confidence interval) for PAD associated with the highest versus lowest tertile of urinary BPA was 2.69 (1.02–7.09); p-trend=0.01.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- Analytical method (SPE LC-MS-MS)
- Quality control, including blanks and quality assurance procedures

Weaknesses:

- Cross-sectional study design
- Single spot urine BPA measurement
- No distinction between unconjugated and conjugated BPA
- Confounding by diet or by concurring exposure factors not considered
- Generalisability to the total population

Overall, the CEF Panel notes that the study suggests that total BPA may be involved in inducing cardiovascular related diseases as a minor risk factor. However, the study has limitations and the cross-sectional design makes it unsuitable for causal inference. The authors acknowledged in their discussion that the study is limited by its cross sectional design, possible residual confounding by socioeconomic status, and the use of single a spot urine sample. Potential confounding by diet is also a limitation.

This paper is included in the WoE table because of its relevance to one or more review questions addressed there.

Teppala S, Madhavan S and Shankar A, 2012. Bisphenol A and Metabolic Syndrome: Results from NHANES. International Journal of Endocrinology, 2012, Article ID 598180, 5 pages. http://www.ncbi.nlm.nih.gov/pubmed/23251154

The authors examined the association between urinary BPA concentrations and metabolic syndrome (MetS) in 2 104 participants (≥18 years) in the National Health and Nutrition Examination Survey 2003–2008 (NHANES) in a cross-sectional study. BPA exposure (total BPA) was measured in spot urine samples by solid phase extraction (SPE) coupled with liquid chromatography tandem mass spectrometry (LC-MS-MS, LOD 0.36 ng/ml). MetS was defined based on the revised Adult Treatment CEF Panel III (ATP III) guidelines. A total of 741 participants were found to be positive for 3 or more of the 5 measured components and were considered to have MetS: (1) abdominal obesity (waist circumference:≥102 cm in men and ≥88 cm in women), (2) hypertension (systolic blood pressure ≥130mm of Hg, diastolic blood pressure ≥85 mm of Hg, use of medications for elevated blood pressure), (3) elevated serum triglycerides (≥150 mg/dl), (4) glucose intolerance (fasting serum glucose ≥100 mg/dl, medications for diabetes), and (5) reduced HDL (<40 mg/dl for men and <50 mg/dl for women). The results showed that increasing levels of urinary BPA were positively associated with MetS, independent of confounders such as age, gender, race/ethnicity, smoking, alcohol intake, physical activity, and urinary creatinine. Compared to tertile 1 (referent), the multivariable adjusted odds ratio (95% confidence interval) of MetS in tertile 3 was 1.51 (1.07–2.12); p-trend was 0.02. The potential biological mechanism suggested by the authors is the endocrine disrupting and estrogen-like effects of BPA reported in animal studies.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:
- Standardised samples (urinary creatinine included in the model as independent variable)
- Analytical method (SPE LC-MS-MS)
- Quality control, including blanks and quality assurance procedures

Weaknesses:
- Cross-sectional study
- Single spot urine BPA measurement
- No distinction between unconjugated and conjugated BPA
- Confounding by diet or by concurring exposure factors not considered
- Generalisability to the overall population

Overall, the CEF Panel notes that this is the first reporting a positive association between BPA and MetS in humans. As acknowledged by the authors, it is not possible to draw cause effects from the

observed associations due to the cross sectional nature of the study. The authors also acknowledged the potential confounding role of diet, as the main source of BPA exposure in humans is consumption of food and beverages known to be associated with MetS.

This paper is included in the WoE table because of its relevance to one or more review questions addressed there.

(ii) Animal studies

Pant J, Pant MK and Deshpande SB, 2012. Bisphenol A attenuates phenylbiguanide-induced cardio-respiratory reflexes in anaesthetized rats. Neuroscience Letters 530, 69– 74.

The study was undertaken to examine the effects of repeated and acute exposure to BPA on cardio-respiratory reflexes elicited by phenylbiguanide (PBG). In chronic experiments, adult female Albino rats of Charles Foster strain were fed with pellets containing BPA (2 µg/kg body weight, (n=6) or without BPA (time-matched control, n=6) for 30 days. Food pellets containing BPA were prepared by dissolving BPA in vegetable oil and then mixed with wheat flour and water. Blood pressure, ECG and respiratory excursions were recorded under urethane anaesthesia. PBG (10 µg/kg bw) was injected through the jugular vein to evoke reflexes in these animals. In acute experiments, BPA was dissolved in 100% ethanol. In these latter experiments, the PBG reflexes were obtained before and after injecting BPA (35 mg/kg body weight dissolved in ethanol) (n=7) with ethanol (0.1%) treated animals (n=5) as controls. Vagal afferent activity was recorded in rats (numbers not given) given 35 mg/kg bw BPA intravenously. Other rats served as controls. Measurements were done pre-BPA-dosing and post-BPA-dosing. In time-matched control rats, PBG produced bradycardia, hypotension and tachypnoea over 60 seconds. Changes were calculated with the value obtained before PBG dosing as reference. The maximal changes were a decrease to 50–65% of the reference value. In BPA treated group, the PBG-induced heart rate and respiratory frequency changes were attenuated. Acute exposure of animals to BPA also attenuated the PBG-induced responses significantly whereby the effect on respiratory rate was identical with the influence of ethanol (control group). The attenuation of the PBG reflex responses by BPA in acute experiments was associated with decreased vagal afferent activity. The present results may suggest that BPA attenuates the protective cardio-respiratory reflexes due to decreased vagal afferent activity.

Comments from the CEF Panel:
The CEF Panel identified the following strengths and/or weaknesses in this study:
Strenghts:

- None

Weaknesses:

- Test performed in one sex only
- Insufficient study reporting, e.g. number of animals tested,
- Animal diet and phytoestrogen content not reported
- Environmental contamination (use of PC cages and/or plastic drinking bottles) not reported

Overall the CEF Panel considers that this study does not contribute to the understanding of BPA effects in humans based on the following. First, the PBG model does not mimic any situation in humans. Second, in the BPA experiment suggesting an influence of BPA on vagal nerve afferent activity an extremely high dose of BPA (35 mg/kg bw) dissolved in ethanol was given intravenously. The authors describe in the text that earlier experiments had shown that doses up to 30 mg/kg bw did not influence vagal nerve afferent activity.

No WoE analysis was carried out for the one animal study that was evaluated by the CEF Panel.

(iii) In vitro studies

Appraisal of strengths and weaknesses and WoE analysis were not carried out for in vitro studies.

Belcher SM, Chen Y, Yan S, Wang HS (2012) Rapid estrogen receptor-mediated mechanisms determine the sexually dimorphic sensitivity of ventricular myocytes to 17β-estradiol and the environmental endocrine disruptor Bisphenol A. Endocrinology, 153, 712-720.

This study extends earlier findings of this group (Yan S, Chen Y, Dong M, Song W, Belcher SM and Wang HS, 2011. Bisphenol A and 17β-oestradiol promote arrhythmia in the female heart via alteration of calcium handling. *PLoS One*, 6:e25455) and reports on concentration-dependent effects of 10^{-12} – 10^{-6} M BPA on the contraction of primary female ventricular myocytes. E_2 (10^{-12} – 10^{-6} M), the selective β-agonist DNP (10^{-11} – 10^{-7} M) and the β-adrenergic agonist isoproterenol (ISO, 10^{-8} M) were used as positive controls. A significant increase in fractional shortening during contraction was detected at all BPA concentrations in myocytes from female rats with a maximum effect at 10^{-9} M (inverse U-shaped curve) comparable to the effects of E_2, ISO and DNP. No effects were detected in myocytes from male rats and gonadectomised animals (male and female). Binding of BPA to membrane ERβ receptors was required to increase the fractional shortening during contraction. Blockade of ERβ abolished the stimulatory effect while a selective ERα agonist decreased the effects. Exposures of female myocytes to BPA and E_2 (10^{-9} M each) in triggered cells resulted in spontaneous after-contractions, determined by intracellular Ca^{2+} transients indicating proarrhytmic effects. An ERβ blocker and an ERα agonist reduced these effects. In addition, BPA and E_2 effects on myocytes were also studied in myocytes from female wild-type (WT) and in ERβ$^{-/-}$ mice: Whilst ISO induced contractility in WT and ERβ$^{-/-}$ myocytes BPA and E2 induction were only observed in WT cells.

The results suggest that induction of contractility and arrhythmogenesis in female myocytes is dependent on the ERβ while ERα has opposite effects. However, the underlying molecular mechanisms related to the balance between ERα/ ERβ and the relevance of this balance in the complex in vivo situation remain to be determined.

O'Reilly AO, Eberhardt E, Weidner C, Alzheimer C, Wallace BA and Lampert A, 2012. Bisphenol A binds to the local anesthetic receptor site to block the human cardiac sodium channel. PLoS One, 7, e41667.

The effect of 10^{-7} – 10^{-4} M BPA on the human cardiac voltage gated Na^+ channel hNav1.5, expressed in HEK293 cells was studied. BPA blocked the channel at and above 10^{-6} M with a K_d of 25.4 µM. All further experiments were performed at 30 µM or 100 µM BPA. The local anaesthetic mexiletine and BPA share the same binding site of hNav1.5. Molecular docking simulations allowed to visualize binding and to identify relevant molecular structures.

The CEF Panel concluded that BPA effects on the voltage gated Na^+ channels did not occur at relevant concentrations and were observed in an artificial system (overexpression of Na^+ channel in a cell line).

Yan S, Chen Y, Dong M, Song W, Belcher SM and Wang HS, 2011. Bisphenol A and β-estradiol promote arrhythmia in the female heart via alteration of calcium handling. PLoS One, 6, e25455.

Rapid arrhythmogenic effects of 10^{-9} M BPA and/or E_2 on the activity of rat hearts and of primary rodent myocytes were investigated. More than 20 % of female rat myocytes showed increased after-contraction in the presence of BPA or E_2. This correlated with Ca^{2+} after-transients. The number of after-contractions increased to 40% in the presence of BPA and E_2. In contrast, BPA induced only infrequently arrhythmias in the isolated heart. However, during catecholamine-induced stress of the heart, BPA and E_2 (at 10^{-9} M each) increased the frequency of ventricular beats by 4 folds. Arrhythmia of the myocytes is based on altered Ca^{2+} handling between the sarcoplasmic reticulum and the cytosol. In addition, using female WT and ERβ$^{-/-}$ mouse myocytes the BPA-effects on spontaneous Ca transients were only observed in ERβ expressing WT myocytes.

The CEF Panel noted that treatment with BPA/E_2 increases arrhythmic contractions in female heart only during catecholamine-induced stress. The discrepancy between the arrhytmogenic BPA effects on the whole organ and the isolated myocytes and the consequences for the in vivo situation are unclear.

Metabolic effects

(i) Human studies

Obesity outcomes

Bhandari R, Xiao J and Shankar A, 2013. Urinary Bisphenol A and Obesity in US Children. American Journal of Epidemiology, 177, 1263–1270.

Using the same data as Trasande et al. (2012) from the National Health and Nutrition Examination Survey (NHANES) 2003–2008, Bhandari et al. examined the cross–sectional association between urinary BPA and obesity in children aged 6–18 years (n=2 200), with special focus on analyzing the associations separately by race/ethnicity and gender. The primary exposure was urinary BPA and the outcome was obesity, defined as the ≥95th percentile of body mass index specific for age and sex. Measures of BPA concentration included BPA parent compound and conjugated metabolites. Urinary BPA was measured by using solid–phase extraction (SPE) coupled with liquid chromatography tandem mass spectrometry (LC–MS–MS, LOD 0.36 ng/ml). Quality assurance and quality control ensured that samples were not contaminated during collection, handling, and analysis. Urinary BPA was categorized into quartiles (<1.5 ng/ml, 1.5–2.7 ng/ml, 2.8–5.4 ng/ml, >5.4 ng/ml) and also analyzed as a continuous variable, after log transformation due to skewed distribution.
A positive association between increasing levels of urinary BPA and obesity was seen, independent of age, sex, race/ethnicity, education, physical activity, serum cotinine, and urinary creatinine. The multivariable adjusted OR was 1.25 (95% CI: 1.09, 1.43 when log BPA was considered). When BPA was ranked into quartiles the results showed: compared with children in the lowest quartile of BPA (<1.5 ng/ml), children in the highest quartile (>5.4 ng/ml) had a multivariable OR for obesity of 2.55 (95% CI: 1.65, 3.95) ($p_{trend} < 0.01$). The observed positive association was predominantly present in boys (OR = 3.80, 95% CI: 2.25, 6.43) ($p_{trend} < 0.001$) and in non–Hispanic whites (OR = 5.87, 95% CI: 2.15, 16.05) ($p_{trend} < 0.01$).

Comments from the CEF Panel:
The CEF Panel identified the following strengths and/or weaknesses in this study:
Strengths:
- Large sample size
- Analytical method (SPE LC–MS–MS)
- Quality control, including blanks and quality assurance procedures

Weaknesses:
- Cross–sectional study design
- Single exposure measurements
- Single spot urine BPA measurement
- Confounding by diet or by concurring exposure factors not considered or not reported
- Inconsistent results amongst different studies (cross sectional studies vs longitudinal study; different gender–related effects in cross sectional studies)

Overall the CEF Panel noted that NHANES data from 2003 to 2008 were used for this study as well as for the study by Trasande et al. (2012), and while Trasande used data for n=2 838, the study sample in the current study consisted of 2 664 children (6–18 years), of which 2 200 had complete data on all covariates.The authors reported differences in association between urinary BPA and obesity reported for gender and race. However, as also noted by the authors, due to the cross–sectional nature, no causal inference can be drawn from this study.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Carwile JL and Michels KB, 2011. Urinary bisphenol A and obesity: NHANES 2003–2006. Environmental Research, 111, 825–830.

Carwile and Michels examined urinary BPA in relation to general and central obesity in 2 747 adults using pooled data from the 2003–2004 and 2005–2006 National Health and Nutrition Examination Surveys. Total (free and conjugated) urinary BPA was measured by solid phase extraction (SPE) coupled with liquid chromatography–tandem mass spectrometry (LC–MS–MS, LOD 0.36 ng/ml) at the Center for Disease Control and Prevention (CDC) in Atlanta. Quality control procedures included reagent blanks and samples of pooled human urine spiked with BPA at low– and high concentrations. For BPA values below the lower level of detection, LLOD/square root of 2 was assigned. Urinary BPA was adjusted for creatinine. Participants weight, height and waist circumference were objectively measured by trained health technicians. Elevated waist circumference was defined according established criteria and BMI was divided into overweight (<25 BMI ≤29.9) and obese (BMI≥30). Relative to those in the lowest BPA quartile, participants in the upper BPA quartiles were more likely to be classified as obese (quartile 2 odds ratio (OR): 1.85, 95% confidence interval (CI): 1.22, 2.79; quartile 3 OR: 1.60, 95% CI: 1.05–2.44; quartile 4 OR: 1.76, 95% CI: 1.06–2.94). Higher BPA concentration was also associated with abdominal obesity (quartile 2 OR: 1.62, 95% CI: 1.11, 2.36; quartile 3 OR: 1.39, 95% CI: 1.02–1.90; quartile 4 OR: 1.58, 95% CI: 1.03–2.42).

Comments from the CEF Panel:
The CEF Panel identified the following strengths and/or weaknesses in this study:
Strengths:

- Large sample size
- Analytical method (SPE LC–MS–MS)
- Quality control, including blanks and quality assurance procedures

Weaknesses:

- Cross–sectional study design
- Single exposure measurements
- Single spot urine BPA measurement
- Confounding by diet or by concurring exposure factors not considered
- Inconsistent results amongst different studies

Overall the CEF Panel notes that although a range of covariates were taken into account, e.g., age, sex, race, education, and smoking, no dietary variables were considered among adjustment variables. Due to the cross–sectional design, reverse causality cannot be ruled out as higher urinary levels of BPA could be a consequence of diet rather than a cause.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Eng DS, Lee JM, Gebremariam A, Meeker JD, Peterson K and Padmanabhan V, 2013. Bisphenol A and chronic disease risk factors in US children. Pediatrics, 132, e637–645.

Eng et al. (2013) examined the cross–sectional association between urinary BPA levels and obesity in 3 370 US children aged 6–18 years, with chronic disease risk factors as endpoints. This study used mostly the same data as Trasande et al. (2012) and Bhandari et al. (2013) (National Health and Nutrition Examination Survey 2003–2008), and in addition data from the latest NHANES wave (2009–2010). Concentrations of BPA were measured at the Atlanta Centers for Disease Control and Prevention (CDC), by using online solid–phase extraction (SPE) coupled with liquid chromatography isotope dilution tandem mass spectrometry with peak focusing (LC–MS–MS). The limit of detection

was 0.4 ng/ml, and the coefficient of variation ranged from 6% to 16% for BPA. In NHANES, BPA concentrations below the level of detection were assigned a value of 0.3 ng/ml. The endpoints measured in children were: BMI, waist–circumference (WC), WC–to–hip–Ratio, percent body fat, cholesterol, HDL, fasting LDL, fasting TG, homeostasis model assessment of insulin resistance (HOMA–IR) and fasting glucose. Adjustments were made for relevant covariates (eg, demographics, urine creatinine, tobacco exposure, and soda consumption).
Height, weight and waist circumference was measured by trained examiners. Total body percent fat was measured by whole body DXA scans conducted on a subset of individuals 8 years and older. Cholesterol, TG, and HDL cholesterol were measured in serum, and LDL cholesterol level was calculated from measured values of TC, TG, and HDL cholesterol based on the Friedewald equation, Fasting glucose and insulin was measured. Homeostasis model assessment was used as a surrogate measure of insulin resistance in nondiabetic children. The primary exposure, urinary BPA level was examined by quartiles. The results showed higher odds of obesity (BMI >95th percentile) with increasing quartiles of BPA for quartiles 2 vs 1 (odds ratio [OR] 1.74, 95% confidence interval [CI] 1.17–2.60, p=0.008), 3 vs 1 (OR 1.64, 95% CI 1.09–2.47, p=0.02), and 4 vs 1 (OR 2.01, 95% CI 1.36–2.98, p=0.001). With increasing BPA quartiles the results also showed higher odds of having an abnormal waist circumference–to–height ratio (quartiles 2 vs 1 [OR 1.37, 95% CI 0.98–1.93, p=0.07], 3 vs 1 [OR 1.41, 95% CI 1.07–1.87, p=0.02], and 4 vs 1 [OR 1.55, 95% CI 1.12–2.15, p=0.01]). No significant associations of BPA were found with any other chronic disease risk factors.

Comments from the CEF Panel:
The CEF Panel identified the following strengths and/or weaknesses in this study:
Strengths:

- Large sample size
- Analytical method (SPE LC–MS–MS)
- Quality control, including blanks and quality assurance procedures

Weaknesses:

- Cross–sectional study design
- Single exposure measurements
- Single spot urine BPA measurement
- Confounding by diet or by concurring exposure factors not considered or not reported
- Inconsistent results amongst different studies (cross sectional studies vs longitudinal study; different gender–related effects in cross–sectional studies)

In addition to the above, the CEF Panel noted that no associations were found between BPA and laboratory measures of cardiovascular and diabetes risk.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Galloway T, Cipelli R, Guralnik J, Ferrucci L, Bandinelli S, Corsi AM, Money C, McCormack P and Melzer D, 2010. Daily bisphenol A excretion and associations with sex hormone concentrations: results from the InCHIANTI adult population study. Environmental Health Perspectives 118, 1603–1608.

This paper was the first to report human exposure to BPA in a large–scale European population. The study comprised 715 adults aged 20 through 74 years in a suburban and rural town population in Italy (the InCHIANTI adult population study). Total (unconjugated plus conjugated) BPA concentrations were measured by solid phase extraction (SPE) coupled with liquid chromatography tandem mass spectrometry (LC–MS–MS, LOQ 0.50 ng/ml) in compliance with Good Laboratory Practice in 24–hr urine samples collected in plastic bottles. Fasting blood samples were drawn and the outcomes examined were sex–hormones: 17β–oestradiol, total testosterone, sex hormone binding globulin (SHBG) and free testosterone. Models were adjusted for age, study site, smoking, BMI, weight, waist

and urinary creatinine concentration. Other potential confounders were also evaluated. A weak association between urinary BPA and testosterone were found in men, in models adjusted for age and study site (p=0.044), and in models additionally adjusted for smoking, measures of obesity, and urinary creatinine concentrations (beta = 0.046; 95% CI, 0.015–0.076; p = 0.004). No associations were found for other serum hormone measures and no associations were found for the primary outcomes among women. However, an association between BPA and SHBG concentrations was seen in the 60 premenopausal women. The authors concluded that higher BPA exposure may be associated with endocrine changes in men. This study reported BPA associations with covariates including parameters indicative of obesity.

Comments from the CEF Panel:
The CEF Panel identified the following strengths and/or weaknesses in this study:
Strengths:

- Large sample size (European population)
- Standardised samples (24–h urine collection)
- Analytical method (SPE LC–MS–MS)
- Quality control, including blanks

Weaknesses:

- Cross–sectional study design
- Single exposure measurements
- Confounding by diet or by concurring exposure factors (drugs) not considered
- Handling of values below LOD not reported
- Inconsistent results amongst different studies

Overall the CEF Panel notes that the 24–hour urine collection is a better measure of BPA exposure than single spot urine samples and covers to some extent the same time period as the time covered by the blood sampled for hormone concentrations. The association with testosterone was weak and the clinical relevance of association is not clear. Higher BPA excretion was associated with increasing waist circumference and weight, but not with overweight or obesity defined by BMI cut–offs as defined by the World Health Organization.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Harley KG, Schall RA, Chevrier J, Tyler K, Aguirre H, Bradman A, Holland NT, Lustig RH, Calafat AM and Eskenazi B, 2013b. Prenatal and postnatal bisphenol A exposure and body mass index in childhood in the CHAMACOS cohort. Environmental Health Perspectives, 121, 514–520.

In this study the authors examined whether prenatal and postnatal urinary BPA concentrations were associated with body mass index (BMI), waist circumference, percent body fat, and obesity in 9 year–old children (n=311) in the CHAMACOS longitudinal cohort study. BPA was measured in spot urine samples collected from mothers twice during pregnancy and from children at 5 and 9 years of age. Of 601 pregnant women enrolled in the study, a total of 527 were followed through the birth of a singleton, live–born infant. BPA measurements in spot urine collected during pregnancy were available for 498 mothers and 402 children had at least one measure of BMI between age 2 and 9 years. Urine samples were collected in polypropylene urine cups, aliquoted into glass vials, and frozen at 80 °C until shipment to the CDC for analysis. Analysis of field blanks showed no detectable contamination by BPA using this collection protocol. Solid phase extraction (SPE) coupled to high performance liquid chromatography–isotope dilution tandem mass spectrometry (LC–MS–MS) was used to measure total urinary BPA concentration (conjugated plus unconjugated). The limit of detection (LOD) was 0.4 µg/l. Concentrations below the LOD for which a signal was detected were reported as measured.

Prenatal urinary BPA concentrations were associated with decreased BMI at age 9 in girls but not boys. Among girls, being in the highest tertile of prenatal BPA concentrations was associated with decreased BMI Z–score (β=–0.47, 95% Confidence Interval (CI): –0.87, –0.07) and percent body fat (β=–4.36, 95% CI: –8.37, –0.34) and decreased odds of overweight/obesity (Odds Ratio (OR) = 0.37, 95% CI: 0.16, 0.91) compared to girls in the lowest tertile. These findings were strongest in pre–pubertal girls. Urinary BPA concentrations at age 5 years were not associated with any anthropometric parameters at age 5 or 9 years, but BPA concentrations at age 9 were positively associated with BMI, waist circumference, fat mass, and overweight/obesity at age 9 in boys and girls. Consistent with other cross–sectional studies, higher urinary BPA concentrations at age 9 were associated with increased adiposity at 9 years. However, increasing BPA concentrations in mothers during pregnancy were associated with decreased BMI, body fat, and overweight/obesity among their daughters at age 9.

Comments from the CEF Panel:
The CEF Panel identified the following strengths and/or weaknesses in this study:
Strengths:

- Prospective study design
- Urine, container specified (PP cups)
- Repeated measurements (>1)
- Analytical method (SPE LC–MS–MS)
- Quality control, including blanks and quality assurance procedures

Weaknesses:

- Small sample size
- Single spot urine BPA measurement
- Confounding by concurring exposure factors not considered
- Unclear clinical relevance (small effect size)
- Generalisability to the overall population (low–income Mexican American population)
- Inconsistent results amongst different studies (cross sectional studies vs longitudinal study; different gender–related effects in cross–sectional studies)

Overall, Overall, the CEF Panel notes that this is a well conducted study that examined both cross–sectional and longitudinal associations between BPA exposure and body mass index in children. Concomitant exposure to other contaminants was considered in the CHAMACOS cohort, a study in the agricultural Salinas Valley California comprising an immigrant Mexican–American population. BPA was measured in child urines at age 5 and 9 and examined in addition to maternal exposure. Dietary factors were also considered among the covariates, including soda consumption during pregnancy, and fast food and sweet snack consumption in children. Contrary to what was expected, higher BPA concentrations in mothers during pregnancy were associated with decreased BMI, body fat and overweight and obesity in children, but only in girls. The results from the longitudinal analyses weaken the hypothesis that BPA exposure leads to overweight in children. Although dietary factors were included among covariates, the study highlights that cross–sectional associations cannot be used for drawing any causal inferences.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Li D–K, Miao M, Zhou Z, Wu C, Shi H, Liu X and Wang S, 2013. Urine Bisphenol A level in relation to obesity and overweight in school–age children. PLoS ONE 8(6): e65399.

The aim of the study was to examine associations between urinary BPA and overweight/obesity in school–age children. The study population comprised 1 326 children in grades 4–12 from three schools (one elementary, one middle, and one high school) in Shanghai, China. Spot urine samples (non–fasting) were collected from each participant. The collection time ranged from 9 am to 4 pm. All urine kits were made of BPA free materials. For each urine sample, the total urine BPA concentration

(free plus conjugated species) was measured using HPLC with fluorescence detection. The limit of detection (LOD) was 0.31 µg/l. Anthropometric measurements including weight, height, hip circumference, waist circumference, and skinfold thickness were taken by trained staff members at the time when urine BPA samples were collected. Body weight was used as the primary measure of overweight/obesity, and other measures including hip circumferences, waist circumference, waist to height ratio, skinfold thickness, and BMI were used as secondary measurements to verify the findings based on weight. The 90th percentile age– and gender–specific distribution for the anthropometric measures was used as a cutoff for overweight.

Median urinary BPA in the study population was 0.98 µg/l. Risk factors for childhood obesity were included as potential confounders. A food frequency questionnaire with 24 questions was administered to all participating students to determine their dietary patterns (e.g., frequency of eating junk food, unbalanced diet such as eating favourite foods only, and habit of eating fruits/vegetables). Information on physical activities (e.g., average daily time on playing video/computer games and participating in sports or other physical activities), parental overweight, and children's current depression status using the published Children's Depression Inventory (CDI) was also collected.

No association between urinary BPA and overweight was found for boys. For girls, increasing urinary BPA concentration was associated with overweight. After adjustment for potential confounders, a higher urine BPA level (>2 mg/l), at the level corresponding to the median urine BPA level in the US population, was associated with more than two–fold increased risk of having weight >90th percentile among girls aged 9–12 (adjusted odds ratio (aOR) = 2.32, 95% confidence interval: 1.15–4.65). The association showed a dose–response relationship with increasing urine BPA level associated with further increased risk of overweight [The adjusted risk of overweight: OR: 5.18 (95%CI: 1.68–15.9) for BPA above>90th percentile vs BPA<50th percentile (p–trend p=0.006).

Comments from the CEF Panel:
The CEF Panel identified the following strengths and/or weaknesses in this study:
Strengths:

- Large sample size
- Urine, container specified (BPA–free)

Weaknesses:

- Cross sectional study design
- Single exposure measurements
- Single spot urine BPA measurement
- Not adjusted urine samples
- No quality control (e.g., blanks) and quality assurance procedures
- No distinction between unconjugated and conjugated BPA
- Invalid outcome (weight)
- Inconsistent results amongst different studies (cross sectional studies vs longitudinal study; different gender–related effects in cross–sectional studies)

Overall, the CEF Panel notes that the outcome variable "weight" used in this study is not appropriate. In the age range under study, BMI z–scores should be used. In any case, also BMI (gender and age–adjusted) would be better than weight. Another weakness is the lack of measures of pubertal status, relevant to the outcome in the age range considered. The authors should be commended for taking into account dietary patterns and other risk factors of childhood obesity. The sample size was fair. Contrary to the results from the US, which showed a stronger association between urinary BPA and overweight in boys, this study found a significant association between urinary BPA levels and overweight only in girls. The study is interesting, but the cross–sectional design is a major limitation and the results cannot be used to infer any causal relationship between BPA exposure and obesity.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Shankar A, Teppala S and Sabanayagam C, 2012b. Urinary Bisphenol A levels and measures of obesity: results from the National Health and Nutrition Examination Survey 2003–2008. ISRN Endocrinology 2012:965243. doi: 10.5402/2012/965243.

Shankar et al. (2012c) examined the association between urinary BPA levels and obesity in 3 967 participants aged greater than 20 years in the National Health and Nutritional Examination Survey (NHANES) 2003–2008. Total BPA was measured in spot urine samples by solid phase extraction (SPE) coupled with liquid chromatography tandem mass spectrometry (LC–MS–MS, LOD 0.36 ng/ml). Height, weight, and waist circumference were measured by trained technicians. Obesity was defined as (1) body mass index (BMI) ≥30 kg/m^2 and (2) waist circumference (WC) ≥102 cm in men and ≥88 cm in women. Urinary BPA levels were examined in quartiles. A positive association was reported for increasing levels of urinary BPA and both measures of obesity, independent of potential confounding factors including smoking, alcohol consumption, and serum cholesterol levels. The adjusted OR for upper quartile compared to the lower quartile (referent) for BMI–based obesity was 1.69 (1.30–2.20); p–trend <0.0001 and for WC–based obesity was 1.59 (1.21–2.09); p–trend=0.0009. The association between BPA and both measures of obesity was consistently present across gender and race–ethnic groups. Of 4792 eligible participants, the authors excluded subjects with self–reported cardiovascular disease (n=495) and also subjects with missing data (n=330) on covariates (including level of education, smoking status, serum glucose levels, systolic or diastolic blood pressure, body mass index (BMI) or cholesterol levels), resulting in 3 967 participants (51.7% women) in the final analysis.

Comments from the CEF Panel:
The CEF Panel identified the following strengths and/or weaknesses in this study:
Strengths:

- Large sample size
- Analytical method (SPE LC–MS–MS)
- Quality control, including blanks and quality assurance procedures

Weaknesses:

- Cross–sectional study design
- Single exposure measurements
- Single spot urine BPA measurement
- Not adjusted urine samples
- Confounding by diet or by concurring exposure factors not considered
- Insufficient study reporting (urinary BPA stratified in quartiles, but no justification provided)
- Inconsistent results amongst different studies

Overall the CEF Panel noted that BPA concentrations in urine were stratified in quartiles, without an explicit justification by the authors. Due to the cross–sectional design and the lack of consideration of dietary variables no conclusions can be drawn as to the causality between BPA exposure and obesity.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Trasande L, Attina TM and Blustein J, 2012. Association between urinary bisphenol A concentration and obesity prevalence in children and adolescents. JAMA, 308, 1113–1121.

This study also used data from NHANES, and the study sample comprised 2 823 children and adolescents (age 6 through 19 years) from 2003–2008. Spot urine BPA was measured by solid phase extraction (SPE) coupled with liquid chromatography–tandem mass spectrometry (LC–MS–MS, LOD

0.3 ng/ml) at CDC and quality control procedures included reagent blanks and samples of pooled human urine spiked with BPA at low– and high concentrations. For BPA concentrations below the level of detection, the value of 0.3 ng/ml was assigned. Body mass index was converted to sex– and age– standardized z–scores and modelled as a continuous variable as well as dichotomized to classify participants as overweight (>85th percentile) or obese (>95th percentile). The results showed that urinary BPA was significantly associated with obesity. Controlling for race/ethnicity, age, caregiver education, poverty to income ratio, sex, serum cotinine level, caloric intake, television watching, and urinary creatinine level, children in the lowest urinary BPA quartile had a lower estimated prevalence of obesity (10.3% [95% CI, 7.5%–13.1%]) than those in quartiles 2 (20.1% [95% CI, 14.5%–25.6%]), 3 (19.0% [95% CI, 13.7%–24.2%]), and 4 (22.3% [95% CI, 16.6%–27.9%]). It should be noted that the relationship with obesity was not dose–dependent (quartile 2–3–4 had similar OR). Similar patterns of association were found in multivariable analyses examining the association between quartiled urinary BPA concentration and BMI z score and in analyses that examined the logarithm of urinary BPA concentration and the prevalence of obesity. In stratified analysis, significant associations between urinary BPA concentrations and obesity were found among whites ($p<0.001$) but not among blacks or Hispanics.

Comments from the CEF Panel:
The CEF Panel identified the following strengths and/or weaknesses in this study:
Strengths:

- Large sample size
- Analytical method (SPE LC–MS–MS)
- Quality control, including blanks and quality assurance procedures

Weaknesses:

- Cross–sectional study design
- Single exposure measurements
- Single spot urine BPA measurement
- Inconsistent results amongst different studies (cross sectional studies vs longitudinal study; different gender–related effects in cross–sectional studies)

Overall the CEF Panel notes that this is a well conducted study that examined BPA exposure both by quartiles and as a continuous variable. Additional strengths of the study include body measurements obtained by trained health technicians. Furthermore, a broad range of variables, including caloric intake, was included in the adjusted analyses. However, the cross–sectional nature of the study makes it inappropriate for drawing any causal inference.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Wang T, Li M, Chen B, Xu M, Xu Y, Huang Y, Lu J, Chen Y, Wang W, Li X, Liu Y, Bi Y, Lai S and Ning G, 2012a. Urinary Bisphenol A (BPA) concentration associates with obesity and insulin resistance. The Journal of Clinical Endocrinology and Metabolism, 97, E223–E227.

Wang et al. examined in a cross sectional study urinary BPA and obesity and insulin resistance in 3 390 Chinese adults in a district from Shanghai, China. Questionnaire, clinical and biochemical measurements, and urinary BPA concentration were determined. Morning spot urine samples were collected and total (unconjugated and conjugated) BPA was measured by liquid chromatography tandem mass spectrometry (LC–MS–MS, LOQ 0.30 ng/ml). The authors report that if urinary BPA concentrations were below the limit of quantification (0.30 ng/ml) they assigned the value of 0.15 ng/ml. The outcome measures were objectively measured. Weight, height, waist circumference and blood pressure were measured by nurses. All participants were subjected to a 75 g oral glucose tolerance test, and blood samples were collected at 0 and 2 hours. Urinary BPA levels measured were divided into quartiles, and logistic regression model analysis revealed a positive association between

the fourth quartile of BPA concentration (>1.43 ng/ml) and generalized obesity with an OR value of 1.50 (CI95%: 1.15–1.97), and a positive association with abdominal obesity (OR: 1.28; CI95%: 1.03–1.60). Furthermore, this study also reported a positive association with insulin resistance (OR: 1.37; CI95%: 1.06–1.77). The associations between BPA and obesity were adjusted for age, sex, urinary creatinine, smoking, alcohol drinking, education, systolic blood pressure, HDL–cholesterol, LDL–cholesterol, total cholesterol, triglycerides, ALT, GGT, CRP, fasting plasma glucose, and fasting serum insulin. The association between BPA and insulin resistance was additionally adjusted for BMI.

Comments from the CEF Panel:
The CEF Panel identified the following strengths and/or weaknesses in this study:
Strengths:

- Large sample size
- Standardized urine samples (morning spot samples)
- Analytical method (SPE LC–MS–MS)

Weaknesses:

- Cross–sectional study design
- Single exposure measurements
- Single spot urine BPA measurement
- No quality control, including blanks or quality assurance procedures reported
- No distinction between conjugated and unconjugated BPA
- Confounding by diet or concurring exposure factors not considered
- Inconsistent results amongst different studies

Overall the CEF Panel notes that this study has a very high sample size and used objectively measured anthropometric data. However, the cross–sectional design hampers the reliability of the study as dietary behaviour could be a common cause of both overweight/insulin resistance and higher BPA concentrations.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Wang HX, Zhou Y, Tang CX, Wu JG, Chen Y and Jiang QW, 2012b. Association between bisphenol a exposure and body mass index in Chinese school children: a cross–sectional study. Environ Health. 2012 Oct 19;11:79. doi: 10.1186/1476–069X–11–79

Wang et al. examined urinary BPA and obesity in a cross–Section study in 259 Chinese children and adolescents (age 8 to 15) in Changning district in Changhai city. All urine samples were morning spot samples. Total (unconjugated and conjugated) BPA was measured by solid phase extraction (SPE) coupled with ultra performance liquid chromatography–tandem mass spectrometry (UPLC–MS–MS, LOD 0.07 ng/ml). Weight and height were objectively measured and body mass index (BMI) was modelled as a continuous outcome. Urinary BPA concentration was associated with increasing BMI as a continuous variable in all subjects (adjusted for age and sex). There were sex and age related variations. The authors claim that adjusting urinary BPA for creatinine is not appropriate and instead they conducted the analyses with and without adjusting urinary BPA for specific gravity. The results did not differ. Furthermore, the authors converted urinary BPA to estimated dietary BPA exposure, which resulted in similar results as for the urinary BPA concentrations. In this sample, the geometric mean (95% CI) urinary BPA corrected by standard gravity was 0.40 ng/ml (0.33, 0.49) and the estimated daily intake was 0.33 µg/day (0.27, 0.45 µg/day). Without correction for standard gravity the values were slightly higher (0.45 ng/ml and 0.37 µg/day) for urinary and estimated dietary intake, respectively.

Comments from the CEF Panel:
The CEF Panel identified the following strengths and/or weaknesses in this study:

Strengths:
- Urine, container specified (glass)
- Standardized urine samples (first morning spot samples)
- Analytical method (SPE LC–MS–MS)

Weaknesses:
- Cross–sectional study design
- Small sample size
- Single exposure measurements
- Single spot urine BPA measurement
- No quality control, including blanks and quality assurance procedures
- No distinction between conjugated and unconjugated BPA
- Confounding by diet or by concurring exposure factors not considered
- Inconsistent results amongst different studies (cross sectional studies vs longitudinal study; different gender–related effects in cross–sectional studies)

Overall the CEF Panel notes that this study showed additional strengths, i.e. the body weight and height were measured by trained technicians and that all spot urine samples were first morning urines, which is preferable to random spot urines. The authors report that they calculated daily BPA intakes based on individual body weights and urinary BPA concentrations, but no equation as to how this is done was provided. The low urinary BPA and low estimated daily intakes (much lower than the recommended TDI) should be noted.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Zhao H, Bi Y, Ma L, Zhao L, Wang T, Zhang L, Tao B, Sun L, Zhao Y, Wang W, Li X, Xu M, Chen J, Ning G and Liu J, 2012. The effects of bisphenol A (BPA) exposure on fat mass and serum leptin concentrations have no impact on bone mineral densities in non–obese premenopausal women. Clinical Biochemistry, 45(18), 1602–1606.

The aim of this study was to examine the relationships between urinary BPA exposure, body composition, hormone levels and bone mineral density in 246 healthy premenopausal women from Shangai aged 20 years and older. The study was cross–sectional and BPA exposure was measured second morning urine spot samples. The serum and urine samples were stored at −80 °C until analysis. Urine samples were available from 251 individuals for BPA measurement, and 246 of these samples had measurable BPA levels above the lowest detection limit (0.3 ng/ml). Urinary BPA levels were determined by enzymatic hydrolysis using a sensitive and selective liquid chromatography tandem mass spectrometry method (LC–MS–MS, LOQ 0.30 ng/ml). None of the subjects enrolled in this study suffered from any diseases or took any medications that were likely to affect bone metabolism or body weight.

Body mass index (BMI), fat mass, fat–free mass and bone mineral density (BMDs) were measured by Dual–energy x–ray absorptiometry (DXA). Independent variables: serum oestradiol, leptin, osteocalcin, urinary BPA and N–telopeptide of type I collagen (NTx).

Urinary BPA was positively associated with fat mass (r=0.193, p=0.006) and leptin (r=0.236, p=0.001) but not with fat–free mass after adjusting for age and BMI. Urinary BPA was not associated with serum oestradiol levels, BMDs or other bone parameters. Mean urinary BPA concentration was 2.27 ng/ml, and women with urinary BPA <LOD were excluded.

Comments from the CEF Panel:
The CEF Panel identified the following strengths and/or weaknesses in this study:

Strengths:

- Standardized urine samples (second morning samples)
- Analytical method (SPE LC–MS–MS)

Weaknesses:

- Cross–sectional study design
- Small sample size
- Single exposure measurements
- Single spot urine BPA measurement
- Not adjusted urine samples
- No quality control, including blanks or quality assurance procedures reported
- No distinction between unconjugated and conjugated BPA
- Confounding by diet or by concurring exposure factors not considered
- Inconsistent results amongst different studies

In addition to the limitations listed above the CEF Panel noted that no adjustment to urinary BPA creatinine was made. Several models were used to understand potential associations between variables, and BPA was considered as independent or dependent variable. Finally, a discussion about the normality of variables (urinary BPA in particular) is lacking.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Hormonal outcomes

Brucker–Davis F, Ferrari P, Boda–Buccino M, Wagner–Mahler K, Pacini P, Gal J, Azuar P and Fenichel P, 2011. Cord blood thyroid tests in boys born with and without cryptorchidism: correlations with birth parameters and in utero xenobiotics exposure. Thyroid, 21, 1133–1141.

The sample comprised 53 boys from the control group in a case–control study of cryptorchidism. The aim of the study was to examine associations between exposure to a range of xenobiotics (including BPA) in cord blood (and maternal breast milk) and birth parameters including cord blood thyroid tests. BPA was measured only in cord blood by RIA and no distinction is given between conjugated and unconjugated BPA. The median (range) unspecified BPA was 0.9 ng/ml (0.2–3.3 ng/ml). A weak negative correlation was reported between cord blood BPA and thyroid stimulating hormone (TSH), with r=–0.25, p=0.077).

Comments from the CEF Panel:
The CEF Panel identified the following strengths and/or weaknesses in this study:
Strenghts:

- None

Weaknesses:

- Case–control study
- Small sample size
- Cord blood BPA measurement (invalid exposure measurement)
- Single exposure measurements
- Analytical method (RIA)
- No quality control (e.g., blanks) and quality assurance procedures
- No distinction between unconjugated and conjugated BPA
- Handling of values below LOQ not reported
- Confounding by diet not considered
- Inconsistent results amongst different studies

Overall the CEF Panel notes that the study has major limitations. It is not clear which form of BPA was measured (total, unconjugated or conjugated BPA). In blood, only unconjugated BPA can be considered a valid measure of BPA exposure. The sample size was small and the finding needs to be confirmed in a larger sample.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Chou WC, Chen JL, Lin CF, Chen YC, Shih FC and Chuang CY, 2011. Biomonitoring of bisphenol A concentrations in maternal and umbilical cord blood in regard to birth outcomes and adipokine expression: a birth cohort study in Taiwan. Environmental Health, 10, 94.

This cross–sectional study was also evaluated in relation to developmental and reproductive effects of BPA. It is included in this Section because maternal BPA concentrations were studied versus maternal hormone expression as well as in relation to prenatal growth retardation. BPA was determined in maternal and umbilical cord blood samples by HPLC with UV detection (LOD 0.13 ng/ml) in 97 mother–newborn pairs in a birth cohort in Taiwan and association with birth outcomes was investigated. In male neonates only, high maternal BPA (upper quartile) was associated with increased risk of low birth weight babies, small for gestational age babies. The results reported for adverse action of leptin (high leptin (HLP) defined as >90th percentile and low adiponectin (LAD) defined as <10th percentile) in highest versus lowest quartile of maternal BPA exposure given in the abstract differed from the results given in the main text. Abstract: HLP: OR: 1.67, 95% CI: 1.12–2.25 and LAD: OR: 1.12, 95% CI: 1.52–3.97. Text: HLP: OR: 3.03, 95% CI: 2.09–4.54 and LAD: OR: 1.67, 95% CI: 1.12–2.25).

Comments from the CEF Panel:
The CEF Panel identified the following strengths and/or weaknesses in this study:
Strengths:

- Quality control, including blanks

Weaknesses:

- Case–control study
- Small sample size
- Maternal blood and umbilical cord blood BPA measurement (invalid exposure measurement)
- Single exposure measurements
- No distinction between unconjugated and conjugated BPA
- Confounding by diet or by other exposure factors not considered
- Insufficient study reporting (discrepancies between the abstract and the text)
- Inappropriate statistics (excessive categorization of continuous variables)
- Unclear clinical relevance

Overall the CEF Panel notes that the study has major limitations. Discrepancy between results reported in the abstract and main text raise question to the results altogether. Furthermore, the study has several statistical limitations including excessive categorization of continuous variables. The results regarding maternal BPA and adverse birth outcomes, including adverse action of leptin and adiponectin, were weak and can only be regarded as preliminary results.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Galloway T, Cipelli R, Guralnik J, Ferrucci L, Bandinelli S, Corsi AM, Money C, McCormack P and Melzer D, 2010. Daily bisphenol A excretion and associations with sex hormone concentrations: results from the InCHIANTI adult population study. Environmental Health Perspectives 118, 1603–1608.

This paper was the first to report human exposure to BPA in a large–scale European population. The study comprised 715 adults aged 20 through 74 years in a suburban and rural town population in Italy (the InCHIANTI adult population study). Participants each collected one 24–hour urine sample. Total BPA (unconjugated plus conjugated) concentration in the 24–h sample was measured by solid phase extraction (SPE) coupled with liquid chromatography tandem mass spectrometry (LC–MS–MS, LOQ 0.50 ng/ml). The BPA collection and analysis was appropriate. Fasting blood samples were drawn and the outcomes examined were sex–hormones: 17β–oestradiol, total testosterone, sex hormone binding globulin (SHBG) and free testosterone. Models were adjusted for age, study site, smoking, BMI, weight, waist, and urinary creatinine concentration. Other potential confounders were also evaluated. A weak association between urinary BPA and testosterone were found in men, in models adjusted for age and study site (p=0.044), and in models additionally adjusted for smoking, measures of obesity, and urinary creatinine concentrations (β=0.046; 95% CI, 0.015–0.076; p=0.004). No associations were found for other serum hormone measures and no associations were found for the primary outcomes among women. However, an association between BPA and SHBG concentrations was seen in the 60 premenopausal women. The authors concluded that higher BPA exposure may be associated with endocrine changes in men.

Comments from the CEF Panel:
The CEF Panel identified the following strengths and/or weaknesses in this study:
Strengths:

- Large sample size (European population)
- Standardised samples (24–h urine collection)
- Analytical method (SPE LC–MS–MS)
- Quality control, including blanks

Weaknesses:

- Cross–sectional study design
- Single exposure measurements
- Confounding by diet or by concurring exposure factors (drugs) not considered
- Handling of values below LOD not reported
- Unclear clinical relevance
- Inconsistent results amongst different studies

Overall the CEF Panel notes that the 24–hour urine collection is a better measure of BPA exposure than single spot urine samples and covers to some extent the same time period as the time covered by the blood sampled for hormone concentrations. The association with testosterone was weak and the clinical relevance of association is not clear. Concomitant drug treatment was not reported.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Mendez W Jr and Eftim SE, 2012. Biomarkers of perchlorate exposure are correlated with circulating thyroid hormone levels in the 2007–2008 NHANES. Environmental Research, 118, 137–144.

This study only marginally examined BPA and the aim of the study was to examine the relationship between biomarkers of perchlorate exposure and serum thyroid hormone levels in 1 887 subjects in the 2007–2008 NHANES. The models included covariates related to gender, age, ethnicity, income, smoking status, medications, BPA and other goitrogenic ions and phthalate ester metabolites. Total (unconjugated plus conjugated BPA) and thyroid hormones were analysed according to NHANES procedures. Subjects who were pregnant, had thyroid disease or used thyroid medication were excluded. The geometric mean BPA in spot urine was 2.0 ng/ml. Urinary BPA was not associated with total thyroxine (T4) in men or in women.

Comments from the CEF Panel:
The CEF Panel identified the following strengths and/or weaknesses in this study:
Strengths:
- Large sample size
- Analytical method (SPE LC–MS–MS)
- Quality control, including blanks

Weaknesses:
- Cross–sectional study design
- Single exposure measurements
- Single spot urine BPA measurement
- Confounding by diet or by concurring exposure factors not considered
- Inconsistent results amongst different studies

Overall the CEF Panel notes that the statistical analyses were sound, but the relevance of the finding is limited by the cross–sectional design.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Volberg V, Harley K, Calafat AM, Davé V, McFadden J, Eskenazi B and Holland N, 2013. Maternal bisphenol a exposure during pregnancy and its association with adipokines in Mexican–American children. Environmental Molecular Mutagenesis, 54, 621–628.

The study examined maternal urinary BPA concentrations from spot urine samples in early and late pregnancy and in children at age 9 years, and plasma leptin and adiponectin at age 9 years. The study sample included 188 mother–child pairs from the CHAMACOS cohort, a study in the agricultural Salinas Valley California comprising an immigrant Mexican–American population. BPA was measured in urinary spot samples by solid phase extraction (SPE) coupled with liquid chromatography tandem mass spectrometry (LC–MS–MS, LOD 0.4 µg/l) during early (12.6±3.9 weeks gestation) and late (26.3±2.5 weeks gestation) pregnancy and in 9–year–old children. The results showed that BPA concentrations during late pregnancy were associated with increased plasma leptin in boys (β=0.06, p=0.01), controlling for maternal pre–pregnancy body mass index (BMI), pregnancy soda consumption, and smoking, years in US prior to pregnancy, maternal education, household poverty status, child BMI and child soda, fast food and sweet snack consumption at 9 years. Furthermore, BPA concentrations during early pregnancy were associated with plasma adiponectin levels in girls (β=3.71, p=0.03). No significant relationships between concurrent BPA concentrations and 9–year child adiponectin or leptin.

Comments from the CEF Panel:
The CEF Panel identified the following strengths and/or weaknesses in this study:
Strengths:
- Prospective study design
- Urine, container specified (PP cups)
- Repeated measurements (n=2, maternal urine)
- Analytical method (SPE LC–MS–MS)
- Quality control, including blanks and quality assurance procedures

Weaknesses:
- Small sample size
- Single spot urine BPA measurement
- Confounding by concurring exposure factors not considered
- Unclear clinical relevance (effects in boys and girls)
- Generalisability to the overall population (low–income Mexican American population)

Overall, the CEF Panel notes that the study is based on the same study population as in the study by Harley et al., 2013b, which examined cross–sectional and longitudinal associations between BPA exposure and BMI in 9 year old children. The results of the current study complement the Harley et al. study. The authors report that plasma adiponectin levels were inversely correlated with 9–year child BMI ($r=-0.38$, $p<0.001$) and plasma leptin levels were positively correlated with 9–year child BMI ($r=0.82$, $p<0.001$). The strengths of this study are the prospective design and that adjustment variables included the dietary variables: soda consumption during pregnancy and child soda, fast food and sweet snack consumption at 9 years. Concomitant exposure to other contaminants was however, not considered. Limitations of the study include the short–term nature of the BPA exposure measurement and the limited generalisability of results obtained from the immigrant, low SES population from an agricultural community.

Wang F, Hua J, Chen M, Xia Y, Zhang Q, Zhao R, Zhou W, Zhang Z and Wang B, 2012c. High urinary bisphenol A concentrations in workers and possible laboratory abnormalities. Occupational and Environmental Medicine. Occupational and Environmental Medicine, 69, 679–684.

This cross–sectional study examined associations between urinary BPA in spot samples and biological markers in blood or urine, including markers of liver function, glucose homeostatis, thyroid function, and cardiovascular disease in 28 workers in two epoxy resin factories in China. Total (free and conjugated) BPA was measured by solid phase extraction (SPE) followed by isotopic dilution liquid chromatography tandem mass spectrometry (LC–MS–MS). The levels of total urinary BPA in exposed workers were about ten times higher than in the general population. The geometric mean BPA concentration was 55.7 ng/ml (geometric standard deviation, GSD: 5.48) or 32.0 µg/g creatinine (GSD: 4.42). The concentrations differed between workers in different positions in the factories, reflecting higher exposure in manual labour workers than in office workers. Higher urinary BPA concentrations were associated with clinically abnormal concentrations of free triiodothyronine (FT3), free thyroxine (FT4), total triiodothyronine (TT3), total thyroxine (TT4), thyroid stimulating hormone, glutamic–oxaloacetic transaminase and gamma–glutamyl transaminase.

Comments from the CEF Panel:
The CEF Panel identified the following strengths and/or weaknesses in this study:
Strengths:

- Analytical method (SPE LC–MS–MS)

Weaknesses:

- Cross–sectional study design
- Small sample size
- Single exposure measurements
- Single spot urine BPA measurement
- No quality control and quality assurance procedures
- No distinction between unconjugated and conjugated BPA
- Confounding by diet or by concurring exposure factors not considered
- Generalisability to the overall population
- Inconsistent results amongst different studies
- Occupational exposure

Overall the CEF Panel notes that the study is limited by a very small sample size. Another concern is occupational exposure to other chemicals in these factory workers. Occupational exposure to BPA warrants further examination.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Diabetes outcomes

Kim K and Park H, 2013. Association between urinary concentrations of bisphenol A and type 2 diabetes in Korean adults: A population–based cross–sectional study. International Journal of Hygiene and Environmental Health, 216, 476–471.

In a cross–sectional study in Korea, associations between urinary BPA and type 2 diabetes was studied in 1 210 adults (age 40–69 years) in Korea, and was based on the 2009 Korean National Human Biomonitoring study. The mean age was 53.4 years. Spot urine samples were collected at different times throughout the day and creatinine levels were used to correct for urine dilution. After hydrolysis and liquid liquid extraction, total BPA was measured by isotopic dilution gaschromatography mass spectrometry (GC–MS, LOD 0.05 ng/ml, LOQ 0.20 ng/ml). The criteria for type 2 diabetes were based on self–reported and doctor–diagnosed type 2 diabetes. The geometric mean urinary BPA concentrations were 2.03 ng/ml among those not diagnosed with type 2 diabetes and 2.40 ng/ml among those diagnosed with type 2 diabetes. However, after adjusting for potential confounders, higher BPA concentrations were not significantly associated with type 2 diabetes. When adjusted for creatinine, age, sex, body mass index, education, cigarette smoking, income, and place of residence, the odds ratio for being in the highest versus lowest quartile of BPA was 1.71 (95%CI: 0.89, 3.26), p=0.374.

Comments from the CEF Panel:
The CEF Panel identified the following strengths and/or weaknesses in this study:
Strengths:

- Large sample size
- Analytical method (GC–MS)

Weaknesses:

- Cross–sectional study design
- Single exposure measurements
- Single spot urine BPA measurement
- No quality control and quality assurance procedures
- No distinction between unconjugated and conjugated BPA
- Confounding by diet or by concurring exposure factors not considered
- Inconsistent results amongst different studies

Overall the CEF Panel notes that the study was well conducted. Height and weight were objectively measured. The fact that spot urine samples were collected at different times throughout the day could give a more correct population median. However, as the cross–sectional design and self–reported outcome measure limits the relevance of the study for risk assessment.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Lakind JS, Goodman M and Naiman DQ, 2012. Use of NHANES Data to Link Chemical Exposures to Chronic Diseases: A Cautionary Tale. PLoS One. 2012;7(12):e51086.

This same study has also been reviewed in the section on cardiovascular effects – human studies.

The authors reanalysed data from four datasets in the National Health and Nutrition Examination Survey (NHANES). Data on urinary BPA and health outcomes from 2003–2004, 2005–2006, 2007–2008, and 2009–2010 were available. The aim was to examine the consistency of the association between urinary BPA measures and diabetes, coronary heart disease (CHD), and/or heart attack across datasets when consistent scientifically and clinically defined criteria were applied. The study sample included n=4811 for CVD, n=4811 for heart attack and n=4823 for diabetes. Samples were analysed

by on line solid–phase extraction (SPE) coupled with liquid chromatography tandem mass spectrometry (LC–MS–MS, LOD 0.4 ng/ml). All multivariable analyses were controlled for a priori selected potential confounders including, but not limited to, those used in the previous studies. The models included the following covariates: creatinine, age, gender, race/ethnicity, education, income, smoking, body mass index, waist circumference, heavy drinking, family history of diabetes (in the analyses of diabetes) or heart attack/angina (in the analyses of CHD and heart attack), hypertension, sedentary activity, blood cholesterol, and daily energy intake. Urinary BPA was not significantly associated with adverse health outcomes for any of the NHANES surveys, with ORs (95% CIs) ranging from 0.996 (0.951–1.04) to 1.03 (0.978–1.09) for CHD, 0.987 (0.941–1.04) to 1.04 (0.996–1.09) for heart attack, and 0.957 (0.899–1.02) to 1.01 (0.980–1.05) for diabetes. When the data from four surveys were pooled, the ORs (95% CIs) for the full model that included all covariates were 1.004 (0.998–1.009) for CHD, 1.002 (0.998–1.007) for heart attack, and 0.995 (0.982–1.007) for diabetes. The choice of covariates had only minor effect on point estimates. The authors concluded that the discrepancy between their findings on diabetes and those reported previously was largely explained by the choice of case definition. For discrepancy between results of this study and previous findings for CHD, the authors concluded that this was in part attributable to differences in inclusion criteria. In the current study, no subjects were excluded based on very high BPA concentrations.
The authors provided an example of reverse causality obscuring possible conclusions from cross–sectional studies: "In all analyses, cholesterol levels were statistically significantly inversely associated with heart attack and CHD. Given the well–documented positive association between cholesterol and heart disease from prospective studies, the most logical explanation for the observed result is reverse causation, i.e. it is likely that diagnoses of heart attack or CHD, which preceded the cholesterol measurements in NHANES, likely triggered changes in lifestyle or use of medications that resulted in lower cholesterol levels."

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- Large sample size
- Urine, container specified
- Analytical method (SPE LC–MS–MS)
- Quality control, including blanks and quality assurance procedures

Weaknesses:

- Cross–sectional study design
- Single exposure measurements
- Single spot urine BPA measurement
- Confounding by diet or by concurring exposure factors not considered
- Inconsistency in results among different studies

Overall, the CEF Panel considers that this study shows how relatively minor decisions made a priori (clinical definition of diabetes and inclusion of participants with higher levels of BPA) affected the previously reported results and conclusions of associations between urinary BPA exposure and chronic disease. This study does not add to the evidence as to whether or not BPA is a risk factor for chronic disease, but highlights that using data from cross–sectional studies like NHANES surveys to draw such conclusions about relations between short–lived environmental chemicals and chronic diseases is inappropriate.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Ning G, Bi Y, Wang T, Xu M, Xu Y, Huang Y, Li M, Li X, Wang W, Chen Y, Wu Y, Hou J, Song A, Liu Y and Lai S, 2011. Relationship of Urinary Bisphenol A Concentration to Risk for

Prevalent Type 2 Diabetes in Chinese Adults: A Cross–sectional Analysis. Annals of Internal Medicine, 155, 368–374.

The association between urinary BPA and diabetes was investigated in 3 423 Chinese adults in Baoshan disctrict in Shanghai. Total (free and conjuagted) urinary BPA was determined in morning spot urine samples by liquid chromatography isotopic dilution tandem mass spectrometry (LC–MS–MS, LOQ 0.30 ng/ml). Overall, BPA concentration was lower (median 0.81 ng/ml) than in data from NHANES (median 2.0 ng/ml). Dividing participants into quartiles of BPA exposure, the data showed that risk of diabetes was higher in people in the second and fourth quartiles of exposure, but not the third. The overall trend was not significant. The adjusted odds ratio (OR) of type 2 diabetes for participants in the second quartile of BPA (0.48 to 0.81 ng/ml) was 1.30 [95% CI, 1.03 to 1.64] and in the fourth quartile (>1.43 ng/ml) was OR, 1.37 [CI, 1.08 to 1.74]. The adjusted odds ratio in the third quartile (0.82 to 1.43 ng/ml) was 1.09 [CI, 0.86 to 1.39], and a test of the trend of the association was not statistically significant.

Comments from the CEF Panel:
The CEF Panel identified the following strengths and/or weaknesses in this study:
Strengths:

- Large sample size
- Analytical method (LC–MS–MS)

Weaknesses:

- Cross–sectional study design
- Single exposure measurements
- Single spot urine BPA measurement
- Not adjusted urine samples
- No quality control, including blanks and quality assurance procedures
- No distinction between unconjugated and conjugated BPA
- Confounding by diet or by concurring exposure factors not considered
- Inconsistent results amongst different studies

Overall the CEF Panel notes that the statistical modelling included adjustment for a wide range of risk factors including blood lipids, blood pressure, waist circumference etc, but as acknowledged by the authors themselves, the study did not take into account any potential confounding by diet such as for example consumption of sugared drinks from plastic bottles. It should be noted that even if significant associations were found for the second and third BPA quintile, the authors conclude that the data do not support the previous finding of an association between urinary BPA and self–reported diabetes in NHANES (Lang et al., 2008). The study sample and BPA measurements seem to be the same as used in Wang et al. (2012a) who reported on urinary BPA concentration in relation to general and abdominal obesity. The main limitation is however the cross–sectional design.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Shankar A and Teppala S, 2011. Relationship between Urinary Bisphenol A Levels and Diabetes Mellitus. The Journal of Clinical Endocrinology and Metabolism, 96, 3822–3826.

Shankar et al. analysed NHANES data (n=3967), and found that pooled data from 2003–2008 showed a positive association between single spot urine BPA concentrations and diabetes, using fasting glucose levels and glycosylated haemoglobin to define diabetes mellitus according to the latest American Diabetes Associations guidelines. The risk of type 2 diabetes (insulin–resistent diabetes) increased with increasing quartiles of BPA in a dose–dependent manner. In the fully adjusted model the OR for highest versus lowest quartile was 1.68, 95%CI: 1.22–2.30. The trend of association was

significant. The association was present among normal weight as well as overweight and obese subjects.

Comments from the CEF Panel:
The CEF Panel identified the following strengths and/or weaknesses in this study:
Strengths:
- Large sample size
- Analytical method (LC–MS–MS)
- Quality control, including blanks and quality assurance procedures

Weaknesses:
- Cross–sectional study design
- Single exposure measurements
- Single spot urine BPA measurement
- Confounding by diet or by concurring exposure factors not considered
- Inconsistent results amongst different studies

Overall the CEF Panel notes that strengths of the study include a large sample size and objective outcome measures. However, it is limited by the cross–sectional design and cannot be considered relevant for establishing a link between BPA exposure and increased risk of diabetes type 2.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Silver MK, O'Neill MS, Sowers MR and Park SK, 2011. Urinary Bisphenol A and Type–2 Diabetes in US Adults: Data from NHANES 2003–2008. PloS One, 6, e26868.

Silver et al. used the 2003–2008 NHANES data, and used a different definition of diabetes 2 by whether or not participants (n=4389) used diabetic medication, or had high long–term blood glucose levels (HbA1c ≥6.5%). Total BPA (free and conjugated) was measured by solid phase extraction (SPE) coupled with liquid chromatography tandem mass spectrometry (LC–MS–MS, LOD 0.36 ng/ml). The results showed an overall weak positive association between BPA and diabetes in 2003–2008 pooled data (adjusted OR for a two fold increase in BPA: 1.08 (95% CI: 1.02, 1.16), while breaking down by year, the association was only significant in 2003–2004 (n=1,364, OR=1.23 (95% CI, 1.07 to 1.42), not 2005–2006 (n=1 363, OR=1.05 (95% CI, 0.94 to 1.18)), or 2007–2008 (n=1,662, OR = 1.06 (95% CI, 0.91 to 1.23)). Similar patterns of associations between BPA and continuous HbA1c were also observed.

Comments from the CEF Panel:
The CEF Panel identified the following strengths and/or weaknesses in this study:
Strengths:
- Large sample size
- Analytical method (LC–MS–MS)
- Quality control, including blanks and quality assurance procedures

Weaknesses:
- Cross–sectional study design
- Single exposure measurements
- Single spot urine BPA measurement
- Confounding by diet or by concurring exposure factors not considered
- Inconsistent results amongst different studies

Overall the CEF Panel notes that as for the study by Shankar et al. (2011) the main strengths of the study are a large sample size and an objective outcome measures. However, the study is limited by the

cross sectional design and cannot be considered relevant for establishing a link between BPA exposure and increased risk of diabetes type 2.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Wang T, Li M, Chen B, Xu M, Xu Y, Huang Y, Lu J, Chen Y, Wang W, Li X, Liu Y, Bi Y, Lai S and Ning G, 2012a. Urinary Bisphenol A (BPA) concentration associates with obesity and insulin resistance. The Journal of Clinical Endocrinology and Metabolism, 97, E223–E227.

See review of the same study above.

Other outcomes

Li M, Bi Y, Qi L, Wang T, Xu M, Huang Y, Xu Y, Chen Y, Lu J, Wang W and Ning G, 2012. Exposure to bisphenol A is associated with low–grade albuminuria in Chinese adults. Kidney International, 81,1131–1139.

In the same population of Chinese adults as the study by Wang et al. (2012a) and Ning et al. (2011), Li et al. examined urinary BPA in relation to renal disease defined by albuminuria in 3 055 adults. Morning spot urine samples were collected and total (unconjugated and conjugated) BPA was measured by liquid chromatography tandem mass spectrometry (LC–MS–MS, LOQ 0.30 ng/ml). The results showed that urinary BPA was an independent determinant of the urinary albumin–to–creatinine ratio significantly associated with an increased risk of low–grade albuminuria. The adjusted odds ratio (OR) and 95% confidence interval (95%CI) for the third quartile of BPA relative to the lowest was OR: 1.20 (1.06–1.37), and for the fourth quartile was OR: 1.23 (1.13–1.34). The association was not modified by conventional risk factors such as age, gender, smoking, alcohol consumption, body mass index, hypertension, diabetes, and the estimated glomerular filtration rate. The univariate correlation between log–BPA and log–albumin was very weak (r=0.09).

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- Large sample size
- Urine, container specified
- Standardised samples (first morning spot samples)
- Analytical method (LC–MS–MS)

Weaknesses:

- Cross–sectional study design
- Single exposure measurements
- Single spot urine BPA measurement
- No quality control, including blanks or quality assurance procedures reported
- No distinction between unconjugated and conjugated BPA
- Confounding by diet or by concurring exposure factors not considered
- Unclear clinical relevance

Overall, the CEF Panel notes that the study is sound and the statistical analysis adequate, but the clinical relevance of the results are not clear. Furthermore, the study is limited by the cross sectional design and single spot urines.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Teppala S, Madhavan S and Shankar A, 2012. Bisphenol A and Metabolic Syndrome: Results from NHANES. International Journal of Endocrinology, 2012, 598180, 5 pages. http://www.ncbi.nlm.nih.gov/pubmed/23251154

The authors examined the association between urinary BPA concentrations and metabolic syndrome (MetS) in 2 104 participants (≥18 years) in the National Health and Nutrition Examination Survey 2003–2008 (NHANES) in a cross–sectional study. BPA exposure (total BPA) was measured in spot urine samples by solid phase extraction (SPE) coupled with liquid chromatography tandem mass spectrometry (LC–MS–MS), and the lower limit of detection (LOD) for BPA concentrations was 0.36 ng/ml. MetS was defined based on the revised Adult Treatment Panel III (ATP III) guidelines. A total of 741 participants were found to be positive for 3 or more of the 5 measured components and were considered to have MetS: (1) abdominal obesity (waist circumference:≥102 cm in men and ≥88 cm in women), (2) hypertension (systolic blood pressure ≥130mm of Hg, diastolic blood pressure ≥85mm of Hg, use of medications for elevated blood pressure), (3) elevated serum triglycerides (≥150 mg/dl), (4) glucose intolerance (fasting serum glucose ≥100mg/dl, medications for diabetes), and (5) reduced HDL (<40 mg/dl for men and <50 mg/dl for women). The results showed that increasing levels of urinary BPA were positively associated with MetS, independent of confounders such as age, gender, race/ethnicity, smoking, alcohol intake, physical activity, and urinary creatinine. Compared to tertile 1 (referent), the multivariable adjusted odds ratio (95% confidence interval) of MetS in tertile 3 was 1.51 (1.07–2.12); p–trend was 0.02. The potential biological mechanism suggested by the authors is the endocrine disrupting and estrogen–like effects of BPA reported in animal studies.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- Large sample size
- Analytical method (SPE LC–MS–MS)
- Quality control, including blanks and quality assurance procedures

Weaknesses:

- Cross–sectional study design
- Single exposure measurements
- Single spot urine BPA measurement
- Confounding by diet or by concurring exposure factors not considered
- Generalisability to the overall population

Overall, the CEF Panel notes that this is the first reporting a positive association between BPA and MetS in humans. The finding of higher BPA exposure in participants with MetS is potentially interesting. However, as acknowledged by the authors, it is not possible to draw cause effects from the observed associations due to the cross–sectional nature of the study. The authors also acknowledged the potential confounding role of diet, as the main source of BPA exposure in humans is consumption of food and beverages known to be associated with MetS.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

You L, Zhu X, Shrubsole MJ, Fan H, Chen J, Dong J, Hao CM and Dai Q, 2011. Renal function, Bisphenol A, and Alkylphenols: Results from the National Health and Nutrition Examination Survey (NHANES 2003–2006). Environmental Health Perspectective, 119, 527–533.

You et al. used the 2003–2006 NHANES data for 2 573 adults without known renal diseases to examine whether urinary excretion of BPA and alkyphenols differed by renal function. Renal function was measured by glomerular filtration rate (eGFR) estimated by using the Modification of Diet in Renal Disease (MDRD) Study equation and newly developed Chronic Kidney Disease Epidemiology

Collaboration (CKD–EPI) equation. Mildly decreased renal function or undiagnosed chronic kidney disease (CKD) was found in 58% of the study population. Total urinary BPA (unconjugated plus conjugated) was measured in spot urine samples by solid phase extraction (SPE) coupled with liquid chromatography isotopic dilution tandem mass spectrometry (LC–MS–MS, LOD 0.36 ng/ml). The adjusted geometric means for urinary BPA excretion decreased with decreasing renal function (decreasing levels of GFR), primarily in females, by using the most widely used equation in the clinic and epidemiologic studies (MDRD equation). On the other hand, through a newly developed CKD–EPI equation, the association was not significant.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- Large sample size
- Analytical method (SPE LC–MS–MS)
- Quality control, including blanks and quality assurance procedures

Weaknesses:

- Cross–sectional study design
- Single exposure measurements
- Single spot urine BPA measurement
- Confounding by diet or by concurring exposure factors not considered
- Unclear clinical relevance (small effect size)

Overall, the CEF Panel notes that despite the high number of subjects considered, the association between BPA urinary levels and renal function impairment was very low, and no threshold doses were proposed. The study is limited by the cross sectional design and single spot urines.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

(ii) Animal studies

Studies involving prenatal exposure

Anderson OS, Peterson KE, Sanchez BN, Zhang Z, Mancuso P and Dolinoy DC, 2013. Perinatal bisphenol A exposure promotes hyperactivity, lean body composition, and hormonal responses across the murine life course. FASEB Journal, 27, 1784-1792.

Anderson et al. (2013) exposed mice during gestation and lactation to 0, 50 ng, 50 µg or 50 mg of BPA/kg of diet. The authors state that the mice were obtained from a colony maintained with sibling mating and forced heterozygosity for the viable yellow agouti (*Avy*) allele for 220 generations, resulting in a genetically invariant background. Following parturition, a subset of *a/a* wild-type animals, 1 male and 1 female/litter, was followed until 10 months of age on standard diet (n = 20 offspring); 50 ng BPA/kg diet (n = 20 offspring); 50 µg BPA/kg diet (n = 21 offspring); or 50 mg BPA/kg diet (n = 18 offspring). Offspring energy expenditure, spontaneous activity, and body composition was assessed at 3, 6, and 9 months of age, and hormone levels were measured at 9 and 10 months of age. The authors found increased energy expenditure as evidenced by increased oxygen consumption and carbon dioxide production in all BPA-treated animals compared with controls. The CEF Panel noted however that the dose-response relationship was inconsistent over the period of the study and overall the results were difficult to interpret. For example, energy expenditure as measured by oxygen consumption was only significantly increased at 3 months in female offspring whose dams had been exposed to 50 mg BPA/kg of diet (p = 0.004, and was still significantly increased at 6 months, while other treated groups showed a non-significant dose related trend, while at 9 months only the animals whose dams had been exposed to 50 ng/kg diet showed a significant increase in oxygen consumption. Males overall showed no such increases until 9 months (significant compared with controls at 50 µg or 50 mg of BPA/kg of diet). Carbon dioxide production was inconsistent in females, but was significant increased compared to controls in males at 3 months only by doses of 50 µg/kg bw per day and 50 mg/kg bw per day. Spontaneous activity was increased, but only in females, and again showed an inconsistent dose-response. Food consumption in females was reduced to a statistically significant extent but without a clear dose:response (significant only in females whose dams had received 50 µg or 50 ng of BPA/kg of diet at 6 months and in the 50 µg BPA/kg of diet group at 9 months, with no significant differences being seen at 3 months. In males the reduction of food intake was not statistically significant at any time period. Body weight and body fat were overall not statistically different from controls and glucose tolerance and insulin release were also unchanged.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:

Strengths:
- Number of doses (≥3)
- Use of non-PC cages
- Phytoestrogen-free diet

Weaknesses:
- Insufficient study reporting (administration via diet but intakes of BPA not specifically calculated)
- Inappropriate statistics

Overall, the CEF Panel noted that the results of this study, in a genetically specific mouse strain, are in contrast to the findings of a number of other studies showing effects on body weight gain and on glucose tolerance and insulin resistance. The study is considered broadly acceptable, with adequate numbers of animals per group, although the intakes of BPA were not specifically calculated. It is not however possible to derive an indication of a dose-response, and no NOAELs or LOAELs can be derived from the study because of the inconsistency of the results. The biochemical basis for the results seen is not obvious. For these reasons, and also because of the specificity of the genetic model,

the general applicability of the results is debatable. It is to be noted that the standard diet for the animals was phytoestrogen free as explicitly stated in the publication.
This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Angle BM, Do RP, Ponzi D, Stahlhut RW, Drury BE, Nagel SC, Welshons WV, Besch-Williford CL, Palanza P, Parmigiani S, Vom Saal FS and Taylor JA, 2013. Metabolic disruption in male mice due to fetal exposure to low but not high doses of bisphenol A (BPA): Evidence for effects on body weight, food intake, adipocytes, leptin, adiponectin, insulin and glucose regulation. Reproductive Toxicology, 42, 256-268.

Angle et al. (2013) orally exposed CD-1 mice in utero to a range of BPA concentrations (5, 50, 500, 5000 and 50000 µg/kg bw per day) from**.**

Angle et al. (2013) orally exposed CD-1 mice in utero to a range of BPA concentrations (5, 50, 500, 5000 and 50000 µg/kg bw per day) from GD9 to GD18. The study duration was 17-19 weeks. The study results were only obtained in males. The authors studied as much as 19 parameters which were measured even at different time points. Statistically significant effects were seen in 11 of the parameters measure using an alternative, non-monotonic dose-response model after the standard ANOVA gave statistical significant results in 10 out of the 19 parameters. Statistical results with the same significantly positive outcome between the two statistical models were obtained in 7 parameters. The effects with statistical significance were those on body weight (at week 3 and week 19), energy intake (at 3-4 and 4-5 weeks), gonadal and abdominal fat pad weights, gonadal adipocytes number and volumes, liver weight, glucose tolerance and serum concentrations of insulin. Non-monotonic dose-response relationships were reported for many outcomes.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strenghts:

- Number of doses (≥3)
- Adequate positive controls included
- Use of non-PC cages

Weaknesses:

- Study design not appropriate to the scope (only males tested for glucose and insulin tolerance tests)
- Statistical analysis (insufficient study reporting)

Overall, the CEF Panel noted that only males were evaluated for glucose and insulin tolerance test. Multiple endpoints were measured which are either time related (e.g. body weight in longitudinal follow up) or mechanism related (e.g. release of hormones such a leptin from fat tissue the weight of which had also been assessed). The authors used two different statistical models for dose-response modelling and they explored which statistical model would fit their data better. From this procedure it is obvious that the study results are not to be seen as confirmatory but as exploratory. In addition, given the multiple outcomes and multiple time periods examined, the statistical adjustment procedure for the multiple comparisons had to be explained. As this information is not provided, the question remains open whether appropriate adjustment was made. There were discrepancies with an effect seen between weeks 4 and 5 e.g. for BPA 500 which disappear between week 5 and 7. Weight at 19 weeks was not statistically significantly changed in both of the statistical models they used in contrast to what is suggested in the article. Discrepancies were also found for effects seen on fat weight. Whereas for renal fat pad weight significant differences versus control were reported for 5 µg/kg bw per day, for 500 µg/kg bw per day and for 5000 µg/kg bw per day but not for 50 µg/kg bw per day and for 50000 µg/kg bw per day the only significant difference reported for cell number in renal fat pad was at the dose of 500 µg/kg bw per day and the only statistical difference reported for renal adipocyte volume

was for 5000 µg/kg bw per day. Serum leptin was statistically significantly elevated only at 500 µg/kg bw per day but serum adiponectin was unchanged at the dose whereas it was decreased at 50 µg/kg bw per day and 5000 µg/kg bw per day. In total, the multiple parameters measured showed an inconsistent pattern with many effects seen for one parameter at a certain dose at which however no effect was observed for a second, pathophysiological related parameter. The interpretation of the results is not clear, in particular a unifying mode of action approach is lacking.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Mackay H, Patterson ZR, Khazall R, Patel S, Tsirlin D and Abizaid A, 2013. Organizational Effects of Perinatal Exposure to Bisphenol-A and Diethylstilbestrol on Arcuate Nucleus Circuitry Controlling Food Intake and Energy Expenditure in Male and Female CD-1 Mice. Endocrinology, 154, 1465-1475.

In the study of MacKay et al. (2013) CD mice were exposed throughout pregnancy (from GD 1) and lactation to diets containing 0, 1 or 20 µg BPA/kg. Diethylstilbestrol (DES) was used as a positive control. The authors estimated that the mice consumed an average of 0.19 and 3.49 µg/kg bw per day of BPA in the low and high BPA treatments prenatally and an average of 0.36 and 7.2 µg/kg bw per day of BPA postnatally. Offspring were weaned initially onto a normal diet, then as adults exposed to either a normal or high-fat diet (HFD). Two males and two females from each litter at each dose level made up cohort 1 (n= 3-5 per sex per treatment and diet), in which whole-body energy expenditure was measured at 3 months of age (before HFD) and again at 5 months of age (after HFD). Glucose tolerance tests were performed in the cohort after 60 days of HFD exposure. The animals were killed at 5.5 months at which time the adipokines IL-6, insulin, leptin, and resistin were measured in blood and perigonadal, retroperitoneal, sc fat pads, and interscapular brown adipose tissue (BAT) were dissected and weighed. Cohort 2 was used for brain histochemical investigations. Female offspring receiving 20 µg BPA/kg diet and fed a high fat diet as adults showed increased body weight gain compared with controls and also the DES positive control, and also ate more. They had increased adiposity and leptin concentrations with reduced proopiomelanocortin mRNA expression in the arcuate nucleus and estrogen receptor α expression patterns similar to those seen in males, which the authors considered was suggestive of a masculinising effect of BPA. Male offspring showed no similar BPA-linked effect on body weight gain, however males at both levels of BPA showed significantly increased weight of the retroperitoneal and intrascapular brown adipose fat pads compared with control and DES-exposed mice, and similar effects were seen in female offspring at the higher dose level of BPA. Effects were more pronounced or only significant in the animals receiving high fat diets. Males exposed to the high dose of BPA showed impaired glucose tolerance on both diets.They also showed reduced proopiomelanocortin fiber innervation into the paraventricular nucleus of the hypothalamus, and when exposed to HFD, they demonstrated increased neuropeptide Y and Agouti-related peptide expression in the arcuate nucleus (ARC). The authors concluded that exposure to BPA leads to sexually dimorphic alterations in the structure of hypothalamic energy balance circuitry, leading to increased vulnerability for developing diet-induced obesity and metabolic impairments, such as glucose intolerance.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:
- Adequate positive controls included
- Use of non-PC cages

Weaknesses:
- Small sample size
- Insufficient study reporting (uncertainty about estimated intakes due to very low (< 1 ng/kg bw per day) dietary level and homogeneity of the diet not checked)
- Statistical analysis (litter effect not completely controlled)

Overall the CEF Panel notes that the actual intakes of BPA were only estimated, the litter effect was not completely controlled, numbers of animals per test group were small. Results between males and females were inconsistent and the magnitude of the effects seen was small. The obesity-inducing diet is a very high-fat diet (60 % Kcal from fat), thus raising doubts about its relevance to the development of human obesity. In addition, as in rodents adipose tissue is developing particularly during the last week of gestation and during early postnatal life and this process essentially takes place before birth in bigger mammals the findings might not be directily relevant fo the human situation. Furthermore, it is to be noted that the phytoestrogen content of the diet was apparently not tested.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Miyawaki J, Sakayama K, Kato H, Yamamoto H and Masuno H, 2007. Perinatal and postnatal exposure to bisphenol a increases adipose tissue mass and serum cholesterol level in mice. Journal of Atherosclerosis and Thrombosis, 14, 245-252.

This study was already considered in the 2010 EFSA opinion, and was used in the 2013 ANSES opinion to derive a LOAEL for BPA based on body weight and cholesterol increase in females. Below the extract from the 2010 EFSA opinion:

"The effects of peri- and postnatal exposure to BPA on adipose tissue mass were investigated by Miyawaki et al. (2007). Groups of 3 pregnant ICR mice were exposed to BPA in drinking water (0, 1 or 10 µg/ml, resulting in 0, 0.26, 2.72 mg/kg b.w./day) from GD 10 to end of lactation. Offspring were exposed up to PND 31 and groups of 16 to 25 offspring per sex and dose group were evaluated. Body weights of female offspring were increased at the low and high dose group, body weights of the males at the high dose group. Adipose tissue weight was increased significantly in females at the low dose and in males at the high dose group. Serum leptin was increased only in females of the low dose group. Total cholesterol was increased only in females with the highest increase in the low dose group. Serum triacylglycerol and non-esterified fatty acid levels were increased and serum glucose levels decreased only in males of the low dose group. The low number of dams per group invalidates this study".

The study has some flaws e.g. the sample size is small, inconsistencies in the results as in females body weight increases at both doses but adipose tissue only at the lower dose. It is to be noted that there is no evidence for a dose-response relationship and in addition, the phytoestrogen content of the diet was apparently not tested.

US FDA/NCTR, 2013. Evaluation of the toxicity of bisphenol A (BPA) in male and female Sprague-Dawley rats exposed orally from gestation day 6 through postnatal day 90. Experiment E02176.01

Delclos KB, Camacho L, Lewis SM, Vanlandingham MM, Latendresse JR, Olson GR, Davis KJ, Patton RE, da Costa GG, Woodling KA, Bryant MS, Chidambaram M, Trbojevich R, Juliar BE, Felton RP and Thorn BT, 2014. Toxicity Evaluation of Bisphenol A Administered by Gavage to Sprague Dawley Rats From Gestation Day 6 Through Postnatal Day 90. Toxicological Sciences, 139, 174-197.

In this study, Sprague-Dawley rats (Sprague-Dawley/CD23/NCTR BR) were used to investigate the effects of BPA on a very wide range of pathological, physiological, endocrine, reproductive and developmental endpoints in a very broad dose interval. Ethinyl oestradiol was used as a positive control of the estrogenic effects of BPA. The dose-matched vehicle control was carboxymethylcellulose, sodium salt. The doses were: (i) BPA 2.5, 8, 25, 80, 260, 840, 2700, 100 000, 300 000 µg/kg bw per day, (ii) Vehicle, (iii) EE_2 0.5, 5 µg/kg bw per day. The study included a naïve

control group and doses were administered by oral gavage. The protocol and methods, including statistical analysis were of the high quality and robust with treatment, body weight and litter randomisation and appropriate inclusion and exclusion criteria established prior to the start of the study. The target unit for analysis was 20 litters and 18-23 were achieved. F0 females were dosed from GD 6 up to labour onset and pups from PND 1 until tissue harvesting, up to PND 90.

Metabolic endpoints were body weight, weekly food consumption. At PND 90 the following parameters were considered: glucose, TG, cholesterol, insulin, leptin, cardiac troponins. 0.5 and 5 µg/kg bw per day ethinyl-oestradiol (EE_2) served as estrogenic controls. At the dose of 300 mg/kg bw day several effects were noted which were similar to those of EE_2: 1. preweaning body weight reduction (12 – 16 % and 9 – 12 % in females and males, respectively), 2. Reduced retroperitoneal fat pad (females only) on PND 90, 3. Reduced body weight on day 90, 4. Reduced leptin in males (33 %) and females (56 %), 5. Reduced cholesterol in males and females. No effects were seen on triglycerides and insulin. A significant dose trend for glucose was due to an 11 % lower mean glucose level in the 2,700 µg BPA/kg bw per day. However, this changed level was not significantly different from the vehicle control group, p = 0.189).

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses the study:
Strengths:

- Large sample size
- Both naïve and vehicle controls available
- Adequate positive controls included
- Measurement of BPA concentration in the serum
- Number of doses (≥3)
- Oral administration by via gavage
- Phytoestrogen-free diet
- Use of non-PC cages
- Study performed according to OECD guidelines, under GLP US FDA/NCTR, 2013 and Delclos et al., 2014

Weaknesses:

- Unintended exposure of the control groups (see Churchwell et al, 2014)

Overall, the CEF Panel noted that this GLP study, performed according to OECD standards, evaluated a wide range of nine dose levels, seven below and two above the dose of 5 mg/kg bw per day in former assessments defined as the reference point. The highest dose had an influence on several parameters. It is to be noted that the study duration was 90 days (13 weeks) with permanent dosing. Phytooestrogen levels in food were monitored to be in the low range. As the number of litters is sufficiently large the study has a fair sensitivity to detect effects.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Somm E, Schwitzgebel VM, Toulotte A, Cederroth CR, Combescure C, Nef S, Aubert ML and Huppi PS, 2009. Perinatal Exposure to Bisphenol A Alters Early Adipogenesis in the Rat. Environmental Health Perspectives, 117, 1549-1555.

This study was already considered in the 2010 EFSA opinion where it is stated "*Perinatal exposure to BPA (1 mg/l in drinking water from GD 6 to PND 21) in rats (Somm et al., 2009) was reported to increase adipogenesis in female offspring at weaning*".

Body weight on PND 1 was increased in both males and females, whereas body weight and pWAT was increased in females only on PND 21. Furthermore, BPA exposed animals fed with high fat caloric diet had increased body weights compared to controls on high fat caloric diet (feeding from

week 4 until week 14) from week 9 to week 14 in males and in week 4 and 5 as well as week 8 to week 11 in females. No effects were seen on glucose metabolism in a sub-study performed only in males.

Comments from the CEF Panel:
The study was performed with a single dose only (app. 70 µg/kg bw per day). Phytoestrogen content in food was measured as was also the water content of BPA. The effects on body weights and pWAT were only seen in females as was expression of genes involved in adipogenesis. The statistical procedures are not clearly described and might be flawed.

Wei J, Lin Y, Li Y, Ying C, Chen J, Song L, Zhou Z, Lv Z, Xia W, Chen X and Xu S, 2011. Perinatal Exposure to Bisphenol A at Reference Dose Predisposes Offspring to Metabolic Syndrome in Adult Rats on a High-Fat Diet. Endocrinology, 152, 3049-3061.

Wei et al. (2011) gave doses of 0, 50, 250 or 1 250 µg BPA/kg bw per day orally by gavage in corn oil to pregnant Wistar rats from GD 0 to PND 21. The offspring (n=16 per group, 2 from each of 8 litters) were maintained on either a normal or a high fat diet for 16 weeks), with monitoring of body weight, blood parameters (triglycerides, cholesterol, low- and high-density lipoprotein), glucose tolerance test, insulin tolerance test were investigated periodically through the experimental period, while morphology and function of the pancreas was assessed at termination at week 27. No effects of BPA were observed at doses of 250 or 1250 µg BPA/kg bw per day. Offspring exposed prenatally to 50 µg BPA/kg bw per day and maintained on a normal diet showed increased weight gain from week 17 (females) or week 19 (males). No significant differences in fasting blood glucose levels were seen in animals exposed to 50 µg BPA/kg bw per day when compared to controls, while serum insulin levels were higher at week 15 and 26 for males and week 26 for females. Effects were more evident in the 50 µg BPA/kg bw per day animals fed a high fat diet, with body weight gain being increased compared with controls at weeks 7 (males) and 9 (males). BPA (50 µg /kg bw per day) was associated with changes in blood lipid parameters compared with controls in both males and females fed a high fat diet and in males fed a normal diet. Serum leptin was elevated in BPA-treated animals compared with controls at week 26. The animals also had a higher body fat percentage and showed hypertrophy of adipocytes. Mitochondrial structure and insulin granule characteristics in pancreatic β-cells were altered by BPA at 50 µg/kg bwper day, both at weaning (week 3) and at termination (week 27) and mRNA expression of islet-associated transcription factors were reduced compared to controls. The authors suggested that BPA exposure predisposed the offspring to metabolic disturbances, possibly indicating the presence of metabolic syndrome, and noted that low dose BPA (50 µg/kg bw per day) was more effective than high doses, suggesting a non-monotonic dose-response relationship.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses the study:
Strengths:
- Number of doses (≥3)
- Oral administration by gavage
- Use of non-PC cages and non plastic water bottles

Weaknesses:
- Small sample size
- Insufficient study reporting (number of animals used for each end-point was variable and not always clear)
- Statistical analysis (litter effect not completely controlled)
- Animal diet phytoestrogen content not reported

Overall the CEF Panel noted that this was a reasonably well designed oral study, but but how litter effect was taken into consideration is not fully described and remains unclear. Results appeared to show a non-monotonic dose-response, with effects only seen at 50 µg BPA/kg bw per day. Mechanisms for leptin and serum insulin increase are not well explained. Statistical analysis was

flawed; in particular, the choice to consider the litter size as a covariate in the ANCOVA analysis was not properly justified. Moreover, the number of animals used for each end-point was variable and not always clear. For some parameters, sample size (n=3) was low. The phytoestrogen content of the diet was apparently not tested.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Xu X, Tan L, Himi T, Sadamatsu M, Tsutsumi S, Akaike M and Kato N, 2011b. Changed preference for sweet taste in adulthood induced by perinatal exposure to bisphenol A-A probable link to overweight and obesity. Neurotoxicology and Teratology, 33, 458-463.

Xu et al. (2011b) suggested that an increased preference of adult rats for a sweet taste, potentially resulting in obesity, could be linked to prenatal exposure to BPA. Female Sprague Dawley rats were exposed to BPA in drinking water at doses of 0.01, 0.1 and 1.0 mg/L from GD 11 to lactation day 21. The sweet preference of the offspring for 0.25 % or 0.5 % saccharin compared with water was assessed on week 7 after birth, while preference for 15 % sucrose compared with water was assessed on PND 42, 70 and 140 (in rats perinatally exposed to 0.1 mg/L compared with controls only). Body fat percentage and tail blood pressure were measured at the end of the study. A significant sex difference in preference for a sweet taste was evident in both BPA-treated and non-BPA treated offspring, with all females showing a preference for saccharin-containing drinking water compared with plain water and no evidence of a treatment-related effect. However male offspring showed an increased preference for 0.25 % (but not for 0.5 %) saccharin compared with male controls. Prenatal treatment of dams with 0.1mg/L BPA in drinking water treatment increased sucrose preference in males at postnatal day (PND) 70 and 140 ($p<0.05$ and $p<0.001$, compared to control respectively) but decreased sucrose preference in females at PND 140 ($p<0.05$, compared to control). This tendency was reversed in BPA-treated females compared with controls, implying the feminization of males and masculinization of females. Male offspring from dams receiving 0.1 mg/L BPA and administered 15 % sucrose in their drinking water postnatally also showed increased body weight gain compared with controls at PND 140 ($p<0.001$), their percentage of body fat as imaged by X-ray CT Scan was higher ($p<0.001$) as was their tail blood pressure ($p<0.05$).

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses the study:

Strengths:
- Number of doses (≥3)
- Use of non-PC cages

Weaknesses:
- Insufficient study reporting (administration via drinking water but no information on consumption)
- Statistical analysis (litter effect not completely controlled)
- Study design not appropriate to the scope (only one BPA dose was assessed postnatally

Overall, the CEF Panel noted that BPA was administered in drinking water, and no information was provided about the actual daily intakes based on drinking water consumption. Pups were selected randomly for assessment, litter effects were therefore not taken fully into account. Numbers of pups investigated for each endpoint was 4-6 pups/gender, which was acceptable. The inconsistency in the response to saccharin (preference for 0.25 % but not for 0.5 % saccharin) is noted, interpretation of the saccharin preference results was difficult, limiting overall the conclusions that can be drawn from the study. The choice of only the middle dose of BPA pups for the sucrose preference test based on the dose-response for saccharin preference is difficult to justify. It is impossible to evaluate the dose-

response effect for body weight because only one BPA dose was assessed postnatally. The phytoestrogen content of the diet was apparently not tested.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Studies in adult mice and rats

Batista TM, Alonso-Magdalena P, Vieira E, Amaral ME, Cederroth CR, Nef S, Quesada I, Carneiro EM and Nadal A, 2012. Short-term treatment with bisphenol-A leads to metabolic abnormalities in adult male mice. PLoS One, 7, e33814.

In the study of Batista et al. (2012), 3-month old Swiss albino OF1 mice (n=6-12 in different tests) were administered a total of 100 µg BPA/kg bw daily by subcutaneous injection (in two injections) for 8 days. Control mice received vehicle (corn oil) alone. Whole body energy homeostasis was assessed with in vivo indirect calorimetry, while responses of insulin sensitive peripheral tissues as measured by plasma insulin levels, glucose tolerance testing, secretion of insulin from isolated pancreatic islets and insulin signaling assays Mice treated with BPA and assessed at the end of the treatment period showed higher plasma insulin concentrations in the fed state and increased glucose-stimulated insulin secretion in isolated pancreatic islet of Langerhans, in addition to changes in insulin signaling. Glucose tolerance testing showed that BPA-treated mice were insulin resistant and had increased glucose-stimulated insulin release. Whole-body energy homeostasis, as assessed by reduced food intake, reduced locomotor behavior and decreased energy expenditure during night, was reduced, although respiratory exchange ratio was unchanged. Insulin-stimulated tyrosine phosphorylation of the insulin receptor b subunit and the mitogen-activated protein kinase (MAPK) signaling pathway was impaired in the skeletal muscle of BPA-treated mice and both skeletal muscle and liver showed an upregulation of IRS-1 protein by BPA. The authors concluded that BPA slows down whole body energy metabolism and disrupts insulin signaling in peripheral tissues, supporting the hypothesis that BPA may be a risk factor for the development of type 2 diabetes.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:

Strengths:
- None

Weaknesses:
- Single dose level study
- Test performed in one sex only
- Insufficient study reporting (number of animals tested is unclear for each endpoint)
- Animal diet and phytoestrogen content not reported.

Overall the CEF Panel noted that this is a single dose study. The results suggest an effect of BPA on insulin homeostasis and signalling, reported also by other authors. The study may be considered supportive for effects of BPA on insulin/glucose metabolism.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Bodin J, Bolling AK, Samuelsen M, Becher R, Lovik M and Nygaard UC, 2013. Long-term bisphenol A exposure accelerates insulitis development in diabetes-prone NOD mice. Immunopharmacology and Immunotoxicology, 35, 349-358.

Bodin and co-workers investigated possible effects of BPA, administered at 0, 1 and100 mg/l BPA in the drinking water of non-obese diabetic (NOD) mice (n = 6-10 per group for different parameters) on the development of type 1 diabetes (T1DM). This mouse strain is used as a animal model of

spontaneous diabetes development, due to a high level of beta cell apoptosis leading to increased insulitis. The authors estimated from parallel measurements of water consumption in non-diabetic and diabetic mice that these levels of BPA in drinking water corresponded to intakes of 0, 150 or 15000 µg/kg bw per day in non-diabetic mice. Intakes in diabetic mice at the end of the study were estimated to reach 200–1650 µg/kg bw per day in diabetic mice in the 1.0 mgBPA/l group due to the higher water consumption in these animals. Plasma glucose was measured at weekly intervals from week 7 to week 30, by which time the mice had become diabetic. The development of insulitis was followed by histological examination of the pancreas at 7 and 12 weeks and changes in serum autoantibodies, cytokines, insulin and thyroxine (T4) levels were also investigated at these time intervals. Serum insulin was additionally measured at week 28 of the study. Incidence and degree of insulitis in the pancreas was comparable between groups at week 7. It was markedly increased compared with controls in 12-weeks-old female mice exposed to 1 mg/l BPA in drinking water, but was less severe in the female animals receiving 100 mg/l and was decreased in male mice exposed to BPA compared with controls. Increased apoptosis and reduced numbers of tissue resident macrophages were seen in the pancreatic islets of female mice prior to the development of insulitis. Serum glucose levels were increased in the 1 mg/ml BPA group indicating an accelerated onset of T1DM, but this was not seen in the animals exposed to 100 mg/l BPA. Insulin levels did not differ significantly between the groups and while T4 levels increased slightly with increasing BPA intake, this was not statistically significant. Serum levels of cytokines and autoantibodies also did not differ between the groups. The authors suggested that the higher level of BPA could be protective against diabetes development in female mice, while a protective effect was seen for male mice for both BPA concentrations. The authors concluded that long-term BPA exposure at a dose three times higher than the tolerable daily intake of 50 mg/kg, appeared to accelerate spontaneous insulitis and diabetes development in NOD mice, with some evidence of a non-monotonic dose-response.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses the study:
Strengths:

- Phytoestrogen-free diet
- Use of non-PC cages

Overall, the CEF Panel concluded that the relevance of the findings in this strain of diabetes-prone mice for development of diabetes in a normal population is debatable, particularly in the light of the inconsistent dose-response and the absence of effects on plasma insulin. Furthermore, the results are quite inconsistent and mainly negative. Indeed, the tendency toward an accelerated development of diabetes, observed only in females exposed to the lower dose BPA is not statistically significant. The immunological markers whose impairment should lead to the development of diabetes, according to the authors' hypothesis are non different between the study groups.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

D'Cruz SC, Judendradass R and Mathur PP, 2012a. Bisphenol A Induces Oxidative Stress and Decreases Levels of Insulin Receptor Substrate 2 and Glucose Transporter 8 in Rat Testis. Reproductive Sciences, 19, 163-172.

D'Cruz SC, Jubendradass R, Jayakanthan M, Amala Rani SJ and Mathur PP, 2012b. Bisphenol A impairs insulin signaling and glucose homeostasis and decreases steroidogenesis in rat testis: an in vivo and in silico study. Food and Chemical Toxicology, 50, 1124-1133.

Note: these papers are reviewed together as they address the same endpoints, the second-listed study providing an extension of the endpoints investigated in the first.

In the studies of D'Cruz et al. (2012a, b) male Wistar rats (n = 6 per group) were given doses of BPA ranging from 0.005, 0.5, 50 and 500 µg/kg bw per day by oral gavage for 45 days. In the first study, levels of plasma glucose and insulin, testicular glucose and peroxide and enzymes involved in glucose metabolism were investigated, together with levels of insulin signaling molecules, glucose transporter-2 (GLUT-2). The second study investigated testicular levels of insulin, insulin signaling molecules, GLUT-2, antioxidant enzymes and steroidogenesis were investigated at the end of the treatment period. 17-β-oestradiol (50 µg/kg bw per day) was used as a positive control. Levels of plasma glucose and insulin were significantly increased down to the lowest level of BPA exposure of 5 ng/kg bw per day, whereas the testicular glucose level significantly decreased, again at all dose levels. Similar responses were seen with the positive control, 17-β-oestradiol. There was also a significant decline in the activities of hexokinase and phosphofructokinase in the testis of rats treated with BPA. Levels of insulin and various insulin signalling molecules as determined by Western blot analysis were significantly decreased in rat testis of BPA-treated rats in a dose-related manner down to an exposure level of 5 ng/kg bw per day. Similarly, a dose-dependent and significant decrease in testicular superoxide dismutase and catalase activities was measured following BPA exposure at all doses, and lipid peroxidation was increased, together with a dose-dependent increase in the level of hydrogen peroxide, decreases in testicular marker proteins and key enzymes of steroidogenesis. The authors reported testicular damage as evidenced by loss of germ cells and decrease in the spermatids in rats treated with 500 µg BPA, as well as in the positive control, and immunolocalization of GLUT-8 protein in the testis revealed decreased expression of this protein in spermatocytes and developing spermatids of rats exposed to BPA. The authors concluded that low doses of BPA affect insulin signaling and glucose, possibly leading to impairment of testicular function.

Comments from the CEF Panel:
Comment: The standard deviation is much smaller than normal variability

The CEF Panel identified the following strengths/weaknesses in the studies:
Strengths:

- Adequate positive controls included
- Number of doses (≥3)
- Oral administration by via gavage
- Use of non-PC cages

Weaknesses:

- Test performed in one sex only
- Small number of animals
- Inappropriate statistics (considering the small number of animals)
- Animal diet and phytoestrogen content not reported

Overall, the CEF Panel noted that, despite the relatively small number of animals used at each dose level and the relative complexity of some of the assays undertaken, the dose-response reported, persisting down to a dose level of 5 ng/kg bw per day, was perfect. The statistics are not properly reported as one-way ANOVA followed by Tukey's post test, but the results of the overall ANOVA are not given. The use of this statistics with such a small sample size is questionable. The reported changes in testicular pathology cannot be related to functional deficits.

These papers are included in the WoE table because of their relevance to one or more review questions addressed there.

Hassan ZK, Elobeid MA, Virk P, Omer SA, ElAmin M, Daghestani MH and AlOlayan EM, 2012. Bisphenol A induces hepatotoxicity through oxidative stress in rat model. Oxid Med Cell Longev, 2012, 194829.

The study investigates at the cellular level whether BPA causes hepatotoxicity by induction of oxidative stress in liver. As indices of oxidative stress liver content of glutathione, superoxide dismutase, glutathione peroxidase, glutathione-S-transferase, glutathione reductase and catalase activity were measured. Five rats per group received BPA (0.1, 1, 10, 50 mg/kg/day) via gavage for four weeks. One additional group of five rats served as control group and received water. In addition, gene expression profile in liver tissue was measured by real-time PCR. The final body weights in the 0.1 mg/kg bw group showed a significant decrease and the 10 mg/kg bw group a significant increase compared to control group. Serum ALT, ALP and bilirubin were significantly elevated in the 10 mg/kg bw and the 50 mg/kg bw group indicating liver cell damage. Levels of reduced glutathione, superoxide dismutase, glutathione peroxidase, glutathione-S-transferase, glutathione reductase and catalase activity were found in the 50mg BPA group compared to controls. Likewise, the activity of antioxidant genes was reduced as confirmed by real time PCR in which the expression levels of these genes in liver tissue were significantly decrease compared to control. The data from this study are compatible with the assumption that BPA generates ROS and reduces antioxidant gene expression that these effects are causative for the observed hepatotoxicity of BPA.

Comment from the CEF Panel:
The study seems well performed although the number of animals per group is small and the statistical testing was performed without adjustment for multiple testing. In addition the description of the methods is not given in detail. The phytoestrogen content of the diets was apparently not tested. Nevertheless, the findings are pointing to a dose dependent hepatotoxic effect of BPA which was observed in other studies with identical doses (50 mg/kg bw per day, Tyl et al., 2008). The mechanism by which the hepatotoxicity is mediated seems to be oxidative stress as evidenced by biochemical indices and by the expression levels of antioxidant genes in the liver.

Indumathi D, Jayashree S, Selvaraj J, Sathish S, Mayilvanan C, Akilavalli N, Balasubramanian K, 2013. Effect of bisphenol-A on insulin signal transduction and glucose oxidation in skeletal muscle of adult male albino rat. Human and Experimental Toxicology, 32, 960-971.

Jayashree S, Indumathi D, Akilavalli N, Sathish S, Selvaraj J, Balasubramanian K, 2013. Effect of Bisphenol-A on insulin signal transduction and glucose oxidation in liver of adult male albino rat. Environmental Toxicology and Pharmacology, 35, 300-310.

Note : these papers are reviewed together as they address the same endpoints in different tissues

Jayashree and co-workers investigated the effects of bisphenol-A on insulin signal transduction and glucose oxidation in skeletal muscle and liver of adult male Wistar rats (Jayashree et al. 2013; Indumathi et al., 2013). BPA was administered orally by gavage once daily for 30 days at dose levels of 0, 20 or 200 mg/kg bw, in corn oil. Group size was six animals. At the end of the treatment period serum insulin was significantly increased in a dose-related manner in both groups of BPA-treated animals (the authors also demonstrated a decrease in serum testosterone levels) but fasting blood glucose level remained unaltered. Glucose oxidation was reduced at both dose levels in liver and in skeletal muscle, and glycogen content of the liver was also reduced. In skeletal muscle, treatment with BPA at both 20 or 200 mg/kg bw significantly decreased the insulin receptor, protein kinase B and glucose transporter-4 levels (both plasma membrane and cytosolic fraction), but did not affect the mRNA levels for these proteins. In the liver both m-RNA and protein levels were significantly decreased at the highest BPA-exposed group. The authors concluded that BPA can affect glucose oxidation and hepatic glycogen reserves through defective insulin signal transduction.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the studies:

Strengths:
- Oral administration by via gavage (Indumathi et al., 2013, Jayashree et al., 2013)
- Use of non-PC cages (Indumathi et al., 2013, Jayashree et al., 2013)

Weaknesses:
- Small sample size (Indumathi et al., 2013, Jayashree et al., 2013)
- Test performed in one sex only (Indumathi et al., 2013, Jayashree et al., 2013)
- Animal diet and phytoestrogen content not reported (Indumathi et al., 2013, Jayashree et al., 2013)

Comments from the CEF Panel:
Overall, the CEF Panel noted that in these studies a relatively small number of animals were used at each dose level, the dose levels were quite high and the diet was apparently not checked for phytoestrogen content.

These studies are included in the WoE table because of their relevance to one or more review questions addressed there.

Marmugi A, Ducheix S, Lasserre F, Polizzi A, Paris A, Priymenko N, Bertrand-Michel J, Pineau T, Guillou H, Martin PG and Mselli-Lakhal L, 2012. Low doses of bisphenol A induce gene expression related to lipid synthesis and trigger triglyceride accumulation in adult mouse liver. Hepatology, 55, 395-407.

Marmugi et al. (2012) administered BPA (0, 0.05, 0.5, 5 or 50 mg/kg diet, estimated by the authors to be equivalent to 0, 5, 50, 500 and 5000 µg/kg b.w. per day) to male CD1 mice (n=6 per group) for 28 days. At the end of the experimental period, the authors measured body weight gain, liver weight and weight of perigonadic white adipose tissue (pWAT), hepatic lipid content and fatty acid composition, plasma levels of insulin, triglycerides, glucose, total cholesterol, low- or high-density lipoprotein (LDL, HDL) cholesterol were measured, and the the effects of BPA on gene expression in the liver was assessed using microarrays. No effect was seen on body weight gain and relative liver weight, but pWAT weight was significantly increased in mice receiving 50 µg/kg bw per day (but not at higher dose levels). Plasma insulin levels were significantly increased following exposure to 5, 50, and 500 µg BPA/kg bw per day, with the greatest effect being seen at the lowest dose. No significant effect was apparent on plasma glucose and total, LDL- or HDL-cholesterol, but mice exposed to 500 µg BPA/kg bw per day showed a significant increase in plasma triglyceride levels. The results of the microarray assays showed a stimulatory effect of BPA on expression of key enzymes involved in lipogenesis, cholesterol biosynthesis and, to a lesser extent, enzymes involved in glucose metabolism. The authors suggest that effects seen showing a non-monotonic dose-response since a stronger response was seen in the liver of mice receiving 50 µg/kg bw per day than those receiving mice receiving 5000 µg/kg bw per day. The authors suggest that exposure to low doses of BPA may influence *de novo* fatty acid synthesis through increased expression of lipogenic genes, thereby contributing to hepatic steatosis.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:
- Number of doses (≥3)
- Protocols according to EU guideline

Weaknesses:
- Test performed in one sex only
- Small sample size
- Feed consumption (BPA given by the diet) not measured
- Inappropriate statistics: insufficient studying reporting
- Animal diet and phytoestrogen content not reported

Overall, the CEF Panel notes that this study appears to confirm the effects reported by several other studies on lipogenesis, in adult animals, but the lack of a dose-response/non-monotonic dose-response has to be further confirmed. The numbers of animals involved per group are considered to be low. In the statistics no mention to multiple comparisons that should have been performed for all the measurements reported in Figure 1 of the study. The TDI dose induced more changes in gene expression as compared to the other doses and control.
This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Ronn M, Kullberg J, Karlsson H, Berglund J, Malmberg F, Orberg J, Lind L, Ahlstrom H and Lind PM, 2013. Bisphenol A exposure increases liver fat in juvenile fructose-fed Fischer 344 rats. Toxicology, 303, 125-132.

Rönn et al. (2013) administered BPA (0.025, 0.25 or 2.5 mg BPA/L, equivalent in drinking water containing 5 % fructose to female F-344 rats (n= 12 per group) from five to 15 weeks of age. The intakes of BPA, according to the authors, were between 4.6 (week 9) and 5.6 (week 2) µg/kg bw per day (mean 5.1 µg/kg bw per day) at the lowest dose, between 46.3 (week 6) and 61.6 (week 3) µg/kg bw per day (mean 54.3 µg/kg bw per day) at the mid dose and 400.3 (week 9) and 595.3 (week 2) µg/kg bw per day (mean 487.3 µg/kg bw per day) at the highest dose. The authors assessed effects on adipose tissue volume and liver fat content in the BPA-exposed groups by magnetic resonance imaging (MRI) compared with a control group also given fructose solution. They also measured cholesterol, triglycerides and apolipoprotein A-1a, changes in body weight and weight of the perirenal fat pad. There were no significant effects of BPA exposure on body weight or weight of the perirenal fat pad and no differences were seen in total or visceral adipose tissue volumes between the groups. However liver fat content was significantly higher in BPA-exposed rats (0.25 and 2.5 mg BPA/L; corresponding to 54.3 µg/kg bw per day 487.3 µg/kg bw per day) than in fructose controls ($p = 0.04$). BPA exposure also increased the apolipoprotein A-I levels in plasma ($p < 0.0001$) which indicates a favourable modification in the lipid profile because it is the main component of the high density lipoprotein (HDL).

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:
- Number of doses (≥3)
- Oral administration by via gavage
- Phytoestrogen-free diet
- Use of non-PC cages

Weaknesses:
- Test performed in one sex only
- Study design not appropriate to the scope (not fully appropriate for obtaining the substance specific data aimed at (BPA exposure combined with fructose)

Overall the CEF Panel notes that this is considered to be quite a robust study, with an adequate number of animals per group, which notably does not show a marked effect of BPA on lipogenesis other than a marginal effect on liver fat levels. However the methodology used (MRI) may have limitations in relation to sensitivity to detect subtle effects. It should be noted that in humans apolipoprotein A I is part of the high density lipoproteins (HDL), for which is agreement that increases have a beneficial effect.
This study is included in the WoE table because of its relevance to one or more review questions addressed there.

(iii) In vitro studies

Appraisal of strengths and weaknesses and WoE analysis were not carried out for in vitro studies.

Huc L, Lemarié A, Guéraud F and Héliès-Toussaint C, 2012. Low concentrations of bisphenol A induce lipid accumulation mediated by the production of reactive oxygen species in the mitochondria of HepG2 cells. Toxicology In Vitro, 26, 709-717.

HepG2 cells (human hepatocellular carcinoma) were treated with 10^{-4}-10^{-12} M BPA for 1-3 days and changes of the mitochondrial membrane potential, oxidative stress, generation of reactive aldehydes (4-hydroxynonenal) and lipid accumulation were determined. In addition, the release of IL-8, IL-6 and TNFα was monitored. Mitochondrial ROS production was detected mainly at 48 and 72 h after treatment in 20-30% of all cells. An inverse U-shaped increase in cytosolic superoxide anions was detected 72 h after treatment with a maximum at 10^{-9} M in up to 20% of all cells. In addition mitochondrial hyperpolarisation was seen in a time and concentration-dependent manner in up to 60% of all cells. In the presence of free fatty acids the number of cells with more lipid droplets increased from 6 % to 15 % within 72 h. Recently it has been shown that BPA increases the lipid synthesis in hepatocytes *in vivo* (Marmugi et al., 2012). In the latter study key enzymes of the lipid metabolism showed an inverse U-shaped kinetic, suggesting that the data of the present study are of physiological relevance. A release of IL-8 and TNFα was detected after 72 h only at a high BPA concentration, i.e. 10^{-5} M.

Lin Y, Sun X Qiu L, Wei J, Huang Q, Fang C, Ye T, Kang M, Shen H and Dong S, 2013. Exposure to bisphenol A induces dysfunction of insulin secretion and apoptosis through the damage of mitochondria in rat insulinoma (INS-1) cells. Cell Death and Disease 4, e460.

The authors studied the effect of $2x10^{-9}$ - $2x10^{-6}$ M BPA on the immortalized rat pancreatic cell line INS-1. A significant time- and concentration-dependent toxicity (MTT assay) was observed - even at the lowest BPA concentration at 48 hours treatment. In addition, BPA at $2x10^{-9}$ M increased insulin secretion at 16.7 mM glucose. A significant increase in early apoptotic cells was observed at and above $2x10^{-8}$ M BPA (48 h) along with a reduction of the mitochondrial mass, disturbed mitochondrial membrane potential, increased cytochrome c release and a reduced ATP concentration. Western blot analysis of Bax and Bcl-2 expression suggests that apoptosis is mediated via caspase-dependent mitochondrial pathway.

INS-1 cells seem to be very sensitive to BPA effects on mitochondrial membranes resulting in apoptosis. The BPA induced effects – except for insulin secretion and expression of two related genes - were concentration-dependent with a maximum at high BPA concentrations which were clearly toxic and not relevant for risk assessment (>10^{-7} M). It remains to be clarified whether or not this cell model is relevant for toxic effects of BPA on pancreatic β-cells *in vivo*.

Linehan C, Gupta S, Samali A and O'Connor L, 2012. Bisphenol A-mediated suppression of LPL gene expression inhibits triglyceride accumulation during adipogenic differentiation of human adult stem cells. PLoS One, 7, e36109.

Triglyceride accumulation and gene expression weres studied during differentiation of 3T3-L1 pre-adipocytes. Whilst the effective BPA concentration ($8x10^{-5}$ M) was too high to be relevant for risk assessment, lower concentrations of BPA of $8x10^{-8}$ and $8x10^{-6}$ M did not affect adipogenic differentiation or triglyceride accumulation in these cells.

Sheng ZG, Tang Y, Liu YX, Yuan Y, Zhao BO, Chao XJ and Zhu BZ, 2012. Low concentrations of bisphenol A suppress thyroid hormone receptor transcription through a nongenomic mechanism. Toxicology and Applied Pharmacology, 259, 133-142.

The authors studied the effect of 10^{-9}-10^{-7} M BPA on the thyroid receptor (TR) activation in the presence and absence of physiological concentrations of T_3 (10^{-10} M) and T_4 (10^{-7} M). These effects were studied on CV-1 cells derived from cercopithecus aethiops monkey kidneys, lacking TR and

293T cells. After transfection gene reporter assays were used to study involved signalling mechanisms. The authors showed that BPA suppressed the T_3/T_4- and the steroid receptor coactivator (SRC-1)-enhanced TR transcription probably by disrupting the T_3 /T_4-mediated activation of the β3integrin/c-Src/MAPK/TR-β1 pathway.

Whilst BPA is known to bind to both the TR-α and TR-β only with low affinity the present findings with low BPA concentrations suggest a BPA-induced suppression of TR transcription by recruitment of nuclear receptor co-repressor (N-CoR) to TR-β. The relevance of the findings from the complex transfection experiments for the *in vivo* situation is unclear.

Song L, Xia W, Zhou Z, Li Y, Lin Y, Wei J, Wei Z, Xu B, Shen J, Li W and Xu S, 2012. Low-level phenolic estrogen pollutants impair islets morphology and β-cells function in isolated rat islets. Journal of Endocrinology, 215, 303-311.

The authors studied the effect of $4.4x10^{-10}$ – $1.1x10^{-6}$ M BPA and 4 phenolic estrogen pollutants on primary rat pancreatic islet cells. A concentration–dependent decrease in islet viability (MTT assay) was detected at $1.1x10^{-8}$ M BPA and higher. At $1.1x10^{-7}$ M BPA the mitochondria of treated β-cells were remarkably swollen and showed a loss of structural integrity, the cytosolic ATP content was reduced. Incubations in the presence of 16.7 mM glucose resulted in a significant increase in the insulin release at $4.4x10^{-10}$ to $1.1x10^{-8}$ M BPA while it was significantly decreased at higher BPA concentrations. Additionally gene expression was studied at $1.1x10^{-7}$ M BPA: except for Ucp2, most of the studied genes showed reduced gene expression. According to recent publications (Chan *et al*., 1999, 2001) overexpression of UCP2 is associated with reduced ATP generation in mitochondria of β-cell lines and has been shown to promote proton keakage across the mitochondrial membrane (Rial et al., 1999).

The CEF Panel noted that except a very slight increase in insulin secretion in the presence of a high glucose concentration (16.7 mM) all other BPA-induced effects, *i.e.* on insulin secretion (at 3 mM glucose), on mitochondria and gene expression, were observed at (sub)toxic BPA concentrations. Therefore, the relevance of these *in vitro* observation remain questionable.

Soriano S, Alonso-Magdalena P, García-Arévalo M, Novials A, Muhammed SJ, Salehi A, Gustafsson JA, Quesada I, Nadal A (2012) Rapid insulinotropic action of low doses of bisphenol-A on mouse and human islets of Langerhans: role of estrogen receptor β. PLoS One, 7, e31109.

The authors studied the effect of 10^{-10} - 10^{-7} M BPA on the insulin secretion of primary human and murine pancreatic β-cells at 8 mM glucose. BPA increased the insulin secretion at all investigated concentrations in mouse islets. For the following investigations 10^{-9} M BPA was used. While BPA increased the insulin secretion (at 8 mM Glc) in islets and reduced the K_{ATP} channels activity in β-cells from three human cadaveric organ donors and wild type (WT) mice, no such effects was observed in β-cells from ERβ$^{-/-}$ mice. This occurred in parallel with an frequency increase of $[Ca^{2+}]i$ oscillations, again in β-cells from WT mice, only. At 3 mM glucose the observed *in vitro* effects were absent. The negative results on insulin release with cells from from ERβ$^{-/-}$ mice and the stimulation of insulin release with the specific ERβ agonist DPN in human cells suggest a crucial role of from ERβ in the BPA-induction of insulin release.

In this study E2 was not included as a positive control was included, however it was claimed in the discussion Section that BPA and E2 were equally potent. With regard to the *in vivo* situation the impact of E2 on the BPA effects in isolated islets/β-cells would be interesting.

The number of experiments/cells is – where indicated at all – low (n=5), however the reported effects are consistent and are in line with earlier observations from the same research group. The impact of such an artificial cell system on the risk assessment of BPA is unclear.

Wang J, Sun B, Hou M, Pan X and Li X, 2013. The environmental obesogen bisphenol A promotes adipogenesis by increasing the amount of 11β-hydroxysteroid dehydrogenase type 1 in the adipose tissue of children. International Journal of Obesity, 37, 999-1005.

The authors studied the effect of BPA on human adipose tissue, obtained after surgical interventions. The expression of 11β-hydroxysteroid dehydrogenase (11β-HSD1), PPARγ, and of lipoprotein lipase (LPL) significantly increased after 24 h of incubation at 10^{-8} M, 10^{-6} M and $8x10^{-5}$ M BPA. In addition, the 11-βHSD1 enzyme activity increased in adipose tissue. After stimulation with BPA the gene expression of 11β-HSD1 show a similar response in human pre-adipocytes and adipocytes. However, expression levels were higher in adipocytes, compared to pre-adipocytes. The presence of BPA during differentiation of pre-adipocytes to adipocytes resulted in significant higher number of lipid droplets at 10^{-8} M and $8x10^{-5}$ M and an increased expression of PPARγ and LPL. The 11β-HSD1 inhibitor CXB significantly reduced the effects of BPA on the increased expression of 11β-HSD1, PPARγ and LPL. In addition, mifepristone (RU486) a glucocorticoid receptor antagonist significantly reduced the expression of 11β-HSD1 in pre-adipocytes.

Genotoxicity

The selection for relevance as well as the review criteria applied to in vitro and in vivo genotoxicity studies differed from those used for animal and in vitro studies on other endpoints, since they were in line with the EFSA scientific opinion on genotoxicity testing strategy principles

(i) In vitro studies

Audebert M, Dolo L, Perdu E, Cravedi JP and Zalko D, 2011. Use of the γH2AX assay for assessing the genotoxicity of bisphenol A and bisphenol F in human cell lines. Archives of Toxicology, 85, 1463-1473.

In this study the authors investigated the capability of established human cell lines, ACHN (human kidney adenocarcinoma cells), HepG2 (human hepatocellular carcinoma cells) and LS174T (human epithelial colorectal adenocarcinoma cells) to biotransform bisphenol A (BPA) and bisphenol F (BPF). The potential genotoxicity of BPA and BPF was assessed using a novel genotoxicity assay based on the detection of phosphorylated histone γ-H2AX, which forms foci that appears immediately after DNA damage and recruit protein responsible for repair of DNA damage. A description of the experimental procedure followed to detected histone γ-H2AX is missing and no information on antibodies employed is reported. BPA was shown to be metabolized by HepG2 and LS174T cell lines. Intestinal cells showed stronger biotransformation capabilities than liver cells, in terms of production of the glucuronide- and the sulphate-conjugates (phase II metabolites). On the other hand, ACHN cell line was not able to metabolize BPA. Relevant metabolites were separated and quantified by radio-HPLC. Following treatment with BPA at dose-levels of 1, 5, 10, 50 and 100 for 24 hours no increases of γ-H2AX were observed in any concentration assayed. To avoid false-positive genotoxic signals induction of histone γ-H2AX was assessed at dose-levels with at least 80 % cell viability.

Comments from the CEF Panel:
Overall the CEF Panel notes that the study was not specifically designed for risk assessment purposes but rather for basic research objectives. The work shows serious uncertainties mainly related to genotoxicity evaluation.

This paper is included in the WoE table because of its relevance to one or more questions addressed there.

Fic A, Žegura B, Sollner Dolenc M, Filipič M and Peterlin Mašič L, 2013. Mutagenicity and DNA damage of bisphenol A and its structural analogues in HepG2 cells. Arhiv Za Higijenu Rada i Toksikologiju, 64, 3-14.

This study was aimed at assessing the mutagenic and genotoxic potential of bisphenol A (BPA) using the Ames test (*Salmonella* typhimurium strains TA98 and TA 100) in either the absence or presence of S9 metabolic activation and the alkaline comet assay in the HepG2 cells treated with BPA at dose-levels of 0.1, 1.0 and 10.0 µM for 4 and 24 hours. BPA was not mutagenic in the *Salmonella* typhimurium strains TA98 and TA 100 both in the absence and presence of S9 metabolism, while in the comet assay it induced a significant, but not dose-related increase in DNA damage only after 24-hour exposure.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in this study:

Strengths:
- Adequate number of concentrations in presence and absence of metabolic activation (S9)

Weaknesses:
- Limitations of the experimental design in the Ames test (e.g. limited number of *Salmonella* Typhimurium strains)
- Inconsistent results in the comet assay (e.g. not dose-related increase in DNA damage)

This paper is included in the WoE table because of its relevance to one or more questions addressed there.

Iso T, Watanabe T, Iwamoto T, Shimamoto A and Furuichi Y, 2006. DNA damage caused by bisphenol A and estradiol through estrogenic activity. Biological & Pharmaceutical Bulletin, 29, 206-210.

This study was aimed at assessing potential DNA damage induced by BPA and oestradiol using the alkaline comet assay and the detection of phosphorylated histone γ-H2AX, a marker for induction of DNA double strand breaks, in human cell lines positive and negative for estrogen receptors (ER) (MCF-7 and MDA-MB-231, respectively). ER-positive and ER-negative cells were treated with BPA at 10^{-4}, 10^{-6} and 10^{-8} M up to 24 hours. In a time course analysis of DNA damage, cells were treated at 10^{-4} M and damage was analysed after 1, 3 and 24 hours. Results obtained indicate significant increases of DNA breakage (increases in tail length) at the two highest levels assayed following 3 hour treatment and in the time course study in any of the sampling time used. Increases were also noted for induction of phosphorylated histone γ-H2AX in MCF-7 cells.

Comments from the CEF Panel:
The CEF Panel identified the following weaknesses in this study:

- Results are not clearly reported
- Inconsistent results in ER-negative and ER-positive cells (different genomic stability)

Overall the CEF Panel notes that the methods implemented are not sufficiently robust to support the results reported in the study.

This paper is included in the WoE table because of its relevance to one or more questions addressed there.

Johnson GE and Parry EM, 2008 Mechanistic investigations of low dose exposures to the genotoxic compounds bisphenol-A and rotenone. Mutation Research, 651, 56-63.

In this mechanistic study the aneugenicity of two known spindle poisons model compounds, namely rotenone and bisphenol A (BPA), has been investigated following low dose-exposure to mammalian cells, using the cytokinesis blocked micronucleus assay (CBMA) and immunofluorescence methods to visualize modifications of the microtubule organizing centers (MTOCs) of the mitotic spindles. For

induction of micronuclei BPA was added over a tight range of very narrowed low concentrations (1.5, 3.1, 6.2, 7.7, 9.2, 10.8, 12.3, 18.5, 24.6, and 37.0 µg/ml) to cultures of human (AHH-1) lymphoblastoid cell line for a complete cell cycle (22-26 hours dependent upon any cell cycle delay) in the presence of 3 µg/ml of the actin-inhibitor cytochalasin-B. A minimum of five separate experiments were performed. For mechanistic evaluation of the aneugenic effects of BPA, fluorescently labelled antibodies were used to visualize microtubules (α-tubulin) and MTOCs (γ-tubulin) in V79 culture because they are fibroblast cells which grow by attachment to the vessel surface which is an important feature in the study of the fidelity of mitoses. BPA in this case was added to V79 cells growing on sterile glass microscope slides placed in Petri dishes at concentrations 4.2, 4.9, 5.6, 7.0, 8.4, 9.8, 11.2 and 14 µg/ml for 20 hours (i.e. one cell cycle for V79). Results obtained for induction of micronuclei indicated dose-related and statistically significant increases of binucleate-micronucleated cells from 12.3 µg/ml with a clear threshold for induction of micronuclei (NOEL at 10.80 µg/ml and LOEL at 12.3 µg/ml). A NOEL and LOEL for percentage of binucleate cells (i.e. relative proportion of mononucleated to binucleate cells, as a measure of cell viability) was also observed at 9.2 µg/ml and 10.8 µg/ml BPA respectively. Similarly for induction of aberrations in the mitotic machinery a NOEL was observed at 7.0 µg/ml and a LOEL at 8.4 µg/ml BPA in V79 cells. Aberrant mitotic divisions, in the form of multiple spindle poles may be the mechanism for the production of chromosome loss into micronuclei.

Comments from the CEF Panel:
The CEF Panel identified the following strengths in this study:

- Sound experimental design and well documented study
- Adequate selection and spacing of dose-levels

Overall the CEF Panel notes that the conclusions of this study are very informative concerning the interaction of BPA with microtubule organizing centers (MTOCs) of the mitotic spindle and consequent induction of micronuclei. Furthermore, this study clearly demonstrates a threshold level for induction of micronuclei (NOEL at 10.80 µg/ml) and for induction of aberrations in the mitotic machinery (NOEL at 7.0 µg/ml) in mammalian cells, thus confirming thresholds of action for the induction of aneuploidy predicted for spindle poisons since multiple targets of the mitotic machinery need to be disabled before a quantitative response can be detected. The results obtained support the concept of a potential threshold-based hazard and risk assessment for BPA.

This paper is included in the WoE table because of its relevance to one or more questions addressed there.

Tayama S, Nakagawa Y and Tayama K, 2008. Genotoxic effects of environmental estrogen-like compounds in CHO-K1 cells. Mutation Research, 649, 114-25.

In this study, the authors evaluated the genotoxicity of some environmental estrogen-like compounds including bisphenol A (BPA) using sister chromatid exchanges (SCEs), chromosome aberration (CA) and DNA strand breaks (comet) assays in CHO-K1 cell line *in vitro*. For CA and SCEs six concentrations of BPA ranging from 0.1 to 0.6 mM were added to CHO-K1 cells for 3 hours. Following treatments cells were further incubated for 27 hours in the presence of 5-bromo-2'-deoxyuridine (BrdU) until preparation of slides for both SCE and CA from the same culture. For comet assay seven concentrations of BPA ranging from 0.1 to 0.7 mM were added to CHO-K1 for 1 hour and following washes of test compound cells were processed for comet assay using a silver-staining method and manual microscopic analysis. Results reported by the authors indicate positive effects for both SCE and chromosome aberrations at the two highest (0.5 and 0.6 mM) and at the highest concentrations (0.6 mM) respectively. Significant increases of c-mitotic effects were also reported at highest concentrations0.3-0.6 mM. For comet assay significant increases of DNA breakage were only reported at the highest concentration (0.7 mM). For chromosome aberrations, the sampling time used (27 hours) far exceeded the recommended 1.5 cell cycle which is 18-21 hours for cell line

used. Furthermore, cells were recovered in the presence of (BrdU) needed to detect SCE in the same cell culture which induces, although at low level, DNA single strand breaks which can influence production of chromosomal aberrations.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in this study:
Strengths:

- Adequate range of concentrations
- Three genotoxic endpoints (DNA breakage, SCE and CA)
- Concentration-related and statistically significant increases of c-metaphases

Weaknesses:

- Limitations in the experimental design (e.g. sampling times, staining procedures, cells recovered in the presence of BrdU)
- Positive effects only at high dose-level in the presence of cytotoxicity which generates false positives

This paper is included in the WoE table because of its relevance to one or more questions addressed there.

(ii) In vivo studies

De Flora S, Micale RT, La Maestra S, Izzotti A, D'Agostini F, Camoirano A, Davoli SA, Troglio MG, Rizzi F, Davalli P and Bettuzzi S, 2011. Upregulation of clusterin in prostate and DNA damage in spermatozoa from Bisphenol A-treated rats, and formation of DNA adducts in cultured human prostatic cells. Toxicological Sciences, 122, 45-51.

This study evaluated a variety of biomarkers, including the analysis of micronuclei in bone marrow cells and evaluation of the degree of DNA breakage by measurement of tail moment in the alkaline comet assay in peripheral blood lymphocytes, in male Sprague-Dawley rats treated with BPA via drinking water for a calculated daily intake of 200 mg/kg bw for 10 consecutive days. Furthermore, they investigated the formation of DNA adducts in two human prostate (PNT1and PC3) cell lines *in vitro* treated with BPA at 200 µM for PNT1 and at 250 µM for PC3 for 24 hours. Results obtained *in vivo* did not show induction of micronuclei in bone marrow cells and DNA breakage as measured by determination of tail moment in the comet assay. *In vitro*, results obtained showed formation of DNA adducts (4.2 and 2.7 fold increases over control in PNT1 and PC3 cells respectively).

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in this study:
Strengths:

- Sound approach and experimental design

Weaknesses:

- Limitations in the experimental design (e.g. single dose level, number of cells examined)

Overall the CEF Panel notes that—despite some limitations (e.g. 1 000 PCE/animal scored for micronuclei instead of 2 000 PCE/animal recommended; 100 cells analyzed for each test point in the comet assay instead of 150 recommended; only one dose-level used)—the methods implemented in the in vivo study to evaluate micronuclei induction in bone marrow cells and the degree of DNA breakage in peripheral blood lymphocytes by comet assay, are sufficiently robust to support the results reported which were judged informative for purposes of risk assessment.

This paper is included in the WoE table because of its relevance to one or more questions addressed there.

Dobrzyńska MM and Radzikowska J, 2013. Genotoxicity and reproductive toxicity of bisphenol A and X-ray/bisphenol A combination in male mice. Drug and Chemical Toxicology, 36, 19-26.

This study investigated the effects of BPA alone or in combination with X-rays on the sperm and induction of DNA strand breaks in somatic and germ cells of mice. Male Pzh:SFIS mice received BPA orally in drinking water for two weeks. Levels in drinking water were designed to achieve BPA intakes of 0, 5, 10, 20 or 40 mg/kg bw per day. Two additional groups received either 5 or 10 mg BPA/kg bw per day via drinking water in combination with daily radiation doses of 0.05 Gy or 0.10 Gy of X-rays. These latter groups were not considered relevant for the present evaluation. For comet assay animals were sacrificed 24 hours after the last treatment and DNA tail moment was employed to assess the levels of DNA breakage induced in cells isolated from liver, spleen, bone marrow, lungs, and kidneys through mechanic disaggregation of organs and filtered by adequate meshes. Results obtained indicate that BPA induced statistically significant increases of DNA tail moment in bone marrow, spleen, kidney and lung cells at any of the dose-level assayed. No DNA breakage was detected in liver cells.

Comments from the CEF Panel:
The CEF Panel identified the following weaknesses in this study:
Strenghts:

- Number of doses (≥3)

Weaknesses:

- Limitations in the experimental design (e.g. cytotoxicity not evaluated, absence of historical control values, inadequate sampling times)
- Poor study report
- No vehicle controls were tested
- Drinking water consumption (containing BPA) not measured
- Animal diet poorly described
- Animal diet and phytoestrogen content not reported

Overall the CEF Panel notes that the methods implemented are not sufficiently robust to support the results reported in the study.

This paper is included in the WoE table because of its relevance to one or more questions addressed there.

Izzotti A, Kanitz S, D'Agostini F, Camoirano A and De Flora S, 2009. Formation of adducts by bisphenol A, an endocrine disruptor, in DNA *in vitro* and in liver and mammary tissue of mice. Mutation Research, 679, 28-32.

This study was aimed a) to investigate the sensitivity threshold of DNA-adduct detection by ^{32}P-postlabelling in an acellular system and b) to evaluate the formation of DNA adducts, detected by ^{32}P-postlabelling in both liver and mammary cells of female CD-1 mice receiving BPA in their drinking water (200 mg/kg bw) for 8 consecutive days. Calf thymus DNA (dissolved in bi-distilled water, final concentration of 200 µg/ml), BPA dissolved in DMSO (and further diluted in bi-distilled water to final concentrations of 6.2 and 100 µM) and an exogenous S9 mix (metabolizing system containing 10 % liver S12 fractions derived from aroclor-1254-pretreated Sprague-Dawley rats) were incubated at 37 °C for 30 minutes. Results obtained indicated that the reaction of BPA in the presence of exogenous metabolizing system S9 mix resulted in a dose-related formation of bulky DNA adducts (two major and five minor DNA adducts detected in autoradiographic plates) with a detection limit of 10 ng for test compound. *In vivo*, administration of BPA to mice via drinking water under the mentioned experimental conditions resulted in the formation of bulky DNA adducts (two major DNA adduct) in the liver (3.4 fold increase over control level) as well as in the target mammary cells (4.7 fold increase over control level).

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in this study:
Strengths:

- Sound approach and experimental design

Weaknesses:

- Speculative conclusions

The results of this study confirm the ability of BPA to form DNA adducts both *in vitro*, in the acellular system previously described, and *in vivo* in liver and in target mammalian epithelial cells (for the first time). The authors attribute the adduct formation to the reactive metabolite BPA-3,4-quinone (BPAQ). BPA is metabolised in humans and in experimental animals, to its glucuronide and to hydroxylated derivatives, mainly 3-hydroxy-BPA (BPA catechol), which is finally oxidized to BPAQ. The conclusions raised by authors, although plausible from a theoretical point of view, in practice are rather speculative since the chemical identity of DNA adducts has not been characterized. This aspect is important for the outcome of this assay since different methods of DNA extraction can generate unspecific covalent binding to DNA. However, the CEF Panel considers that the methods implemented are sufficiently robust to support the results reported in the study.

This paper is included in the WoE table because of its relevance to one or more questions addressed there.

Masuda S, Terashima Y, Sano A, Kuruto R, Sugiyama Y, Shimoi K, Tanji K, Yoshioka H, Terao Y and Kinae N, 2005. Changes in the mutagenic and estrogenic activities of bisphenol A upon treatment with nitrite. Mutation Research, 585, 137-146.

In this study, the authors investigated the possible generation of genotoxicity from the reaction of BPA and nitrite under acidic conditions to simulate stomach environment. Genotoxicity of BPA alone at a concentration of 1 mM was also evaluated in an Ames test using TA 98 and TA 100 tester strains in either the absence or presence of S9 metabolic activation and in an *in vivo* micronucleus test in male ICR mice using peripheral blood reticulocytes at 228 mg/kg bw once by oral gavage. Peripheral blood was collected at 24, 48 and 72 hours after administration of test compound. Results obtained indicated that BPA alone did not exhibit any mutagenicity in the Ames test and did not induce any increase in micronucleated erythrocytes at any sampling time.

Comments from the CEF Panel:
The CEF Panel identified the following weaknesses in this study:
Weaknesses:

- Ames test limited to two strains
- Limitations in the experimental design (e.g. single concentration/dose)

Although the experiment was performed with the use of a single dose-level, the CEF Panel considers the negative results obtained at reasonably high dose-levels (228 mg/kg bw) in the in vivo peripheral blood micronucleus assay as informative for risk assessment purposes.

This paper is included in the WoE table because of its relevance to one or more questions addressed there.

Naik P and Vijayalaxmi KK, 2009. Cytogenetic evaluation for genotoxicity of bisphenol-A in bone marrow cells of Swiss albino mice. Mutation Research, 676, 106-112.

This study evaluated potential genotoxic effects of BPA by induction of chromosomal aberrations and micronuclei in bone marrow cells of Swiss albino mice. To further assess for potential interference of BPA with mitotic spindle apparatus, induction of c-mitoses was also performed. The test compound was administered orally in a 2 % acacia gum suspension at dose-levels of 10, 50 and 100 mg/kg bw to

groups of three male and three female mice, as single acute dose. Cumulative dose-level experiments were also performed at the lowest (10 mg/kg bw) dose-level for 5 consecutive days. In single treatment schedule, sampling of bone marrow was performed at 6, 24, 48 and 72 hours from beginning of treatment for both micronucleus and chromosome aberration assays. In cumulative treatment schedule, bone marrow was sampled in both assays 24 hour after the last administration of BPA. For induction of c-mitoses, the same dose levels used for micronucleus and chromosome aberration assays were applied as single dose and sampling of bone marrow was performed at 2, 6, 12, 24, 48 and 72 hours. Results showed that no significant increases of chromosomal aberrations or micronuclei were induced at any dose-level and sampling time used. On the other hand, significant increases in the frequencies of gaps were observed in all dose-levels assayed at the 48 and 72 hour sampling time and at the two higher dose-levels (50 and 100 mg/kg bw) at the 24 hour sampling time. The authors did not provide a suitable explanation for increase of gap frequencies but discussed findings from literature. One of the most relevant quoted by Xu and Adler (1990) considered such a finding not relevant for clastogenicity but associated this effect with potential interference with chromosome condensation along with potential effect on the mitotic spindle apparatus. In addition, BPA also induced c-mitotic effects through increases of mitotic indices and decrease in anaphase for both higher dose-level at 24, 48 and 72 hour sampling times.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in this study:
Strengths:

- Sound approach and experimental design

Weaknesses:

- Minor limitations in the experimental design (e.g. top dose too low, sub-optimal dose and exposure to colchicine)

The study complies with current recommendations with the exception that the highest dose-level selected was much lower than the feasible one (2000 mg/kg bw). In addition, the number of six animals employed (three male and three females) compensated the fact that a minimum of 5 males should have been used. Treatment with colchicine to accumulate cells at metaphase stage was shorter (1.5 hours) than recommended (5-6 hours). However, the CEF Panel considers that the methods implemented are sufficiently robust to support the results reported in the study and concluded that BPA under the reported experimental conditions was not clastogenic and did not elicit micronuclei induction thus excluding potential aneugenic effects at dose-levels employed. Furthermore, significant increases of achromatic lesions (gaps) are not considered relevant for clastogenicity and in this case could have been the result of different plausible factors which include flaming of cytogenetic slides during their preparation and use of lower concentration of colchicine (2.5 mg/kg bw instead of 4) for shorter time (1.5 hours instead of recommended 5-6 hours). Induction of c-mitotic effects may be related to interference of BPA with microtubule organising centres (MTCOs) of mitotic spindles in mammalian cells as reported by Johnson and Parry (2008).

This paper is included in the WoE table because of its relevance to one or more questions addressed there.

Pacchierotti F, Ranaldi R, Eichenlaub-Ritter U, Attia S and Adler I-D, 2008. Evaluation of aneugenic effects of bisphenol A in somatic and germ cells of the mouse. Mutation Research, 651, 64-70.

This study aimed to assess potential aneugenic effects of BPA on mouse male and female germ cells and somatic cells (male bone marrow cells), following acute *in vivo* exposure following different time periods. Cytogenetic effects on first and second meiotic divisions in the oocytes were evaluated following administration with BPA to female mice by oral gavage once at 0.2 and 20 mg/kg bw for 7 days with daily dose-levels of 0.04 mg/kg bw or for 7 weeks in drinking water at concentration of 0.5 mg/l. To further assess potential aneugenic effects of BPA on the second meiotic division of mouse

oocytes, analysis of chromosome complement of zygotes generated from mating of similarly BPA-treated females with untreated males was also performed. Evaluation of induction of aneuploidy in the first and second meiotic division of mouse spermatocytes was performed on the 22nd day after treatment of male mice with BPA by oral gavage on 6 consecutive days at 0.002, 0.02 and 0.2 mg/ kg bw. This study design was based on previous experiments with 5-bromo-2'-deoxyuridine (BrdU) to assess meiotic delay in spermatocytes. Furthermore, evaluation of potential aneugenic effects on somatic cells was performed by analysis of micronuclei in bone marrow cells of male mice treated on two consecutive days by oral gavage with 0.002, 0.02 and 0.2 mg/ kg bw and collected 24 hours after last administration of test compound. Results obtained for female animals indicated no significant induction of hyperploidy or polyploidy in oocytes and zygotes at any dose-level and treatment condition employed. Significant increases in the number of metaphase II oocytes with prematurely separated chromatids proved to be of no consequences in terms of fidelity of chromosome segregation during the second meiotic division as shown by normal chromosome complements of zygotes obtained under the same experimental conditions. In male mice no delay of meiotic divisions was observed following six daily administration of 0.2 mg/kg bw BPA in the BrdU assay. Similarly, no induction of hyperploidy or polyploidy in epydidimal sperms hybridized with DNA probes specific for mouse chromosome 8, X and Y 22 days after six oral doses of BPA. Furthermore, no induction of micronuclei in the bone marrow polychromatic erythrocytes of m male mice was observed following treatment on two consecutive days by oral gavage with 0.002, 0.02 and 0.2 mg/kg bw of BPA.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in this study:
Strengths:

- Sound approach and experimental design

Weaknesses:

- Inappropriate dose selection: high dose-levels for single or 7 daily administration apparently low (20 and 0.2, mg/kg bw respectively)

Overall the CEF Panel notes that the study is well conducted. The CEF Panel also notes that the dose-levels used in this study were selected to further evaluate increases of meiotic abnormalities observed in untreated female mice from an experimental colony which was temporally correlated with the accidental release of BPA from polycarbonate cages and bottles damaged by inadvertent treatment with harsh alkaline detergents as reported by Hunt et al. (2003) not according to recommendations for genotoxicity testing. However, information provided by this study is important in terms of risk assessment based on potential human exposure levels, since for aneugenic effects which are reported for BPA in *in vitro* studies, a non genotoxic effect level (NOGEL) can be defined.

This paper is included in the WoE table because of its relevance to one or more questions addressed there.

Tiwari D, Kamble J, Chilgunde S, Patil P, Maru G, Kawle D, Bhartiya U, Joseph L and Vanage G, 2012. Clastogenic and mutagenic effects of bisphenol A: An endocrine disruptor. Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 743, 83-90.

This study was aimed to assess potential genotoxic effects of Bisphenol A (BPA) in rats following oral administration of test compound once a day for 6 consecutive days at dose-levels of 2.4 µg, 10 µg, 5 mg and 50 mg/kg bw by measuring induction of micronuclei and structural chromosome aberrations in bone marrow cells and primary DNA damage in blood lymphocytes using single cell gel electrophoresis (comet assay). Furthermore, plasma concentrations of 8-hydroxydeoxyguanosine (8-OHdG), lipid peroxidation and glutathione activity were evaluated to assess potential induction of oxidative DNA damage. In the same study, mutagenicity was evaluated in the standard *Salmonella* plate test (Ames test) strains TA98, TA100 and TA102 at increasing concentrations from 6.25 µg to 200 µg both in the absence and presence of rat liver S9. No mutagenic response was observed in any of the tester strains at the various concentrations tested in absence and presence of metabolic activation. Results obtained for genotoxicity endpoints show marked dose-related increases of both

micronuclei and structural chromosome aberrations in bone marrow cells of male and female rats exposed to BPA. The observed increases achieved statistical significance at dose-levels as low as 10 µg/kg bw per day. Similarly, primary DNA damage evaluated by comet assay, in isolated peripheral blood lymphocytes showed marked and dose-related increases which were statistically significant at dose-levels as low as 10 µg/kg bw per day.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in this study:
Strengths:

- Ames test well conducted

Weaknesses:

- Ames test: limitations in the experimental design (e.g. three strains only)
- Micronucleus: limitations in the experimental design (e.g. inappropriate staining procedures)
- Chromosomal aberrations: experimental procedures questionable (e.g. inappropriate selection of sampling time; mitotic index as a measure of cytotoxicity not determined; sub-optimal exposure to colchicine)
- Incidence and type of chromosome aberrations generally not compatible with those seen with other chemical agents
- Comet assay: limitations in the experimental design (e.g. number of cells examined, cytotoxicity not evaluated/reported) and poor reporting (e.g. sampling times not reported)
- Plasma 8-OHdG concentrations: inconsistent results when compared with the comet assay outcome (significant increases were observed in the comet assay at dose-levels as low as 10 µg/kg bw but not in plasma concentration of 8-OHdG), low sensitivity of the analytical method (ELISA)

Based on the listed observations, the CEF Panel concludes that the methods implemented are not sufficiently robust to support the results reported in the study.

This paper is included in the WoE table because of its relevance to one or more questions addressed there.

Tiwari D and Vanage G, 2013. Mutagenic effect of Bisphenol A on adult rat male germ cells and their fertility. Reproductive Toxicology, 40, 60-68.

This study investigated the induction by BPA of dominant lethal mutations in the different stages of spermatogenesis in the rat. Furthermore, the effects of BPA on male reproductive functions and potential DNA damage induced in epydidimal sperm, assessed by the alkaline comet assay were investigated. The male rats were treated by oral gavage with BPA at dose-levels of 10 µg/kg bw and 5 mg/kg bw over a period of six days. Negative control were treated with vehicle. Each male of a specific treatment group (e.g. vehicle, 10 µg/kg bw, 5 mg/kg bw) was cohabited with two female per week (e.g. a total number of fourteen per group per mating interval) over a period of eight weeks. The mated females were then sacrificed on the day 15th of their gestation. The authors concluded that BPA induced dominant lethal mutations during the fourth and sixth weeks after BPA exposure, thus indicating its sensitivity to mid-spermatid and spermatocyte stages of spermatogenesis, at the highest (5 mg/kg bw) dose-level employed and that, the positive findings obtained were corroborated by DNA damage observed in the epydidimal sperm cells by the alkaline comet assay.

Comments from the CEF Panel:
The CEF Panel identified the following weaknesses in this study:

- Limitations in the experimental design (e.g. limited number of animals, absence of negative and positive controls, only two dose levels employed and lack of rationale for dose selection)

- Results potentially biased by high background/variability for rodent sperm in the alkaline assay
- No dose-related increases in dominant lethal mutations
- Absence of negative historical control data

Overall, the CEF Panel noted that the conclusion drawn by authors is not supported by experimental data. Similarly, positive findings obtained in the epididymal sperm using the alkaline comet assay appear to be biased by high background/variability for sperm, which might have been influenced by the elevated number of alkali labile sites present in rodent sperm as shown in the literature (see also Speit et al., Mutat Res. 2009; 681, 3-12). On this basis, and in the absence of negative historical control values, significant increases in DNA breaks over concurrent control values might not be indicative of genotoxic activity of BPA in sperm cells.

This paper is included in the WoE table because of its relevance to one or more questions addressed there.

Ulutaş OK, Yıldız N, Durmaz E, Ahbab MA, Barlas N and Çok I, 2011. An in vivo assessment of the genotoxic potential of bisphenol A and 4-tert-octylphenol in rats. Archives of Toxicology, 85, 995-1001.

The authors aimed to assess potential genotoxicity of bisphenol A (BPA) in peripheral blood nucleated cells of rats by comet assay. Groups of 6 rats were dosed orally for 4 weeks at dose-levels of 125 and 250 mg/kg bw. Control group animals (5 animals) were administered orally with corn oil for four weeks. At the end of treatment peripheral blood cells were collected via cardiac puncture and stored at 4°C until preparation of slides for comet assay. Authors showed significant increases of both tail length and tail moment for BPA only at the highest dose-level (250 mg/kg bw per day) employed,

Comments from the CEF Panel:
The CEF Panel identified the following weaknesses in this study:

- Limitations in the experimental design (e.g. inappropriate/not clearly reported sampling times, number of cells examined, cytotoxicity not evaluated/reported)

On this basis, the methods implemented were thought not to be sufficiently robust to support the results reported in the study.

This paper is included in the WoE table because of its relevance to one or more questions addressed there.

Carcinogenicity

(i) Human studies

Duan B, Hu X, Zhao H, Qin J, and Luo J, 2012. The relationship between urinary bisphenol A levels and meningioma in Chinese adults. International Journal of Clinical Oncology,18, 492-497.

The study represents a small case-control study in which urinary BPA concentrations in 243 male and female patients with neuroradiology-confirmed diagnosis of meningioma were compared with those in 258 matched healthy controls undergoing medical examinations at the same hospital in Wuhan, China. The specimens and data of patients were collected from 2009 to 2010. Total urinary BPA were measured using solid-phase extraction (SPE) coupled with high-performance liquid chromatography–mass spectrometry (LC–MS, no details given). A comprehensive quality control system, including reagent blanks, was used to ensure that samples were not contaminated during handling, storage, and analysis. The authors reported a positive association between increased concentrations of BPA in spot

urine samples (unadjusted) and meningioma independent of confounding factors that they identified: gender, age, race, body mass index (BMI), hormone replacement therapy (HRT) use and family history of cancer. Compared to quartile 1 (referent), the multivariate-adjusted odds ratio of meningioma associated with quartile 4 was 1.45 (95 % CI, 1.02–1.98) (p trend=0.03).

Comments from the CEF Panel:
The CEF Panel identified the following strengths and weaknesses in this study:
Strengths:
- Quality control, including blanks and quality assurance procedures
- Analytical method (LC-MS)

Weaknesses:
- Case-control study
- Potential selection bias (details on the selection of patients and controls not provided)
- Single exposure measurements
- Single spot urine BPA measurement
- Not adjusted urine samples
- No distinction between conjugated and unconjugated BPA
- Handling of values below LOD not reported
- Confounding by diet or by concurring exposure factors (medication) not reported
- Unclear clinical relevance

Overall the CEF Panel notes that the study by Duan and colleagues is very small and there are uncertainties about the selection of patients and controls. Additional confounding factors other than those considered, e.g. age, gender, BMI and HRT cannot be excluded. Some of their cases of meningioma had been treated therapeutically but no details are given. No detailed data about the concentrations of BPA measured in urine are provided and numbers are very small for comparisons of single measurement of BPA concentration in a random urine sample. The results of this small case-control do not significantly add to the information about factors involved in the development of meningioma, which is unlikely to be linked to chemical exposure.

No WoE analysis was carried out for the one human case-control study that was evaluated by the CEF Panel.

(ii) Animal studies

To note: in this section, strengths and weaknesses of studies published after 2010 and not previously reviewed by EFSA have been provided as "Comments from the CEF Panel" under each study. For studies reviewed in previous risk assessments of EFSA, the comments of the reviewing Panel (EFSA 2006; EFSA CEF Panel, 2010) have been provided in the format used at that time, without always specifically listing strengths and weaknesses. These are however included in Appendix B in summary form.

Acevedo N, Davis B, Schaeberle CM, Sonnenschein C and Soto AM, 2013. Perinatally Administered bisphenol A acts as a mammary gland carcinogen in rats. Environmental Health Perspectives, 121, 1040-1046.

BPA (0; 0.25; 2.5 or 250 µg/kg bw per day) was administered s.c. to Sprague-Dawley rats (dams N=9-12/dose/exposure period) via osmotic pumps prenatally (GD 9 – GD 23) and pre- and perinatally (GD 9 – PND 21). Cages, water bottles, and bedding tested negligible for estrogenicity by the E-SCREEN assay. Food was supplied ad libitum. Estrogenicity of the feed (Harlan Teklad 2018 Rodent Diet, Harlan Teklad, Indianapolis, IN) was measured at 8–15 fmol of estrogen equivalents per gram. Mammary glands from BPA-exposed offspring were examined at four time points for preneoplastic and neoplastic lesions. To assess circulating BPA levels, pregnant rats were exposed to vehicle or 250 µg BPA/kg bw per day during gestation only or during gestation/lactation and sera were analyzed from dams, fetuses, and nursing pups for total and unconjugated BPA. Serum was either treated with β-

glucuronidase/sulfatase to estimate the concentration of total BPA (conjugated plus unconjugated), or processed without enzymatic treatment to estimate the concentration of unconjugated BPA. Serum concentrations were quntified using on-line solid phase extraction coupled to high performance liquid chromatography–isotope dilution tandem mass spectrometry (LOD: 0.3 ng/mL; LOQ: 0.9 mg/mL).

The authors reported that total and unconjugated BPA were detected in sera from 100% of dams and fetuses and 33% of pups exposed to 250 µg BPA/kg bw per day. Mammary gland tissue was collected at PND 50 (N=5-6), PND 90, PND 140 and PND 200 (N=27-33). The incidences of ductal hyperplasias were assessed as described by Murray et al (2007). TEB, intraductal hyperplasias, atypical ductal hyperplasias and ductular CIS were identified. At PND50 some animals of the group exposed during gestation (except in the 25 BPA group) showed atypical ductal hyperplasia. Incidences of atypical ductal hyperplasias (ADH) were highest at the lowest BPA dose (0.25 µg/kg bw per day) after gestational exposure (no dose-response relationship), whereas the 0.25 BPA group exposed during gestation and lactation did not develop such lesion. Incidences of proliferative lesions and tumours did not increase statistically significantly in the treated offspring (n=23-35) at PND 90, 140 or 200 following gestational or gestational + lactational exposure. However, single adenocarcinoma were observed in most groups, except in controls. One adenocarcinoma was already observed at PND 90 in the 2.5 BPA group.

Comments from the CEF Panel:
The CEF Panel identified the following strengths and/or weaknesses in this study:
Strengths:

- Number of animals per group at PND 90, 140 and 200
- Number of doses (4)
- Phytoestrogen-content of the diet measured and low
- Cages, water bottles, and bedding tested and negligible for estrogenicity
- BPA exposure determination by LC-MS-MS (dams, fetuses and pups)

Weaknesses:

- Low number of rats/group at PND 50 when proliferation was to be assessed
- No quantitative proliferation measurement (only hystopathological examination)

The CEF Panel noted that a low number of rats showing ductal hyperplasia (1-3) and a very low number (1/5) demonstrated a ductular CIS at PND 50 or carcinomas at PND 90, 140 or 200.

Serum levels of unconjugated BPA in dams of the 250 BPA group were 1.25 ng/ml. This value is about 5 fold higher than serum levels after oral administration of BPA, i.e. 0.1 ng/ml in rats treated with 100 µg/kg bw (Doerge et al., 2010a; 2011b) and more than 2 fold higher than in mice and monkeys treated with 400 µg/kg bw (Taylor et al., 2011). Mean serum levels were 0.6 ng/ml in fetuses and < LOD in pups after additional lactational exposure in the present paper. The authors consider the levels of free BPA in the dams comparable to those in humans. However, Teeguarden et al. (2013) calculated the free BPA levels in human serum to be in the (sub)picomolar range. Thus there is a discrepancy of at least a factor of 1000.

This paper is included in the WoE table because of its relevance to one or more questions addressed there.

Ayyanan A, Laribi O, Schuepbach-Mallepell S, Schrick C, Gutierrez, M Tanos, T Lefebvre, G Rougemont, J Yalcin-Ozuysal O and Brisken C, 2011. Perinatal exposure to bisphenol A increases adult mammary gland progesterone response and cell number. Molecular Endocrinology 25, 1915-1923.

In this study designed to investigate whether exposure to low doses of BPA during pregnancy and lactation has the potential to alter mammary gland hormone response of female offspring later on in life, C57Bl/6 mice (breeding pairs) were administered BPA (prediluted in dimethylsulphoxide) in their

drinking water at doses ranging from 2.5 µg/L to 5000 µg/L. Based on selected measurement of drinking water intake this range corresponded to doses of 0.6, 3, 6, 12, 120, 600 and 1200 µg/kg/day. Diethylstilboestrol at doses of 0.12 or 1.2 µg/kg/day was used as a positive control (mode of dosing not given). The resultant female offspring (exposed in utero and postnatally through milk) were transferred to a BPA- and DES-free environment at weaning (day 24). For each BPA concentration, four different mothers were used to achieve a final n=18-20 female offspring for evaluation during the study. C57BL6/J mice were bred in a BPA-free environment using polysulfone cages and bottles, autoclaved water, and no paper towels.

Two inguinal mammary glands were taken from female offspring at post-natal day 30, one for microscopical analysis of a whole mount preparation, the other for measurement of mRNA expression of progesterone receptor, amphiregulin and SLP1. One offspring from each of four mothers was examined in each dose group, in triplicate. Total mammary epithelial cell numbers were measured using a cell counter from pooled mammary tissue from mice at 3 months.

The authors reported that intakes of 3, 120 or 1200_µg BPA/kg bw per day BPA resulted in dose-dependent increases in PR and SLPI mRNA expression, statistically significant and comparable in magnitude to DES (0.12 µg/kg bw per day) only in the offspring of mothers exposed to 1200 µg BPA/kg bw per day. Neither BPA nor DES affected mRNA expression, while amphiregulin mRNA expression showed a non-significant trend toward a nonmonotonic response. A significant increase in terminal end buds (TEB) was measured in the 3 µg BPA/kg bw per day offspring only, with some evidence of a non-monotonic dose-response over all BPA groups. Total mammary cell numbers were significantly increased (approximately 50% higher) compared with controls in the offspring of mothers receiving both low doses of BPA (6 or 12 µg/kg bw per day) and high doses (600 or/1200 µg/kg bw per day). Mammary glands from the offspring of DES-exposed females (1.2 µg/kg bw per day) showed a 70% increase in cell number. Finally PR-positive cells within the luminal epithelial population were significantly increased in the offspring of mothers receiving 6 µg BPA/kg bw per day, as well as mRNA expression of Wnt-4, but not RANKL.

Overall, in this complex experiment these authors showed small statistically significant increases in the mammary terminal end buds in BPA-treated mice, together with increases in mammary cell numbers and the mRNA encoding mediators implicated in control of cell proliferation. The changes were reported to be analogous to those seen in the DES-treated mice.

The authors concluded that perinatal exposure to low doses of BPA can have long-term, measurable biological effects on the mouse mammary gland of the offspring, which could facilitate the development of breast neoplasia in later life. The authors state that specific groups of mice, followed up for over a year did not develop palpable mammary tumours, indicating that BPA exposure is not sufficient to cause mammary carcinomas, but no further details are provided in the publication.

Comments from the CEF Panel:
The CEF Panel identified the following strengths and/or weaknesses in this study:
Strengths:

- Large sample size
- Adequate positive control included (DES)
- Environment BPA free from weaning onwards

Weaknesses

- Insufficient data reporting (e.g. DES administration)
- Study design not appropriate to the scope (low number of animals tested for histological examination)
- Insufficient study reporting (mode of dosing for diethylstilboestrol not given)
- Individual drinking water consumption not measured (doses calculated on average body weight and water consumption)

The CEF Panel noted that offspring were killed at ages when reproductive cycling occurs and this was not assessed or controlled. Cycling can be variable dependant on a number of factors including housing conditions, whether housed singly or in groups and this was also not detailed in the methods. This could significantly influence outcomes. Microscopic analysis was performed on a very limited number of glands in relatively few animals. Whilst the neonatal mouse model may show developmental alterations to mammary gland growth with oestrogenic agents (Bern et al., 1987), the relevance of the findings for the assessment of mammary cancer risk in humans is unclear. The actual exposure to BPA was not measured but only estimated from the average water intake and average body weight, and the mode of administration of diethylstilboestrol not given so it is difficult to compare BPA treated groups with those of the positive control.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Betancourt AM, Eltoum IA, Desmond RA, Russo J and Lamartiniere CA, 2010. *In utero* Exposure to Bisphenol A Shifts the Window of Susceptibility for Mammary Carcinogenesis in the Rat. Environmental Health Perspectives, 118, 1614-1619.

Betancourt et al. (2010) also used the model of DMBA-induced mammary carcinogenesis, administering BPA *in utero* by gavaging pregnant Sprague-Dawley rats with 0, 25 or 250 µg BPA/ kg b.w./day (GD 10-21). Female offspring of BPA treated dams did not differ from controls with respect to body weight development, vaginal opening and on PND 50, serum concentration of 17ß-oestradiol, progesterone as well as estrus cyclicity. On PND 50, expression of oestrogen receptor (ER)-α, PR-A and bcl-2 was reduced. On PND 100, ER-α and bcl-2 were upregulated, PR-A was at similar levels to controls. As to SRC-1,-2 and -3, only SRC-3 was increased on PND 50, but all members of the SRC-family were up-regulated on PND 100. Cell proliferation (n=6/group) and apoptosis (n=5/group) were measured in the mammary gland of the offspring of controls and the high BPA-dose group on PND100 (before DMBA treatment). Upon prenatal BPA exposure, proliferation of epithelial cells was increased but apoptosis was not affected. Consistent with increased cell proliferation expression of the following proteins was increased in the high dose group at PND100: EGFR, phosphorylated -IGF-1R, phosphorylated –c-Raf, phosphorylated pERKs 1/2, phosphorylated ErbB2, phosphorylated Akt. For tumourigenesis studies, one female offspring per litter was given a single gavage of 30 mg DMBA/kg b.w. on PND 50 (31, 29 and 33 rats in the control, low- and high-dose groups, respectively) or on PND 100 (30 and 28 rats in the control, and high-dose groups, respectively). Offspring were palpated twice weekly to monitor tumour development and underwent necropsy at 12 month of age or when tumour burden exceeded 10% of body weight. DMBA administration at PND50 of rats which received prenatal BPA-treatment (both 25 and 250 µg/kg bw) did not result in an increase in the number of tumours per animal (2.94 ± 0.48, 2.38 ± 0.42, and 2.88 ± 0.4 for control, low and high BPA groups, respectively). The tumour latency was not reduced (109 ± 11, 116 ± 14, 106 ± 14 days for control, low and high BPA groups, respectively). DMBA administration at PND 100 caused a significant increase in tumour (benign and malignant combined) incidence (53 to 83%) along with a non-significant increase in tumour multiplicity (1.96 ± 0.53 to 2.53 ± 0.55). The latency period was reduced from 267 to 189.5 days. Finally, a significant greater proportion of DMBA-induced tumours classified as grade II (Bloom-Richardson system for human breast tumours; control: 3 of 13 tumours (23%); BPA high dose group: 9 of 20 tumours (45%)) was observed. The authors concluded that the high BPA dose (250 µg BPA/kg b.w.) enhanced cell proliferation in mammary glands of the offspring, associated with an increased cancer susceptibility and shift of the window for susceptibility for DMBA-induced tumourigenesis in rat mammary gland from PND 50 to PND 100. Cell proliferation was increased in the epithelial cells of mammary tubular ducts of 100 day old rats prenatally exposed to 250 µg BPA (not 25 µg BPA) compared to controls ($p<0.05$). Apoptosis in these rats was not altered.

Comments from the CEF Panel:
The CEF Panel identified the following strengths and weaknesses in this study:

Strengths

- Large sample size (not applicable to proliferation)
- Oral administration by gavage
- Use of non-PC cages and of non plastic bottles

Weaknesses

- Low sample size for proliferation assessement
- Only one dose-level tested for cell proliferation
- Insufficient study reporting (e.g timing of necrospy) (not relevant for proliferation)
- Animal diet and phytoestrogen content not reported

The study revealed similar shortcomings in design and reporting as the study by Jenkins et al. (2009), i.e. measurement uncertainties involved in tumour data collection by palpation, time of necropsy of individual animals not exactly reported but given as "at 12 months of age or when tumour burden exceeded 10% of body weight". The CEF Panel further noted that only a single tumour from each animal was randomly selected for histopathological analysis and concluded that thus not all tumours were histologically characterised and graded according to the Bloom Richardson grading system, which is rather unusual. The CEF Panel concluded that these data can be used as supporting evidence of the induction of proliferation by BPA.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Durando M, Kass L, Piva J, Sonnenschein C, Soto AM, Luque EH and Munoz-de-Toro M, 2007. Prenatal bisphenol A exposure induces preneoplastic lesions in the mammary gland in Wistar rats. Environmental Health Perspectives, 115, 80-86.

Pregnant Wistar rats were exposed to 25 µg BPA/kg bw per day or to DMSO (vehicle control) from GD 8 (corresponding to the beginning of organogenesis in the fetus) by s.c. implantation of Alza Osmotic pumps. Both BPA and DMSO were released continuously for 14 days (GD 8 up to GD 23). Offspring were delivered on GD 23 and weaned from their mothers on PND 21. According to the design of the study, exposure to BPA was only prenatally and not during lactation. Animals were sacrificed at PND 30 and PND 50 and at adulthood (PND 110 and PND 180). In a separate experiment, female offspring from the DMSO group received at PND50 a single i.p. dose of either 25 mg N-nitroso-N-methylurea (NMU) or 50 mg/kg bw NMU, and female offspring from the 25BPA group received 25 mg NMU. This resulted in 3 groups: DMSO + 25 mg/kg MNU (n=16); DMSO + 50 mg/kg MNU (n=10) and 25 BPA + 25 mg/kg MNU (n=21). Cell proliferation was determined by i.p. injection of all rats with BrdU (6 mg/100 g bw). Apoptosis was determined by TUNEL technique. At PND 50, but not at PND 30, cell proliferation was increased and apoptosis decreased in mammary gland epithelium. At PND 110 and 180, a significant increase in hyperplastic ducts in rats in utero exposed to BPA was observed in comparison to DMSO-treated controls. Tumour incidence after NMU administration at PND180 was 0% for the DMSO 25 MNU group and 83% for the DMSO 50 MNU group. Females treated in utero with DMSO and at PND50 with 25 mg/kg MNU showed no changes in number of hyperplastic ducts at PND 110, whereas at PND 180 a significant increase was found. Furthermore, in rats in utero treated with BPA, the 25NMU dose at PND 50 caused a significant increase in hyperplastic ducts at PND180, but not at PND 110. Moreover 2/15 of the BPA NMU group developed cribiform CIS. Rats treated with DMSO and 50 mg/kg NMU developed invasive adenocarcinomas in 7/10 animals. The authors concluded that prenatal exposure to BPA increases the sensitivity to endogenous estrogen. At PND 180, BPA exposed rats treated with 25 mg NMU at PND 50 exhibited a significantly higher number of ductal hyperplasias compared to animals not exposed to BPA and treated with NMU. Based on this observation the authors suggested that in utero BPA exposure increased the susceptibility of the mammary gland to develop preneoplastic and neoplastic lesions as a response to NMU exposure. The authors further concluded that the results obtained with this widely accepted surrogate model of human breast carcinogenesis, strengthen the

arguments linking the increased incidence of endocrine-dependent human tumours, to in utero exposure to minimal doses of xenoestrogens such as BPA.

Comments from the CEF Panel:
The CEF Panel identified the following strengths and weaknesses in this study:
Strengths:

- Use of non-PC cages and of non plastic bottles

Weaknesses:

- Single dose level study
- Animal diet and phytoestrogen content not measured
- Low No of animals tested for histological examination

The CEF Panel noted that that only one dose of BPA is used and, moreover, the exact dose to which the offspring was exposed during gestation was not determined. The CEF Panel also noted that it is unclear whether the animal model used is relevant for the human situation, taking the differences in toxicokinetics of BPA in experimental animals and humans into consideration. (see also the serum levels in BPA exposed monkeys in the study of Tharp). The CEF Panel agreed with the conclusion of the authors that rats which are in utero exposed to BPA exhibited an increase in cell proliferation and a decrease in apoptosis in mammary gland epithelium at PND 50, which in the present study lead to an increased number of hyperplastic ducts at PND110 and PND180. Therefore the CEF Panel concluded that the results of this study can be used as supportive evidence of the induction of proliferation by BPA.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Jenkins S, Raghuraman N, Eltoum I, Carpenter M, Russo J and Lamartiniere CA 2009. Oral exposure to bisphenol A increases dimethylbenzanthracene-induced mammary cancer in rats. Environmental Health Perspectives, 117, 910-915.

In a study examining the effect of lactational exposure to BPA on dimethylbenzanthracene (DMBA)-induced mammary cancer in female offspring, Jenkins et al. (2009) gavaged nursing Sprague-Dawley rats with BPA (0, 25 or 250 µg/kg b.w./day) from lactation day 2 to 20. All female offspring (5-8 per litter) and enough males were retained to yield 10 offspring/litter. Cell proliferation and apoptosis were measured in the mammary gland of the female offspring (n=5/group) at 21 days of age (at end of BPA treatment) and at 50 days of age (before DMBA exposure). Increased cell proliferation and reduced apoptosis in the mammary gland of female offspring were observed in the high dose group at 50 days of age but not at 21 days of age. Consistent with increased proliferation and reduced apoptosis, expression of the following proteins was increased in the high dose group: Akt and phosphorylated Akt (pAkt; proteins linked with apoptosis), progesterone receptor (PR)-A, steroid receptor activator (SRC) 1 to 3, and erbB3. The expression of the oestrogen receptor (ER)-α was slightly reduced. At 50 days of age, one female offspring from each litter of each treatment group was given a single dose of DMBA (30 mg/kg b.w.) by gavage which was expected to result in a low number of mammary adenocarcinomas. In total, 32, 34, and 24 female offspring in the control (no BPA during lactation), low and high BPA group, respectively, received DMBA. Offspring were palpated twice weekly to monitor tumour development and underwent necropsy at 12 month of age or when tumour burden exceeded 10% of body weight. BPA-treatment increased the number of tumours (not further specified between adenoma and carcinoma) per animal (2.84 ± 0.31, 3.82 ± 0.43, and 5.00 ± 0.88 for control, low and high BPA groups, respectively) with the effect at the high dose group being statistically significant. The authors reported that there was "no change in the carcinomas score". Tumour latency was also reduced (65, 53, 56.5 days for control, low and high BPA groups, respectively) with statistically significance at the high dose group.

Comments from the CEF Panel:

The CEF Panel noted the following: (a) the toxicokinetic studies showed that only minimal fraction of BPA administered to dams is transferred to breast milk. Therefore, the exposure of the pups to BPA under this condition is anticipated to be very low, however, information on internal BPA levels is lacking. (b) The score of carcinoma formation which would indicate tumour progession is not changed. (c) When considering the results of the study on tumour latency, the measurement uncertainties involved in the data collection (palpation) should be taken into account. In conclusion, the shortcomings in the study design, and the absence of a significant dose: response, the uncertainty regarding the exposure of the offspring to BPA, and the limitations in reporting preclude these results to be used for risk assessment of BPA and the re-evaluation of the existing TDI. However, the CEF Panel noted that a dose-related response of BPA on cell proliferation and apoptosis in the mammary gland was reported in the study and this deserves further considerations. Consistent finding: cell proliferation was increased and apoptosis decreased by BPA at PND 50 but not after PND 21 days. The CEF Panel concluded that the present study cannot be used for the assessment of cancer risk but as supporting evidence of the induction of proliferation by BPA following lactational as well as in utero exposure.

The CEF Panel identified the following strengths and weaknesses in this study:
Strengths:

- Oral administration by gavage
- Phytoestrogen-free diet
- Use of non-PC cages and of non plastic bottles

Weaknesses:

- Only one dose level tested for proliferation measurement
- Low No of animals tested for histological examination

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Jenkins S, Wang J, Eltoum I, Desmond R and Lamartiniere CA, 2011. Chronic oral exposure to bisphenol A results in a nonmonotonic dose-response in mammary carcinogenesis and metastasis in MMTV-erbB2 mice. Environmental Health Perspectives, 119, 1604-1609.,

The effect of chronic, oral exposure to BPA during adulthood was investigated on the development of mammary tumours in a transgenic mouse model which spontaneously develops tumours through over-expression of wild type erbB2 (MMTV-erbB2). Female MMTV-erbB2 mice (n= 36-76, control n=94) were administered BPA at levels of 0, 2.5, 25, 250, 2500 µg BPA/L in drinking water, from PND 56 until PND 112 (for mechanism of action) or PND 252 (for tumorigenesis). The authors roughly estimated that the intakes of BPA were 0.5, 5, 50 or 500 µg/kg bw per day, in the absence of actual consumption data, based on their pilot data that showed that 20 g mice drink 4 ml of water per day. Drinking water of all groups, including the control group, contained 0.05% by volume of the vehicle, ethanol. A positive (oestrogenic) control was not included. Animals were fed AIN-76A diet (phytoestrogen-free; Dyets, Inc., Bethlehem, PA), housed in polypropylene cages, and provided glass water bottles.

To assess tumour development, mice were killed at 252 days of age or before when tumours exceeded 10% of body weight. The tumour endpoints used in the study were number of tumours/animal and tumour volume/animal. Tumours were assessed histologically and lung metastases were also counted in a blinded manner. The authors assessed tumour volume by measurement of tumour dimensions. Selected mice were sacrificed at 112 days of age to measure mammary cell proliferation, apoptosis and protein expression (n = 5-17 mice/treatment) of a number of growth factor-related proteins in mammary epithelial cells.

The authors reported a statistically significant increase in numbers of tumours/mouse (multiplicity) and also in the percentage of mice with lung metastases at estimated intakes of 0.5 or 5 µg BPA/kg bw

per day. In addition latency time to first tumour was also reduced at both dose levels and tumour volume was significantly increased in mice receiving 5 µg BPA/kg bw per day. No such effects were reported at the higher dose levels. The evaluation of histopathology showed no difference in tumour differentiation. Cell proliferation was stimulated at intakes of 5 µg BPA/kg bw per day and above, while a significant increase in apoptosis was reported at the highest dose level of 500 µg/kg bw per day only. The ratio of cell proliferation index to apoptotic index was significantly increased at the 5 µg BPA/kg bw per day dose level only. At the molecular level, doses of 5 µg BPA but not 500 µg BPA/kg bw per day were reported to increase phosphorylation of erbB2, erbB3, insulin-like growth factor 1 receptor, and Akt in the mammary gland. The authors concluded that oral administration of BPA accelerated mammary cancer development and progression in the mouse in a non-monotonic fashion and that the ratio of cell proliferation and apoptosis indices and alterations in protein expression were predictive of the potential of varying doses of BPA to alter tumorigenesis in the experimental model.

Comments from the CEF Panel:
The CEF Panel identified the following strengths and/or weaknesses in this study:
Strengths:

- Adequate number animals/group
- Number of doses (4)
- Slides were blind-evaluated
- Phytoestrogen-free diet
- Use of non-PC cages and of non plastic bottles

Weaknesses:

- Low number of animals for some dose groups
- Insufficient data reporting (e.g. data on tumour incidence and histopathology incomplete)
- Exposure via drinking water: exact doses received are not therefore known

The CEF Panel additionally noted several uncertainties, outlined as follows. The transgenic model is reported to develop mammary tumours in 50% of animals at 205 days of age and over 70% of all tumour bearing mice develop lung metastases if they survive to 240 days of age (data from Jackson Laboratories). The authors did not include a positive control group, and used non-standard measurements of tumour endpoints not usually used in animal cancer studies. In a model with such rapidly growing tumours tumour volume, tumour numbers and metastases are highly variable and difficult to measure because individual tumours rapidly become confluent. Measurement of volume of tumours embedded within the mammary fat pad is also subject to significant error. The exact number of animals that developed tumours and those that remained tumour free is not given and an analysis that took into account the different times at which the animals were killed was not conducted. Thus the small differences seen may simply be the result of usual variability in this model, albeit that the authors reported a non-monotonic dose-response for tumour induction, particularly as they gave no indication of how they randomised entry of mice into the study, crucial when animals are obtained from small colonies. According to the authors, the evaluation of histopathology showed no difference in tumour grade (stage of differentiation). No mention is made of hyperplasia and pre-neoplastic (dysplastic) mammary changes that are important in any histopathological evaluation of neoplasia. Interestingly the results of cell proliferation and apoptosis assays showed a simple dose-response with higher doses showing greater responses particularly of apoptosis, quite different from the reported tumour data. These increased responses were not however statistically significant, other than the apoptotic response at the highest dose, while the increased proliferative responses showed a plateau. The relevance of these findings for mammary cancer development is uncertain.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Jones LP, Sampson A, Hang HJ, Kim HJ, Yi YW, Babus JK, Wang A and Bae I, 2010: Loss of BRCA1 leads to an increased sensitivity to bisphenol A. Toxicology Letters, 199(3), 261-268.

This study used a mouse model of breast cancer susceptibility gene 1 (BRCA1) related mammary cancer as well as MCF7 cells with the BRCA1 gene knocked down by interfering RNA. The aim was to study whether loss of BRCA1 function in mammary epithelium would enhance BPA-mediated cell proliferation and whether the effects were mediated through the ERα signalling pathway. Three month old Brca1 knockout mice maintained on a C57Bl/6 genetic background were used along with non-transgenic C57Bl/6 controls. Mice were implanted with osmotic pumps to deliver either 50% dimethyl sulphoxide vehicle or 250 ng BPA/kg/day dissolved in vehicle for four weeks at a flow rate of 0.22 µL/h. There were 13 mice/group, although the number of wild type mice was not clarified. Mammary glands were processed for whole mount analysis and histology and immunohistochemistry for cell proliferation (PCNA). The proliferative index was determined on one section from each mouse counting a total of 1000 cells. Seven mice/group were used for this assessment. The authors also treated MCF7cells with loss of BRCA1 function with 1 µM of BPA for 0, 1, 2, 3 days or various concentrations (0, 10, 100, or 1000 nM) of BPA for 72 h. Cell proliferation with or without tamoxifen or ICI182780 and ERα target gene expression was studied in these cells.The results suggested that exposure to BPA in vitro enhanced proliferation in cells with loss of BRCA1 in a dose-and time-dependent manner more that in cells without BRCA1 loss and this was linked to ERα signalling. Additionally, BPA exposure in vivo at 250 ng/kg increased mammary epithelial cell proliferation and hyperplasia in adult Brca1 knockout mouse mammary glands more than in wild type mice.

Comments from the CEF Panel:
The relevance to cancer development of these differences in proliferation between BPA and vehicle treated cells and mammary glands in these mice are unclear. Whilst BPA led to increased growth response in MCF7 cells with loss of BRCA1 and mammary epithelium in mice with a deficiency in Brca1, both ordinary MCF7 cells and wild type mice were also affected. Whilst mechanistically interesting, the data do not add significantly to the assessment of the carcinogenic potential of BPA based on the NTP two year studies.

The CEF Panel identified the following strengths and weaknesses in this study:
Strengths:
- Use of phytoestrogen-free diet and of non plastic bottles

Weaknesses:
- Low number of animal/group for proliferation
- Single dose level study
- Type of cages not reported

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Kass L, Altamirano GA, Bosquiazzo VL, Luque EH, and Munoz-de-Toro M, 2012: Perinatal exposure to xenoestrogens impairs mammary gland differentiation and modifies milk composition in Wistar rats. Reproductive Toxicology, 33, 390-400

This study was aimed at evaluating the effects of perinatal (gestation + lactation) exposure to BPA or DES on F1 mammary gland differentiation. Pregnant Wistar rats were given BPA or DES in drinking water from gestational day 9 through to weaning. The concentration of BPA in drinking water was 2.5 µg/L or 250 µg/L or 25 µg DES/L, corresponding to theoretical doses of 0.5 µg BPA/kg bw per day, 50 µg BPA/kg bw per day or 5 µg diethylstilboestrol/kg bw per day, respectively. The control group was exposed to a vehicle solution (0.001% ethanol in water). 10-12 dams/group were used and litters of eight F1 pups (four males and four females) were left with F0 lactating mothers until weaning on LD21, when the female offspring (exposed in utero and postnatally through milk) were transferred to a BPA- and DES-free environment. Randomly chosen 90-day-old F1 females from each BPA group were then bred to non-BPA-exposed males, and after pregnancy confirmation, one F1 female per litter from each treatment group was assigned to a particular experimental time point group (GD18, GD21

and lactation day 14). Reproductive performance parameters of the F1 dams was assessed, mammary gland samples were taken on GD 18 and GD 21, and during lactation, milk yield and milk protein composition were assessed. Blood was also taken at these time intervals for hormone analysis. The mammary glands of mated offspring were investigated on gestational days 18 or 21. Conventional histology and immunohistochemistry for levels of progesterone receptor (PR), oestrogen receptor α and β and phosphorylated Stat5a/b (pStat5a/b) were used as well as assessment of lactation, milk yield, milk protein composition. The authors reported a decrease in α-lactalbumin and β-casein levels in milk, accompanied by reduced prolactin receptor and Stat5a/b expression on gestational day 18. On gestational day 21, slightly delayed histological mammary gland differentiation was reported in both BPA and diethylstilboestrol-treated groups compared with controls. No effect of BPA was observed on any of the reproductive parameters investigated in the female offspring, including numbers of corpora lutea, implantation sites and resorption sites.

Comments from the CEF Panel:
The CEF Panel concluded that this study displays many potential experimental variables that render the result difficult to interpret. The actual doses of BPA are uncertain for there was no monitoring of water intake or serum BPA levels. A subjective method was used to histologically examine single mammary glands from each animal. This is very limited as mammary glands in rodents are very dispersed in the abdominal and thoracic fat and show a variable distribution. The illustrations provided are unconvincing of a significant effect because all appear to show histology that is within the limits to be expected in normal lactating rats. Moreover, the relevance of any putative changes in lactating glands for cancer assessment is unclear. In the view of the CEF Panel, the data do not in themselves lend support to an effect of treatment of mammary gland differentiation and any implications for assessment of the carcinogenic potential of BPA.

Markey CM, Luque EH, de Toro MM, Sonnenschein C and Soto AM, 2001. In utero exposure to bisphenol a alters the development and tissue organization of the mouse mammary gland. Biology of Reproduction, 65, 1215-1223.

Munoz-de-Toro M, Markey CM, Wadia PR, Luque EH, Rubin BS, Sonnenschein C and Soto AM, 2005. Perinatal exposure to bisphenol-A alters peripubertal mammary gland development in mice. Endocrinology, 146, 4138-4147.

As reported in EFSA, 2006, in a series of publications from Markey, Munoz-de-Toro et al. the effects of perinatal exposure to BPA (25 and 250 ng BPA/kg bw per day, but initially reported as 25 and 250 ug/kg bw per day), administered sc by Alzet mini-pumps from day 9 of pregnancy for 14 days through postnatal day 4, n = 6 -10 per group) on the peripubertal development of the mammary gland, the genital tract and on brain sexual differentiation was investigated in CD-1 mice

Markey et al. (2001) reported that mammary glands of BPA-exposed mice compared with controls showed differences in the rate of ductal migration into the stroma at 1 month of age and a significant increase in the percentage of ducts, terminal ducts, terminal end buds, and alveolar buds at 6 mo of age. The percentage of cells that incorporated BrdU was significantly decreased within the epithelium at 10 days of age and increased within the stroma at 6 months of age. The response was very similar at 25 and 250 µg/kg bw per day, with 25 µg/kg bw per day showing a slightly greater response.

Strenghts:
- Cages and bedding tested negative for estrogenicity
- Use of use of phytoestrogen-free diet

Weaknesses:
- Low number of animals/group for proliferation

In **Munoz-de-Toro et al. (2005)**, it was reported that BPA exposure enhanced the mammary gland sensitivity to oestradiol in ovariectomized CD-1 mice. At 30 d of age in intact mice, there was a

significant increase in the number of TEBs relative to the area occupied by the ductal tree in the animals exposed to 250 ng BPA/kg bw·d, compared with that in the vehicle-treated controls (P = 0.008), whereas the increase in the 25 ng BPA/kg bw·d approached significance (P = 0.054). Similarly, when these data were expressed as TEB area relative to ductal tree area, a significant increase was observed at 250 ng BPA/kg bw·d with respect to the vehicle-treated control ($P < 0.05$). a significant decline in the number of apoptotic cells in TEBs of both treated groups (25 ng BPA/kg bw·d, $P < 0.001$; 250 ng BPA/kg bw·d, $P < 0.05$) was seen relative to the controls.There was a positive correlation between ductal length and the age at first proestrus. The age at first proestrus was reduced by BPA. A significant increase of progesterone receptor-positive ductal epithelial cells localised in clusters was also reported in BPA-treated animals. Lateral branching was significantly enhanced at 4 months of age in mice exposed to 25 ng BPA/kg bw per day. A decreased wet weight of the vagina, small increases in the incorporation of bromodeoxyuridine into the DNA of endometrial gland epithelial cells, and increased expression of oestrogen receptor-alpha (ERalpha) and progesterone receptor in the luminal epithelium of the endometrium and subepithelial stroma were reported in BPA-exposed animals. Changes were in the range of 20 to 40 % of control values, but positive controls and controls without Alzet pumps were not included and the selection of animals for assessment was based on ooestrous cyclicity. Apparently, results on other parameters determined in this animal study were reported separately.

Comments from the AFC Panel (EFSA, 2006):
The AFC Panel noted absence of dose-response for many changes reported or the evaluation of samples for only one dose level.

Strenghts:
- Food tested negative for estrogenicity
- Cages and bedding tested negative for estrogenicity

Weaknesses:
- Low/unclear number of animals for the dose-groups

These studies are included in the WoE table because of their relevance to one or more review questions addressed there.

Moral R, Wang R, Russo IH, Lamartiniere CA, Pereira J and Russo J, 2008. Effect of prenatal exposure to the endocrine disruptor bisphenol A on mammary gland morphology and gene expression signature. Journal of Endocrinology 196, 101–112.

In this study pregnant Sprague-Dawley rats were gavaged with 25 µg BPA/kg bw or 250 µg BPA/kg bw on days 10-21 post conception. Controls were given sesame oil vehicle only and there were 10 animals per group. The 4th pair of mammary glands from the offspring (8-10/group) was assessed for morphological changes in whole mount preparations and for cell proliferation in sections at days 21, 35, 50 and 100. Frozen mammary tissue was pooled from controls and each treatment group for gene expression analysis using microarrays and real-time (RT)-PCR.

High-dose BPA exposure was reported to induce small architectural modifications in the mammary glands, mainly in the number of undifferentiated epithelial structures but the proliferative index (as determined by BrdU) was not affected. Low and high doses of BPA were reported to alter the gene expression profile of mammary tissue but in a somewhat inconsistent manner: low dose had the highest effect by 50 days, while high dose had the most influence on gene expression by 100 days. At the low dose, up-regulated genes were related to the immune system and at the high dose, genes related to differentiation were upregulated.

Comments from the CEF Panel:
The CEF Panel identified the following strengths and weaknesses in this study:

Strengths:

- Large sample size
- Oral administration by gavage
- Phytoestrogen-free diet

Weaknesses:

- Types of cages and drinking bottles not reported
- Time-points for BrdU is not indicated

As with other studies of this type it is difficult to assess the relevance of these observations. The differences reported were small, and there was neither control of dosing nor evidence of actual exposure achieved. The authors did not report any precautions to avoid BPA exposure from containers or other environmental sources. However, the study results were used for the evaluation of proliferation induced by BPA.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Murray TJ, Maffini MV, Ucci AA, Sonnenschein C and Soto AM, 2007. Induction of mammary gland ductal hyperplasias and carcinoma in situ following fetal bisphenol A exposure. Reproductive Toxicology 23, 383–390.

As reported in the EU RAR (2008), "Murray *et al.* (2007) examined the effect of prenatal BPA exposure on in situ induction of mammary tumours in rats. From GD 9 (GD 1 = day of vaginal sperm) through PND 1 (PND 0 = day of birth) Wistar-Furth rat dams received subcutaneous osmotic pumps of 0, 0.0025, 0.025, 0.250, or 1 mg/kg bw per day BPA. Number of dams treated was not reported. Based on a limited amount of information provided on the number of offspring examined, it appears that ≤ 6 dams/group were treated. Pup viability was assessed on PND 1. On PND 2 pups were sexed and litters were culled to 8 pups. Anogenital distance was measured on PND 4. Litters were weighed during the lactation period. Female offspring were monitored for body weight and vaginal opening in the post-weaning period. Female offspring were killed on PND 50 or PND 95. Mammary glands were collected and whole-mounted or sectioned for histopathological examination. Morphometric analyses were conducted to examine possible presence of preneoplastic lesions. Mammary glands were examined for ER-α and Ki-67 protein by an immunohistochemistry technique. One female/litter was included in the histological examinations. Apparently, ≤6 offspring/group were examined histopathologically. The number of offspring examined for the other endpoints was not reported. It was not clear if dams or offspring were considered the statistical unit. BPA exposure did not affect offspring viability, sex ratio, age at vaginal opening, or female anogenital distance. Anogenital distance was reduced on PND 4 in males from the 0.250 mg/kg bw per day group. Cribriform structures classified as carcinomas-in-situ were observed in the 0.25 and 1 mg/kg bw per day groups. The incidence of these structures in the controls and lower dose groups were not reported".

Comments from the Panel (current CEF Panel, EFSA, 2014):

The CEF Panel noted that although the study authors classified the cribriform structures as carcinoma in situ because of their hallmarks, it is difficult to establish whether or not these histopathological findings are clear neoplastic lesions of the mammary gland. The study authors concluded that fetal BPA exposure at dose levels of 0.250 and 1 mg/kg bw per day in rats is able to induce development of preneoplastic and neoplastic mammary lesions. The CEF Panel does not agree with this conclusion. Putative preneoplastic lesions have been observed but no neoplastic lesions. Moreover, due to the small sample size, lack of clarity on the statistical analysis, absence of a dose-response relationship and uncertainty about the incidence of the cribriform-like lesions in the controls it is difficult to establish whether the effects reported were due to chance or were real treatment-related effects. In addition, because of the uncertainty about the significance of the cribriform structures, it is unclear whether real neoplasia actually occurred. The incidence of hyperplastic ducts was increased in all dose groups at PND 50 (at PND 95 the incidence of hyperplastic ducts was overall lower than at PND 50, only the incidence of hyperplastic ducts in the 2.5 BPA group was significantly higher than controls

p=0.038); the study authors noted that the effect at PND 50 was quantitatively similar in all dose groups (i.e. 3–4-fold increase). Notwithstanding the lack of dose-response relationship the CEF Panel concluded that the results of this study can be used as supporting evidence of the induction of proliferation by BPA.

The CEF Panel identified the following methodological strengths and weaknesses in this study:
Strengths:

- Number of doses (4)
- Phytoestrogen-free diet
- Use of non-PC cages and of non plastic bottles

Weaknesses:

- Insufficient study reporting (No of animals)
- Inappropriate statistics (lack of clarity)

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Nanjappa MK, Simon L, and Akingbemi BT, 2012. The industrial chemical bisphenol a (BPA) interferes with proliferative activity and development of steroidogenic capacity in rat Leydig cells. Biology of Reproduction, 86(5): 135, 1-12

This is a study in which pregnant and lactating Long-Evans rats were given BPA in olive oil vehicle via gavage at 2.5 and 25 µg/kg bw per day from gestational day 12 to postpartum day 21, followed by examination of the testicular Leydig cells of the male offspring. Although no exposure measurements were performed the authors estimated based on previous data that maternal exposures to BPA at 2.5 and 25 µg/kg body weight represent BPA doses to the offspring of about 8 and 80 pg/kg body weight. Proliferative activity of Leydig cells was assessed using [^{3}H]thymidine incorporation using progenitor Leydig cells isolated from male rats at the end of BPA exposure. Leydig cell proliferation was also examined at 90 days in tissue sections from 3 to 5 rats in the model where rats are treated with the Leydig cell toxin ethane dimethylsulfonate.

Perinatal exposure to BPA did not affect litter size, birth weights of pups and pup sex ratio. Body weights, measured at 21, 35 and 90 days of age, were equivalent in BPA-exposed and control animals. Similarly, paired and relative testes weights (proportion to body weights) were not affected by BPA. However Leydig cell division was stimulated in the prepubertal period and increased Leydig cell numbers were shown in the testes of adult male rats at 90 days.

Comments from the CEF Panel:
The CEF Panel identified the following strengths and weaknesses in this study:
Strengths:

- Phytoestrogen free-diet
- Use of non-PC cages and of non plastic bottles

Weaknesses:

- Results interpretation (biological relevance debatable)

The CEF Panel noted that this rat strain is highly disposed to Leydig cell proliferation. Also particular caution is required when extrapolating findings in rat Leydig cells to humans. A detailed review of comparative physiology and pathology indicated that rats are quantitatively far more sensitive to the development of Leydig cell tumours than men as Leydig cell luteinizing hormone releasing hormone (gonadotropin-releasing hormone) receptors are unique to rats. Rats also have over 10 times more luteinizing hormone receptors than men (Cook et al. 1999). Thus this study is unlikely to have any relevance for assessment of the carcinogenic potential of BPA.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Nikaido Y, Yoshizawa K, Danbara N, Tsujita-Kyutoku M, Yuri, T, Uehara N and Tsubura A. 2004. Effects of maternal xenoestrogen exposure on development of the reproductive tract and mammary gland in female CD-1 mouse offspring. Reproductive Toxicology, 18, 803-11.

Nikaido Y, Danbara N, Tsujita-Kyutoku M, Yuri T, Uehara N and Tsubura A, 2005. Effects of prepubertal exposure to xenoestrogen on development of estrogen target organs in female CD-1 mice. In Vivo, 19, 487-494.

As reported in EFSA, 2006,"Nikaido *et al.* (2004) compared the effects of genistein (GEN), resveratrol (RES), zearalenone (ZEA), BPA and diethylstilboestrol (DES) on reproductive and mammary gland development in female CD-1 mice. Beginning on GD 15, pregnant mice (n = 6) were administered 0.5 or 10 mg/kg/day GEN, RES, ZEA or BPA, and 0.5 or 10 microg/kg/day of DES by daily subcutaneous injection for four consecutive days. Vaginal opening was monitored, 6 animals per group of offspring were autopsied at 4, 8, 12 and 16 weeks of age and ooestrous cyclicity was monitored from 9 to 11 weeks of age. Maternal exposure to BPA did not accelerate puberty onset or modify the ooestrous cycle. Mammary gland differentiation was accelerated in mice after BPA at 4 weeks of age, According to the publication, mice treated with GEN, RES, BPA or DES spent more time in diestrus; but BPA did not induce statistically significant changes. A publication from the same author apparently using the same protocol reported absence of BPA-effects on mammary gland and oestrous cycle when given as 4 daily subcutaneous injections (dose of 10 mg BPA/kg bw per day) to female CD-1 mice beginning at 15 days of age. (Nikaido *et al*., 2005)."

Comments from the AFC Panel (2006):
The AFC Panel noted that a statistical evaluation of the BPA effects on the mammary gland was not performed, and that conflicting results were obtained in the two studies

These studies are included in the WoE table because of their relevance to one or more review questions addressed there.

Prins GS, Ye SH, Birch L, Ho SM and Kannan K, 2011. Serum bisphenol A pharmacokinetics and prostate neoplastic responses following oral and subcutaneous exposures in neonatal Sprague-Dawley rats. Reproductive Toxicology, 31, 1-9

This study reports the effects of subcutaneous injection or oral dosing of 10 μg BPA/kg bw on post-natal days 1, 3 and 5 on the development of prostate cancer in a rat model. In this model rats are given both testosterone and oestradiol-17β (by implants of Silastic capsules packed with both the hormones) for 16 weeks from postnatal day 90 to drive prostatic intra-epithelial neoplasia (PIN) lesions in the prostate lobes. BPA was dissolved in 95% ethanol and solubilized in α-tocopherol stripped corn oil at a final administered concentration of 10 μg/ml. Solutions were made and stored using glass containers and any plastic products used for the collection and storage of blood and sera samples were polypropylene. Serum BPA in PND3 rats was measured using HPLC–MS–MS. Unconjugated and total BPA at Cmax were 1.77 and 2.0 ng/ml, respectively following injection and 0.26 and 1.02 ng/ml, respectively following oral exposure. The AUC_{0-2} for unconjugated and total BPA was 4.1-fold and 1.8-fold greater, respectively, in s.c. vs. oral delivery. Twenty pregnant rats were used to obtain 180 male pups divided equally between treatment groups which allowed 15-25 males per sub group. At 28 weeks of age, the animals were killed and prostate glands were conventionally fixed and serially sectioned at four levels for each organ so that 12-16 sections analysed for prostatic intraepithelial neoplasia for each animal.

Care was taken to avoid contamination by using non-polycarbonate cagesand double-deionized water was supplied from glass bottles. Animals were fed ad libitum a soy-free, phytoestrogen-reduced diet. The author report that post-natal BPA treatment increased prostatic intraepithelial neoplasia (PIN)

equally in both subcutaneous and orally dosed animals compared to controls. Prostate glands from treated animals also had a higher incidence of inflammation than controls.

Comments from the CEF Panel:
The CEF Panel identified the following strengths and/or weaknesses in this study:
Strengths:

- Phytoestrogen-free diet
- Use of non-PC cages and of non plastic bottles
- BPA determination in animal samples

Weaknesses:

- A single dose level study
- Possible confounding (BPA exposure was followed by testosterone and oestradiol-17β)

The CEF Panel also noted that it would be uncommon for true neoplasia to develop within 28 weeks in rats treated with hormones or non-genotoxic chemicals. In a previous study of this model by Bosland and colleagues, early neoplastic change occurred much later - at least after one year (Bosland et al., 1995). Moreover, so called PIN is difficult to distinguish from reactive epithelial alterations. The photomicrographs of PIN reported in this study are unconvincing evidence of true neoplastic change as they do not show sufficient degree of cytological atypia. The rat prostate normally shows variable cytological patterns and the reported findings are much more likely to be reactive responses to the prostatic inflammation also reported in rats in this study.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Tharp AP, Maffini MV, Hunt PA, VandeVoort CA, Sonnenschein C and Soto AM, 2012. Bisphenol A alters the development of the rhesus monkey mammary gland. Proceedings of the National Academy of Sciences of the United States of America, 109, 8190-8195.

This paper describes the effects of dosing BPA at a low dose to pregnant rhesus monkeys from gestational day 100 to term and the examination of the mammary glands of offspring. It was part of an on-going study to investigate the effects of BPA on ovarian function, and this study involved examination of the mammary glands from five control neonates and four neonates from mothers dosed with BPA. The dose of BPA given to mothers was 400 µg/kg/day given orally in a solution in ethanol, delivered within the centre of a small piece of fruit. Control mothers received fruit treated with vehicle (100 µl ethanol) only. The authors showed that this gave rise to average unconjugated BPA in maternal serum, near the time of spontaneous birth, approximately 4 hours after oral dosing, of 0.68 ± 0.312 ng/ml. Both neonatal mammary glands were surgically excised 1-3 days after birth from each of the four offspring of treated mothers and five control offspring, and one gland was whole-mounted for morphometric analysis, while the other was processed for histological analysis, using immunohistochemical staining for smooth muscle actin, keratin 14, keratin 18, ERα and ERβ in conventional sections. All examinations were carried out without prior knowledge of the prior treatment of the animals.

Morphometric analysis revealed a larger epithelial area, more ductal extension and branching points and a higher number of buds per ductal unit in treated compared with controls in the whole mount preparations. Only the difference in the number of buds/ductal mammary unit was statistically significant ($p=0.027$). These differences were ascribed to treatment with BPA because they were morphologically similar to those reported in mice by the same workers. No differences were observed in receptor status between controls and treated as indicated by the immunohistochemical staining

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:

Strengths:

- Oral administration
- BPA measurements in biological fluids

Weaknesses:

- Small sample size
- Single dose level study
- Statistics (because of limited sample size)
- Animal diet and phytoestrogen content not measured

The CEF Panel acknowledged that a primate study is judged to have particular relevance for humans however also noted that very little is known about the mammary gland at birth in rhesus monkeys. So few individuals were used in this study and the variability among the measurements was such that it seems inappropriate to conclude the results were the result of BPA administration and not simply individual biological variation within the context of a high and changing endogenous sex hormone environment. The role of sex steroid receptors has not been explored with respect to in utero mammary development in monkeys but given the high exposure of the primate fetus to oestrogens, progestogens, prolactin, and placental lactogen, it is likely that the fetal mammary gland is relatively insensitive to these hormonal stimuli (Cline 2007; Cline and Wood 2008). Moreover, following parturition there is a rapid reduction in sex hormone stimuli so that the neonatal mammary gland shows rapid regression. The mammary tissue was collected 1-3 days after birth, which is sufficiently non-standardised to represent another uncertain variable. Gestation periods may vary by a day or two between pregnancies and organ development may have been slightly different between the animals used.The results therefore can only be considered very preliminary and their relevance for the assessment of carcinogenic potential of BPA cannot be assessed. The CEF Panel concluded nonetheless that the results of this study can be used as supportive evidence of the induction of proliferation by BPA.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

US FDA/NCTR, 2013. Evaluation of the toxicity of bisphenol A (BPA) in male and female Sprague-Dawley rats exposed orally from gestation day 6 through postnatal day 90. Experiment E02176.01, Technical report of March 2013

Delclos KB, Camacho L, Lewis SM, Vanlandingham MM, Latendresse JR, Olson GR, Davis KJ, Patton RE, da Costa GG, Woodling KA, Bryant MS, Chidambaram M, Trbojevich R, Juliar BE, Felton RP and Thorn BT, 2014. Toxicity Evaluation of Bisphenol A Administered by Gavage to Sprague Dawley Rats From Gestation Day 6 Through Postnatal Day 90. Toxicological Sciences, 139, 174-197.

In this study, Sprague-Dawley rats (Sprague-Dawley/CD23/NCTR BR) were used for a dose-response approach to investigate the effects of BPA on a very wide range of pathological, physiological, endocrine, reproductive and developmental endpoints. Ethinyl oestradiol was used as a positive control of the estrogenic effects of BPA. The dose-matched vehicle control was carboxymethylcellulose, sodium salt. The doses were: (i) BPA 2.5, 8, 25, 80, 260, 840, 2700, 100 000, 300 000 µg/kg bw per day, (ii) Vehicle, (iii) EE_2 0.5, 5 µg/kg bw per day. The study included a naïve control group and doses were administered by oral gavage. The protocol and methods, including statistical analysis were of the high quality and robust with treatment, body weight and litter randomisation and appropriate inclusion and exclusion criteria established prior to the start of the study. The target unit for analysis was 20 litters and 18-23 were achieved. F0 females were dosed from GD 6 up to labour onset and pups from PND 1 until tissue harvesting, up to PND 90.–Additional groups were exposed from GD 6 to PND 21 for histopathological examination of the mammary glands.

Mammary gland duct hyperplasia of minimal severity was reported in the female groups examined at PND 21. The incidence of hyperplastic lesions was statistically significant by at least one of the three statistical methods used when compared with the vehicle control group in the 2 700 and 100 000 µg/kg bw per day groups, but not in the 300 000 µg/kg bw per day group. This observation was considered possibly treatment-related by the study authors but not by the original study pathologist. Mammary gland duct hyperplasia was also reported in the high dose female BPA groups examined at PND 90. Using the Poly-k test, the increase in minimal severity mammary gland duct hyperplasia was statistically significant in the 300 000 µg/kg bw per day group compared with vehicle controls. A significant increase in incidence of mammary gland duct hyperplasia compared with vehicle control was seen in the 2700, 100 000 and 300 000 µg/kg bw per day groups when analysis was carried out using the JT/SW or RTE statistical tests. Both of these tests incorporate lesion severity, but only the RTE method does not explicitly assume a monotonic dose-response curve (CFSAN, 2013a). Taking the incidences, the statistical testing results, and all pathologists and study authors opinions together, the authors of the NTP report (Gu and Mitkus, 2013), concluded that the evidence for duct hyperplasia in the mammary gland of females on either PND 21 or PND 90 was weak. They considered it an equivocal finding that may be the reflection of normal variability and/or a reflection of limits in tissue processing. BPA did not cause duct hyperplasia in the mammary glands of male rats, while conversely the reference estrogen EE_2 induced hyperplasia in the male but not the female mammary gland. A single mammary gland ductal adenocarcinoma (1 out of 260 female rats in the entire study) was seen in the 2.5 µg BPA/kg bw per day dose group at PND 90. The CEF Panel considered that the observation of mammary hyperplasia in female rats in this study albeit of minimal severity was relevant for the risk assessment of BPA, given the findings in other studies reported above. In the 100 000 and 300 000 µg/kg bw per day female BPA groups, significantly higher plasma levels of oestradiol and prolactin were found whereas the EE_2 values were only mildly elevated in comparison to controls. The CEF Panel concluded that this may point to a BPA treatment-related effect in females.

Comments from the CEF Panel:
The CEF Panel identified the following strengths/weaknesses in the study:
Strengths:

- Large sample size
- Both naïve and vehicle controls available
- Adequate positive controls included
- Measurement of BPA concentration in the serum
- Number of doses (9)
- Oral administration by gavage
- Phytoestrogen-free diet
- Use of non-PC cages
- Study performed according to OECD guidelines, under GLP

Weaknesses:

- Inconsistent results within groups (females not sensitive to EE_2 effects)
- Unintended exposure of the control groups (see Churchwell et al, 2014)
- No quantitative proliferation measurement (only hystological examination)

Overall, the CEF Panel noted that this GLP study, performed according to OECD standards, evaluated a wide range of dose levels, seven below and two above the dose of 5 mg/kg bw per day in former assessments defined as the reference point. The highest dose had an influence on several parameters. It is to be noted that the study duration was 90 days (13 weeks) with permanent dosing. Phytooestrogen levels in food were monitored to be in the low range. As the number of litters is sufficiently large the study has a fair sensitivity to detect effects.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Vandenberg LN, Maffini MV, Wadia PR, Sonnenschein C, Rubin BS and Soto AM, 2007. Exposure to environmentally relevant doses of the xenoestrogen bisphenol-A alters development of the fetal mouse mammary gland. Endocrinology, 148, 116-127.

Female CD-1 mice (8 wk of age) were exposed to BPA from gestational day 8 (GD8) until GD18 via s.c. implanted Alzet osmotic pumps designed to deliver either 50% dimethylsulfoxide (vehicle control) or 0.25 µg BPA/kg bw per day dissolved in the vehicle. At GD18, the fetuses were delivered singly by cesarean section and decapitated. One mammary gland was whole mounted and its contralateral gland was embedded in paraffin for light microscopy and immunohistochemistry (Ki67 for cell proliferation and Bax for apoptosis analyses). Exposure of dams to 0.25 µg BPA significantly increased ductal area (increased size of the epithelial cords) and ductal extensions. There was no statistically significant difference in the number of terminal end buds and cell proliferation (Ki67) between BPA-exposed and control mice. A significant decrease in apoptosis was seen in the inner cord cells of BPA-exposed mice relative to controls, whereas there was no such an effect in the outer cells of the BPA-exposed animals.

In the stroma, BPA exposure promoted the maturation of the fat pad and altered the localization of collagen, which is suggested by the authors to be responsible for the altered growth and cell size observed in the epithelium.

*Comments from the CEF Panel:*The CEF Panel concluded that the results indicate that exposure of dams to 0.25 µg BPA from GD8 until GD18 did not cause epithelial proliferation or an increase in the number of TEBs in the mammary glands of female fetuses.

The CEF Panel identified the following strenths and weaknesses in this study:
Strenghts:

- Sample size (40 controls and 36 BPA treated fetuses) for histological parameters
- Cages, bedding and food tested negligible for estrogenicity

Weaknesses:

- Small sample sizes for proliferation (8 controls and 7 BPA-treated) and apoptosis assessment (n=4)
- Only one dose
- Short exposure period

The study is included in the WoE table because of its relevance to one or more review questions addressed there.

Vandenberg LN, Maffini MV, Schaeberle CM, Ucci AA, Sonnenschein C, Rubin BS and Soto AM, 2008. Perinatal exposure to the xenoestrogen bisphenol-A induces mammary intraductal hyperplasias in adult CD-1 mice. Reproductive Toxicology, 26, 210-219.

CD-1 mice were exposed to BPA from gestational day 8 (GD8) until PND16 via s.c. implanted Alzet osmotic pumps designed to deliver dimethylsulfoxide (DMSO vehicle control). Exposure groups comprised: 0, 0.25 (0.25BPA); 2.5 (2.5BPA) or 25 (25BPA) µg BPA/kg bw per day. At 3, 9, and 12-15 months of age, female offspring were killed. Morphometry was performed on whole-mount mammary glands. Cell proliferation was determined by Ki67 staining. The ERα and progesterone receptor (PR) was determined in normal and "beaded ducts" (ducts with a beaded aspect caused by epithelial cells in the lumen; comparable with the intraductal hyperplasias as described by Murray et al, 2007).
3-Month old mice of the 0.25BPA group showed a significant increase in the volume of alveolar buds compared to controls. At 9 months of age the volume frcation of the alveolar buds was significantly increased in the 2.5BPA group. The volume fraction of TD did not vary regardless of treatment.
Immunohistochemical analysis of Ki67, ERα or PR did not indicate quantitative differences among the groups. At 9 months of age (but not at 3 or at 12-15 months of age, where the number of animals

examinded was ranging between 4 and 11) the incidence of beaded ducts was significantly increased in all BPA treated groups compared to controls, however without a dose-response relationship (5/20; 3/12 and 6/20 vs 0/18 in 0.25; 2.5 and 25BPA groups vs controls, respectively). ERα staining beaded ducts was not qualitatively different form non-beaded ducts. However, the epithelial cells in intraductal hyperplasias were often positive for PR. Cell proliferation was almost 5x higher in beaded ducts than in normal ducts and alveolar ducts. The authors concluded that the results at 3 months of age indicate that exposure from GD8 up to PND16 to BPA alters the development of the mammary gland. Specifically, 0.25BPA females showed an increase in alveolar buds compared to controls. The authors further concluded that the most novel observation reported was the development of intraductal hyperplasias in BPA treated mice.

Comments from the CEF Panel:
The CEF Panel concluded that the results can be used as supporting evidence of proliferation induced by BPA.
The CEF Panel identified the following strengths and weaknesses in this study:
Strengths:

- Number of doses (3)
- Phytoestrogen-free diet
- Use of non-pc cages and of non plastic bottles

Weaknesses:

- Small sample size in some of the dose groups
- Unclear reporting of the results

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Vandenberg LN, Schaeberle CM, Rubin BS, Sonnenschein C and Soto AM, 2013c. The male mammary gland: a target for the xenoestrogen bisphenol A. Reproductive Toxicology, 37, 15-23.

In this study, BPA was given to pregnant (GD8 up to delivery) and lactating mice (PND1 up to PND16) and mammary glands were examined at several time points in the adult offspring. In this experiment doses given to pregnant female CD-1 mice were 0.25, 2.5, 25 or 250 µg/bw per day via osmotic mini-pumps. Dams were allowed to litter and litters culled to 8 pups per mother. A single male offspring was killed at 3-4, 7-9 and 12-16 months of age and one mammary gland from each (from 5 to 20 animals/time point) was whole mounted and examined following Carmine-alum staining. In some cases where no visible gland was seen another sample was collected from a litter mate. Glands from animals exposed to 0.25 and 2.5 µg/bw per day had significantly more branching points and males exposed to 2.5 µg/bw per day had increased ductal area relative to controls. In the most severely affected group (2.5 µg/bw per day BPA), the mean number of branching points and ductal area represented 4.5- and 7.7-fold increases, respectively, compared to controls. At 7-9 months of age, a non-monotonic relationship between dose and mammary gland morphology was still present, but had shifted such that animals in the 2.5 µg/bw per day and 25 µg/bw per day groups were significantly different from controls; however, lower doses (0.25 µg/bw per day) and higher doses (250 µg/bw per day) were statistically indistinguishable from controls. The authors suggest that mammary glands of male offspring treated with BPA showed changes in ductal area and branching points compared with controls and that the response was non-monotonic. They suggested this might have relevance for gynaecomastia reported in men.

Comments from the CEF Panel:
The CEF Panel identified the following strengths and/or weaknesses in this study:
Strengths:

- Number of doses (4)
- Phytoestrogen-free diet
- Use of non-PC cages and of non plastic bottles

Weaknesses:

- Number of animals low
- Limited sampling methodology
- Unclear reporting of the results

The CEF Panel noted that the authors draw conclusions based on minor statistical significant differences in a study that used few animals and very limited sampling. The CEF Panel noted in particular the considerable individual variability in the measured effects as reflected in large standard errors around the mean (SEM).Moreover, a dose-response relationship (based on the nominal doses) was not observed. In some cases where no visible gland was seen another sample as collected from a litter mate, which the CEF Panel considered as inappropriate.

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Weber Lozada K and Keri RA, 2011. Bisphenol A increases mammary cancer risk in two distinct mouse models of breast cancer. Biology of Reproduction, 85, 490-497.

Female FVB/N mice were administered by gavage, vehicle (mineral oil), 25 or 250 µg BPA/kg/day from postcoital day 8 and until parturition. The first experiment studied vaginal opening and mammary gland development using groups of 5 mice from at least three litters. In the second experiment, mammary cancer susceptibility was studied in female offspring given (n = 10) 7,12-dimethyl benz[a]anthracene (DMBA) (1 mg/100µl corn oil) at week 5 and 6 weeks of age. These mice were palpated weekly to detect mammary tumours. Tumour latency was measured using Kaplan-Meier survival analyses. Upon the detection of tumours, mice were killed and tumours collected. Mice that were sick or died during the experiment from other causes than mammary cancer were censored from the study. In the third experiment NCR nu/nu female mice (n > 5) were ovariectomized at 8 weeks of age and implanted with a placebo (37.5 mg/60 day release), 17β-oestradiol (1.7 mg/60 day release), or low dose BPA pellet (37.5 mg pellet/60 day release). After recovery from surgery, 1 x 106 oestrogen sensitive cancer MCF-7 cells were subcutaneously injected into the flanks of the mice. Tumour latency was then assessed by weekly palpation and tumour growth was monitored by weekly measurement with callipers. A small group (n = 3) of the mice with injections of MCF-7 cells were also treated with the oestrogen receptor modulator tamoxifen (1 mg/mouse/day) by oral gavage for 5 continuous days per week.

Female FVB/N mice exposed prenatally to vehicle control exhibited vaginal opening on day 22-24, while mice exposed to BPA exhibited accelerated vaginal opening on day 21-22. The difference in vaginal opening was statistically significant. At no time point examined was there any notable morphological difference in mammary gland development observed between the BPA and vehicle treated offspring. The high dose BPA group exhibited a mean tumour latency of 50.8 weeks, and the lower BPA dose group exhibited a mean tumour latency of 69.3 weeks. Only one vehicle treated mouse developed a DMBA-induced tumour at week 111. A statistically significant difference in tumour latency in both the low and high BPA dose treatment group was reported. Numbers of animals that developed tumours in each group (incidence) were not reported, or the numbers of animals that died from other reasons than mammary tumours.

From the experiment with injection of MCF-7 cells, 5 of 7 mice in the 17β-oestradiol treated group, 5 of 6 mice in the BPA treated group and 0 of 7 mice in the placebo treated group formed tumours. On average the tumours form in the 17β-oestradiol treated group were 3 times larger in volume than the tumours from the BPA treated group 9 weeks post tumour cell implantation. Tumour regression was observed by tamoxifen in all mice from both 17β-oestradiol and BPA treated groups.

Comments from the CEF Panel:
The CEF Panel identified the following strengths and/or weaknesses in this study:

Strengths:

- Adequate positive control included
- Phytoestrogen-free diet
- Use of non-PC cages and of non plastic bottles

Weaknesses:

- Small sample size
- Insufficient study reporting (e.g. tumour incidence, no information on the number of animals that died of other causes than mammary cancer)

The CEF Panel noted uncertainties related to interpretation of the results of this paper, since it does not report the tumour incidence, a more relevant measure of effects on carcinogenicity. In addition no information is given on the number of animals that died of other causes than mammary cancer. This may have influenced results as it is probable that most animals in the control group died of other causes as only one animal in this group developed a carcinoma. Although tumours were verified histologically, they were all squamous carcinomas whereas DMBA given orally to mice and rats is reported to produce mainly adenocarcinomas that resemble those occurring in women (Qing et al. 1997; Costa et al., 2004; Mansour et al., 2012). There is no explanation for this. Moreover, histological evaluation was incomplete as it did not include study of preneoplastic changes that also occur in the mammary gland in the DMBA rat model. Such neoplasms therefore may have developed from the skin rather mammary tissue and would be inappropriate for mammary tumour risk assessment. It should also be noted that BPA is not genotoxic and that any observed effect on tumour latency or growth is a threshold phenomenon (EFSA CEF Panel, 2010).

This study is included in the WoE table because of its relevance to one or more review questions addressed there.

Weinhouse C, Anderson OS, Bergin IL, Vandenbergh DJ, Gyekis JP, Dingman MA, Yang J and Dolinoy DC, 2014. Dose-Dependent Incidence of Hepatic Tumors in Adult Mice following Perinatal Exposure to Bisphenol A. Environmental Health Perspectives, 122, 485-491.

Isogenic mice characterized by a resistance to hepatic tumors (genetically 75-93% C57BL/6J) were used in this study to evaluate the incidence of hepatic lesions following perinatal exposure to BPA. Ten wild type Agouti a/a female mice/group were exposed to BPA 2 weeks before mating (with males heterozygote Agouti A^{vy}/a briefly exposed to diets containing BPA during the mating period), during gestation and lactation (PND 21) through the diet (phytoestrogen-free AIN-93G diet (diet 95092, with 7% corn oil substituted for 7% soybean oil) supplemented with 0, 50 ng, 50 µg or 50 mg BPA/kg diet, corresponding to approximately 0, 10 ng, 10 µg, or 10 mg BPA/kg bw per day (calculated based on dam bw). One male and one female wild type (a/a) per litter, which were then given a standard phytoestrogen-free control diet, were followed until 10 months of age and tested for known risk factors for hepatocellular carcinoma, including bacterial and viral infections.
The authors report statistically significant trends in the dose-response in neoplastic lesions (adenomas + carcinomas) with or without preneoplastic lesions in females, but not in males. Furthermore, there was a statistically significant trend in adenomas, in neoplastic lesions (adenomas + carcinomas) and in neoplastic combined with preneoplastic lesions in all mice (males and females together) exposed to 10 mg/kg bw per day.

Comments from the CEF Panel:
Comment:_ The relevance of the isogenic mouse model for human health may be low.

The CEF Panel identified the following strengths and/or weaknesses in this study:
Strengths:

- Number of BPA doses (3)
- Phytoestrogen-free diet
- Use of non-PC cages

- BPA measurement in biological samples (total liver measurement)

Weaknesses:

- Small sample size (low number of animals/group)
- Insufficient reporting (no reporting on historical incidence of liver adenomas and carcinomas in isogenic mice)

The CEF Panel noted that the tumours already developed at an early time point, therefore raising the question as to the possible outcome at later stages (> 20 months).

(iii) In vitro studies related to proliferation

Appraisal of strengths and weaknesses and WoE analysis were not carried out for in vitro studies.

Dairkee et al., 2012/2013. Bisphenol-A-induced inactivation of the p53 axis underlying deregulation of proliferation kinetics, and cell death in non-mallignant human breast epithelial cells. Carcinogenesis 34, 703-712

The study extends previous work of this group using non-cancerous human high risk donor breast epithelial cells (HRBEC) (Goodson et al., 2011). BPA (only one concentration: 10^{-7} M) induced in sponaneously immortalized HRBEC lines and the ER-positive breast cancer cell line, T47D, molecular changes associated with reduced apoptosis (downregulation of p53, $p21^{WAF1}$ and BAX) and increased proliferation (PCNA, cyclins and phosphorylated pRb) and the ERα:ERβ ratio. Additionally, BPA reduced tamoxifen-induced apoptosis in these cell lines and induced proliferation in the cell lines and primary HRBEC cultures resulting in extended proliferation of the latter cells. The observed effects were inhibited by concomitant treatment of HRBEC cells by curcumin (10^{-7} M).

Comments from the CEF Panel:
In conclusion, the results demonstrate the antagonistic interaction of BPA and the anti-oestrogens tamoxifen and curcumin. Considering also potential interactions of BPA with the hormonal environment in the body, the expression of BPA effects *in vivo* is complex and difficult to simulate *in vitro.* The use of only one relatively high BPA concentration is an additional limiting factor in the present study.

Goodson WH , Luciani MG, Sayeed SA, Jaffee IM, Moore DH and SH Dairkee, 2011. Activation of the mTOR pathway by low levels of xenoestrogens in breast epithelial cells from high-risk women. Carcinogenesis 32, 1724-1733.

This *in vitro* study used pairwise comparisons of 16 independent epithelial cells from the unaffected breast of patients at high-risk of breast cancer with and without BPA exposures (10^{-10} M to 10^{-7} M). The authors report induction of genes and proteins in the PI3K-mTOR pathway—AKT1, RPS6 and 4EBP1 and a concurrent reduction in the tumour suppressor, phosphatase and tensin homolog gene protein. The altered regulation of these mTOR pathway proteins in BPA-treated cells led to marked resistance to rapamycin, the defining mTOR inhibitor, as observed in 17β-oestradiol ($5x10^{-9}$ M)-treated cells. Moreover, these cells pretreated with BPA were reported to surmount anti-oestrogenic effects of tamoxifen showing dose-dependent apoptosis evasion and induction of cell cycling.

Comments from the CEF Panel:
Whilst this study has the merit of using normal human breast epithelial cells taken from cancer patients, the interpretation of relevance for humans still suffers from all the constraints inherent in *in vitro* studies. This is particularly difficult in this context where dosing xeno-oestrogenic agents takes place in an artificial environment devoid of the normal oestrogenic and sex hormone environment. Whilst this study suggests that BPA at very low concentrations may have the potential to affect these pathways in mammary epithelial cells, the relevance to the *in vivo* situation is not clear.

Hall JM, Korach KS, 2012. Endocrine disrupting chemicals promote the growth of ovarian cancer cells via the ER-CXCL12-CXCR4 signaling axis. Molecular Carcinogenesis, 52, 715-725.

The authors studied the effect of 10^{-5}-10^{-9} M BPA on cell proliferation and CXCL12 chemokine expression using a human epithelial ovarian cancer cell line (BG-1). BPA induced cell proliferation, increased the expression of CXCL12 and its release into the culture medium. Previously it has been shown that the CXCR4 receptor activation after binding of CXCL12 leads to cell proliferation. Using different biochemical approaches and a relatively high BPA concentration (10^{-7} M) the authors demonstrated that proliferation of this cell line is linked to the ER-CXCL12-CXCR4 signalling axis.

Lee HR, Hwang KA, Park MA, Yi BR, Jeung EB and Choi KC, 2012b. Treatment with bisphenol A and methoxychlor results in the growth of human breast cancer cells and alteration of the expression of cell cycle-related genes, cyclin D1 and p21, via an estrogen receptor-dependent signaling pathway. International Journal of Molecular Medicine, 29, 883-890.

In this study with the oestrogen responsive human brest cancer cell line MCF-7 BPA or methoxychlor were used to follow the proliferative responses and cell-cycle-related genes. Both compounds were shown to induce cell proliferation by the up-regulation of genes that promote the cell cycle and the downregulation of anti-proliferative genes, especially ones affecting the G1/S transition via oestrogen receptor α signalling.

The authors argue that these results confirm the carcinogenicity of these endocrine disrupting chemicals *in vitro*. However these results merely illustrate an *in vitro* effect of these agents in a cell line that originates from a cancer. Its relevance to cancer development in the complex *in vivo* situation is speculative.

Pupo M, Pisano A, Lappano R, Santolla MF, De Francesco EM, Abonante S, Rosano C and Maggiolini M, 2012. Bisphenol A Induces Gene Expression Changes and Proliferative Effects through GPER in Breast Cancer Cells and Cancer-Associated Fibroblasts. Environmental Health Perspectives, 120, 1177-1182.

SKBR3 breast cancer cells and cancer-associated fibroblasts, which lack the classical estrogen receptors, were used to study the involvement of the G protein-coupled receptor (GPR30/GPER) pathway. Induction of ERK1/2 phosphorylation was shown in both cell types only by high concentrations of BPA (10^{-7} and 10^{-6} M) and was abolished by silencing GPER (by shGPER). BPA (10^{-7} M) induction of the expression of GPER target genes (c-FOS, EGR-1 and CTGF) was also inhibited by shGPER. This rapid activation of the GPER signalling pathway has also been reported in other cell-types (human seminoma cells, mouse spermatogonial cells). These data expand the knowledge of BPA signalling via membrane G-proteins.

Qin X-Y, Fukuda T, Yang L, Zaha H, Akanuma H, Zeng Q, Yoshinaga J and Sone H, 2012a. Effects of bisphenol A exposure on the proliferation and senescence of normal human mammary epithelial cells. Cancer Biology Therapy 13, 1-11.

This study reports the effect of BPA cellular proliferation and senescence in a human mammary cell line derived from normal mammary epithelial cells (HMEC). Oestradiol (10^{-9} M) served as positive control. Exposure to BPA (10^{-8} M and 10^{-7} M) for 1 week at the early stage at passage 8 increased the proliferation and sphere size of these cells at the later stage up to passage 16, suggesting that BPA has the capability to modulate cell growth in breast epithelial cells comparable to the treatment with 17β-oestradiol (E2, 10^{-9} M). The number of human heterochromatin protein-1γ positive cells, which is a marker of senescence, was also increased among BPA-treated cells. Consistent with these findings, the protein levels of both p16 and cyclin E, which are known to induce cellular senescence and promote proliferation, respectively, were also increased at 10^{-7} M BPA. DNA methylation levels of a number of genes related to development tumours were also increased in treated cells. DNA methylation levels of

genes related to development of most or all tumor types, such as *BRCA1, CCNA1, CDKN2A (p16), THBS1, TNFRS F10C* and *TNFRS F10D,* were increased in BPA-exposed HMEC. The authors concluded that the findings in the HMEC model suggested that the genetic and epigenetic alterations by BPA might damage HMEC function and result in complex activities related to cell proliferation and senescence, playing a role in mammary carcinogenesis.

Whereas the study shows genetic and epigenetic alterations induced by BPA in this cell model, its *in vivo* relevance is uncertain. The conclusion from the results of this study is hampered by the use of only two BPA concentrations and only one time point for expression of mRNA and protein (passage 11, i.e. 3 weeks after treatment), which is insufficient to obtain a maximal response.

Wu S, Wei X, Jiang J, Shang L and Hao W, 2012. Effects of bisphenol A on the proliferation and cell cycle of HBL-100 cells. Food and Chemical Toxicology, 50, 3100-3105.

Proliferation, progression through cell cycle and cyclin D1 expressio were studied in normal human breast cells (HBL-100). Surprisingly, BPA induced growth at a 100-fold lower concentration, *i.e.* at 10^{-10} M, in these cells than E2. The BPA effect was not completely blocked by the ER antagonist ICI 182780. Additionally BPA induced cyclin D1 expression but no ERα expression.

Zhang W, Fang Y, Shi X, Zhang M, Wang X and Tan Y, 2011. Effect of bisphenol A on the EGFR-STAT3 pathway in MCF-7 breast cancer cells. Molecular Medicine Reports, 5, 41-47.

This study explored the effect of BPA on the EGFR-STAT3 pathway in MCF-7 breast cancer cells. It was shown that the optimal concentration and time point of BPA-induced proliferation in MCF-7 cells was 10^{-6} M and 24 hours, respectively (However, due to poor data presentation the dose-response curve cannot be interpreted). BPA significantly increased the expression of STAT3 at a concentration of 10^{-6} M following treatment for 48 h and the expression of STAT3 was down-regulated after blocking EGFR. It was argued that STAT3 expression, which is a major factor in the pathway of BPA-induced proliferation and STAT3 activation, contributes to BPA-induced breast cancer cell proliferation.

Comments from the CEF Panel:
Again whilst this study shows the potential of BPA to induce cellular changes *in vitro*, it does not provide evidence of their potential to do so *in vivo.*

In vitro studies/Mechanisms of action

Appraisal of strengths and weaknesses and WoE analysis were not carried out for in vitro and/ or mechanistic studies.

Brannick KE, Craig ZR, Himes AD, Peretz JR, Wang W, Flaws JA and Raetzman LT, 2012. Prenatal Exposure to Low Doses of Bisphenol A Increases Pituitary Proliferation and Gonadotroph Number in Female Mice Offspring at Birth. Biology of Reproduction, 87, 82.

This study investigated whether prenatal exposure of mice to low doses of BPA results in changes in pituitary development and cellular specification. Pregnant female mice (described as from a mixed FVB, C57BL/6 background, with up to 8 mice per treatment group) were dosed orally (by gavage) with 0, 0.5 or 50 µg/kg bw per day of BPA dissolved in ethanol and diluted in corn oil. Dosing took place from GD 10.5 to GD 18.5 and pups were examined at PND 1 for effects of BPA on cell proliferation, cell differention and parameters of hormone synthesis. Six to eight individual pituitaries were examined from each treatment, obtained from pups from five to seven different litters per treatment group. BPA induced cell proliferation in the pituitary of female, but not male, offspring as evidenced by the results of quantitative histochemistry to detect mKi67-immunoreactive cells and

measurement of mKi67 mRNA levels. The effect was more marked at 0.5 µg/kg bw per day of BPA compared with 50 µg/kg bw per day of BPA. The number of gonadotrophs (as measured by cells expressing LHb and FSHb) also increased in female offspring from BPA-treated females; female mice exposed to 0.5 µg/kg bw per day BPA had increased mRNA levels of gonadotropins and the gonadotropin-receptor hormone (GNRH) receptor (Gnrhr), while a decrease in gonadotropin mRNA levels, Gnrhr and Nr5a was seen in females that had been exposed to 50 µg/kg bw per day of BPA. Proliferating cells, expressing mKi67 did not also express LHb and FSHb, as demonstrated by double-labelling immunohistochemistry, but proliferating progenitor cell s were demonstrated to frequently be SOX2-positive. No changes were seen in mRNA levels of marker hormones produced by corticotropes, somatotropes, and thyrotropes, and notably no effect of BPA was seen on prolactin (PRL) expression on PND 1. The authors demonstrated however (using CD-1 mice) that PRL expression did not commence until PND 5 and was not fully expressed until adulthood. The authors conclude that exposure to BPA affects pituitary gonadotroph development in female mice but not in males, and postulate that this may be due to an effect of BPA on the sexually dimorphic development of the anteroventral periventricular nucleus (AVPV) of the hypothalamus, leading to altered pituitary function.

The results of this study are mechanistically interesting, suggesting a BPA-mediated effect on pituitary development which is sexually dimorphic and may explain/underlie some of the effects seen on reproductive parameters in female rodents exposed prenatally to BPA. The authors suggest that the effect of BPA on the pituitary may be oestrogenic – use of a positive control would have helped understand the results of the study. Although the number of animals used was relatively small, the methodology appears robust.

Goodson WH, Luciani MG, Sayeed SA, Jaffee IM, Moore DH and Dairkee SH 2011. Activation of the mTOR pathway by low levels of xenoestrogens in breast epithelial cells from high-risk women. Carcinogenesis, 32, 1724-1733.

Non-malignant breast epithelial cells were obtained in this study by random periareolar fine needle aspiration from the unaffected contralateral breast of high-risk women undergoing breast surgery. Sixteen independent samples were expanded *in vitro* and exposed to BPA at concentrations between 10^{-10} M and 10^{-7} M or to 17β-oestradiol ($5x10^{-9}$ M). There was a dose-dependent inhibition of tamoxifen-induced apoptosis by BPA – even at the lowest concentration in these cells. The dose-dependent reversal of tamoxifen-induced growth inhibition by BPA could also be observed using BrdU labelling of the cells. Additionally, BPA-induced molecular changes in the mammalian target of rapamycin (mTOR) pathway were associated with significant reduction in rapamycin-induced apoptosis. Similar changes were observed with the xenoestrogen methylparaben. The finding with BPA supports other observations that BPA increases the cell proliferation/apoptosis ratio in normal tissue as well as preneoplastic lesions of rat mammary gland (see EFSA CEF Panel, 2010, p75: reports by Betancourt et al., 2010 and others). The authors observed also a decline of endogenously accumulated reactive oxygen species (not dose-dependent) in these cells, while usually an induction of oxidative stress by BPA is reported (e.g. Rashid et al., 2009 cited in EFSA CEF Panel, 2010). Considering that the lowest BPA concentration (10^{-10} M) was still active (LOEC) this *in vitro* model using human breast epithelial cells can be regarded as very sensitive to xenoestrogens.

Hall JM and Korach KS, 2012. Endocrine disrupting chemicals promote the growth of ovarian cancer cells via the ER-CXCL12-CXCR4 signaling axis. Molecular Carcinogenesis, 52, 715–725.

The authors studied the effect of 10^{-5}-10^{-9} M BPA on cell proliferation and CXCL12 chemokine expression using the BG-1 cell line (human epithelial ovarian cancer). The authors reported that BPA induces cell proliferation, increases the expression of CXCL12 and its release into the culture medium. Previously it has been shown that the CXCR4 receptor activation after binding of CXCL12 induces also cell proliferation. Using different biochemical approaches and a BPA concentration of 10^{-7} M the

authors reported that proliferation of this cell line is regulated also throught the ER-CXCL12-CXCR4 signalling axis.

Huc L, Lemarié A, Guéraud F and Héliès-Toussaint C, 2012. Low concentrations of bisphenol A induce lipid accumulation mediated by the production of reactive oxygen species in the mitochondria of HepG2 cells. Toxicology In Vitro, 26, 709-717.

See study description in Appendix B under section "Metabolic effects – In vitro studies"

Hwang K-A, Park SH, Yi BR and Choi KC, 2011. Gene alterations of ovarian cancer cells expressing estrogen receptors by estrogen and bisphenol a using microarray analysis. Laboratory Animal Research, 27, 99-107.

This study presents a microarray analysis supported by examination of mRNA levels of selected genes in ovarian adenocarcinoma cell line after exposure to exposed to 17β-oestradiol (10^{-7} M) or BPA (10^{-5} M). This cell line expresses oestrogen receptor α. Altered genes reported included RAB31_member Ras oncogene family, cyclin D1, cyclin-dependent kinase 4, insulin-like growth factor-binding protein 4 and anti-mullerian hormone. This paper presents an in vitro method for screening chemicals with weak oestrogenic properties.

Jung J-W, Park S-B, Lee S-J, Seo M-S, Trosko JE and Kang K-S, 2011. Metformin represses self-renewal of the human breast carcinoma stem cells via inhibition of estrogen receptor-mediated OCT4 expression. PLoS ONE 6(11): e28068.

This mechanistic study investigated the potential of metformin, an oral anti-diabetic drug to reduce the risk to breast cancers using a human breast carcinoma cell line, MCF-7, grown in 3-dimensional mammospheres, representing breast cancer stem cells. The cells were also treated with TCDD, BPA or 17-β-oestradiol or the anti-oestrogen ICI182,780. Using OCT4 expression (which functions as a transcription factor) as a marker for the cancer stem cells, the number and size were measured in these cells. TCDD (100 nM), BPA and the oestrogen (10 nM) increased the number and size of the mammospheres. Metformin reduced the expression of OCT4 in 17-β-oestradiol & TCDD treated mammospheres but not in those treated with BPA, suggesting different mechanisms of action of the BPA on human breast carcinoma cells.

Lee HK, Kim TS, Kim CY, Kang IH, Kim MG, Kyung Jung K, Kim HS, Han SY, Yoon HJ and Rhee GS, 2012d. Evaluation of in vitro screening system for estrogenicity: comparison of stably transfected human estrogen receptor-α transcriptional activation (OECD TG455) assay and estrogen receptor (ER) binding assay. The Journal of Toxicological Sciences, 37, 431-437.

The authors compared 4 different in vitro screening systems for estrogenicity using 7 different industrial chemicals: "Yeast assay", "E-screen assay", "ER binding assay" and the "STTA assay" (OECD TG455). All assays gave comparable results. The authors concluded that the OECD TG455 might be a useful screening test for endocrine disruptors.

Li Y, Burns KA, Arao Y, Luh CJ and Korach KS, 2012a. Differential Estrogenic Actions of Endocrine-Disrupting Chemicals Bisphenol A, Bisphenol AF and Zearalenone through Estrogen Receptor α and β in Vitro. Environmental Health Perspectives, 120, 1029-1035.

Three different cell lines, HepG2 (human hepatocellular carcinoma), HeLa (human cervix epitheloid carcinoma) and Ishikawa (human endometrical adenocarcinoma) were used to study effects of 10^{-6}-10^{-9} M BPA on signalling through ERα and ERβ. The authors concluded that the estrogenic activity is cell type and concentration dependent. In some experimental set-ups 10^{-9} M BPA increased ERα activity. Also antagonizing effects of BPA in combination with E_2 (17β-oestradiol) were detected.

Using specific kinase inhibitors the authors concluded that BPA activates not only the MAPK pathway. Other signalling pathways like src might also be relevant. Finally the authors used different ERα constructs to study the mechanism of receptor binding and gene activation.

Nanjappa MK, Simon L, and Akingbemi BT, 2012. The industrial chemical bisphenol A (BPA) interferes with proliferative activity and development of steroidogenic capacity in rat Leydig cells. Biology of Reproduction 86, 135, 1-12.

The study indicates that low perinatal BPA doses (2.5 and 25 µg/kg bw per day at GD 12 to PND 21) given orally (gavage) to pregnant and lactating Long Evans rats (n=14?) stimulated growth of Leydig cells in male offspring (^{3}H-thymidine incorporation). This was associated with an up-regulation of the expression of cell cycle proteins (e.g., PCNA, cyclin D3). The mitogenic BPA effect is possible mediated in part also by protein kinases (e.g., MAPK3/1), growth factor receptors (IGF1RB, EGFR) and Sertoli cell-secreted paracrine factor anti-Mullerian hormone. A slight induction of proliferation was also confirmed *in vitro* using 10^{-8} M but not with 10^{-11} M BPA. The effects on cell number and PCNA expression were not dose-dependent. A decreased Leydig cell testosterone production was observed at PND 21, 35 and 90 but changes in serum testosterone levels were not significant. The reduced hormone production was associated with a BPA induced suppression of LH receptors and the hydrosteroid dehydrogenase enzyme (HSD17B3) in Leydig cells. The authors suggest that BPA impaired postnatal Leydig cell differentiation but the effect on serum testosterone levels might be counterbalanced by a higher proliferation of Leydig cells. The unchanged testosterone serum levels observed in this study are not in line with earlier findings of Akingbemi et al. (2004) using rats treated with 2.4 µg BPA/kg bw per day from PND 21 – 35.

The limited effect (<40%) on adult (PND 90) Leydig cell testosterone production was not dose-dependent and is not expected to have an impact on sperm production in accordance with the absence of such effects at low doses in the Tyl-study (2002). Despite some limitations (two BPA doses only, no positive control) the consistent effects of BPA on proliferation and the associated biochemical changes at the low dose, which is relevant for human exposure, are challenging. It is noted that BPA at low doses (given perinatally) is frequently reported to stimulate proliferation in different tissues or cells.

Nanjappa MK, Simon L and Akingbemi BT, 2012. The Industrial Chemical Bisphenol A (BPA) Interferes with Proliferative Activity and Development of Steroidogenic Capacity in Rat Leydig Cells. Biology of Reproduction, 86, 135, 131-112.

This is an *ex-vivo* study which describes the effects of developmental exposure of male rats to BPA via gavage of pregnant and lactating Long Evans dams at 2.5 and 25 µg/kg body weight from gestational day 12 to postpartum day 21. Although no exposure measurements were performed the authors estimated based on previous data that maternal exposures to BPA at 2.5 and 25 µg/kg body weight represent BPA doses to the offspring of about 8 and 80 pg/kg body weight. Perinatal exposure to BPA did not affect litter size, birth weights of pups and pup sex ratio. Body weights, measured at 21, 35 and 90 days of age, were equivalent in BPA-exposed and control animals ($P > 0.05$). Similarly, paired and relative testes weights (proportion to body weights) were not affected by BPA. However Leydig cell division was stimulated in the prepubertal period and increased Leydig cell numbers were shown in the testes of adult male rats at 90 days.

It is difficult to judge the biological significance of small statistically differences in the sophisticated measurements made in this study in the context of totally normal pregnancies and littering. Particular care has to be taken in extrapolating findings in rat Leydig cells to humans. A detailed review of comparative physiology and pathology indicated that rats are quantitatively far more sensitive to the development of Leydig cell tumours than men as it appears that Leydig cell luteinizing hormone releasing hormone (gonadotropin-releasing hormone) receptors are unique to rats. Rats also have over 10 times more luteinizing hormone receptors than men[8]. However LH (and indeed AGD, a

masculinisation read-out) was not measured which is a strange omission given the findings presented, and the adaptability of the reproductive axis to small changes in driving signals. It is unlikely that this study confirms an adverse effect of BPA exposure on human male reproductive function as being likely or not without further work (e.g. determination of whether these rats are in fact less fertile).

Sangai NP, Verma RJ and Trivedi MH, 2012. Testing the efficacy of quercetin in mitigating bisphenol A toxicity in liver and kidney of mice. Toxicology and Industrial Health, 28, 28.

The study was performed to evaluate the effect of quercetin (a flavone) on the toxicological effects of bisphenol A in liver and kidney of mice. Groups of Swiss albino mice (adult, males) received 120 mg/kg bw per day and 240 mg/kg bw per day BPA for 30 days with and without quercetin. In the context of this evaluation the results obtained with quercetin are not of interest but the findings with 120 mg/kg bw per day and 240 mg/kg bw per day BPA are of interest. Oral administration of BPA for 30 days caused significant and dose-dependent decrease in body weight. Absolute and relative organ weights increased in liver and kidney of mice compared with vehicle control. Histopathological findings included hepatocellular necrosis, cytoplasmic vacuolization and decrease in hepatocellular compactness in liver and distortion of the tubules, increased vacuolization, necrosis and disorganization of glomerulus in the kidney.BPA treatment caused, when compared with vehicle control, a statistically significant reduction in the activities of a series of enzymes, such as catalase, superoxide dismutase, glutathione peroxidase, glutathione reductase, glutathione-S-transferase. The content of glutathione and total ascorbic acid was reduced whereas significant increase was found in malondialdehyde levels. The results show that high doses of BPA (120 mg/kg bw per day and 240 mg/kg bw per day) caused oxidative damage in liver and kidney of mice. The phytoestrogen content of the diet was apparently not tested.

The study is well performed and gives some insight into the toxicological effects of BPA in liver and kidney. Based on the findings of the authors oxidative damage is one of the mechanisms/mode of action playing a role in BPA organ toxicity. However, the mechanisms remain far from being elucidated.

Sun H, Si C, Bian Q, et al., 2012. Developing in vitro reporter gene assays to assess the hormone receptor activities of chemicals frequently detected in drinking water. Journal of Applied Toxicology, 32, 635-641.

The authors studied the effect of $4.4x\ 10^{-9}$ – 10^{-6} M BPA on the activation of the estrogen receptor (ERα), the androgen receptor (AR) and the thyroid hormone receptor (TR) using transfected Vero (African green monkey kidney) cells. No toxic effects of BPA were detected at the investigated concentration range. A significant activation of the ERα was detected at and above $4.4x\ 10^{-7}$ M BPA. The authors calculated that 20 % of the maximal ERα activation was reached at $2.8x10^{-6}$ M BPA.

A significant anti-androgenic activity was detected at $4.4x10^{-6}$ M BPA. The authors calculated that $1.3x10^{-6}$ M BPA would result in a reduction of the AR receptor by 20%, which was activated with 50 ng/l testosterone. Similarly, $4.4x10^{-6}$ M BPA would result in 20% reduction of the TR activity, which was activated by 0.5 µg/l T_3. No receptor activation/antagonism was observed at relevant BPA concentrations.

Peretz J, Craig ZR and Flaws JA, 2012. Bisphenol A inhibits follicle growth and induces atresia in cultured mouse antral follicles independently of the genomic estrogenic pathway. Biology of of Reproduction, 87, 1-11.

The study aimed at determining whether BPA can affect cell cycle regulators and/or induce atresia in ovarian antral follicles and whether this is via genomic estrogenic signalling. FVB mice, both ESR1 over-expressing and control were used, with 2-3 mice/experiment and 8-16 follicles/treatment/experiment. BPA exposure was in-vitro using well established antral follicle culture methods. BPA was diluted in culture medium to achieve final concentrations in media = 1, 10, 100 µg

BPA/ml. Treatments included co-treatments of BPA variously with E2 (10 nM) and ICI 182,780 ESR antagonist. Culture duration was 24-120 hrs. Endpoints were follicle growth and atresia, expression of cell cycle proliferation and apoptosis transcripts. BPA inhibited follicle growth and induced follicle atresia, effects that were not reversed by oestradiol or ESR antagonist and not increased in ESR-overexpressing follicles. The study concludes that the genomic estrogen signalling pathway is not involved in transducing the adverse effects of BPA.

The concentrations used in this study are higher than those relevant in vivo and those at which most effects were observed were far above human exposure levels.

Pupo M, Pisano A, Lappano R, Santolla MF, De Francesco EM, Abonante S, Rosano C and Maggiolini M, 2012. Bisphenol A Induces Gene Expression Changes and Proliferative Effects through GPER in Breast Cancer Cells and Cancer-Associated Fibroblasts. Environmental Health Perspectives 120, 1177-1182.

See study description in Appendix B under Section "Carcinogenicity – In vitro studies".

Qin XY, Kojima Y, Mizuno K, Ueoka K, Muroya K, Miyado M, Zaha H, Akanuma H, Zeng Q, Fukuda T, Yoshinaga J, Yonemoto J, Kohri K, Hayashi Y, Fukami M, Ogata T, Sone H, 2012b. Identification of novel low-dose bisphenol A targets in human foreskin fibroblast cells derived from hypospadias patients. PLoS One, 7, e36711.

DNA microanalysis was used to identify novel targets of low concentrations of BPA (10^{-8} M) in human foreskin fibroblasts cells derived from child hypospadias patients. In addition to BPA E2 (10^{-11} M) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD at 10^{-9} M) were used. Among the 71 genes differentially expressed after BPA treatment only a small subset was also affected by E2. Using real-time PCR it could be confirmed that the expression of one of the most effectively down-regulated genes, i.e. metallopeptidase 11 (MMP11) was only 40% in BPA-treated cells. While MMP11 was shown to be overexpressed in several human cancers, the authors speculated that its down-regulation might be associated with abortive penile development.

Tilghman SL, Bratton MR, Segar HC, Martin EC, Rhodes LV, Li M, McLachlan JA, Wiese TE, Nephew KP and Burow ME, 2012. Endocrine disruptor regulation of microRNA expression in breast carcinoma cells. PLoS ONE 7(3): e32754.

This study that uses the human MCF-7 breast cancer cell line, which is oestrogen receptor positive and hormone sensitive investigate the cellular effects of both DDT and BPA. It shows that DDT and BPA can potentiate oestrogen receptor transcriptional activity, resulting in an increased expression of receptor target genes, including progesterone receptor, bcl-2, and trefoil factor 1. While these compounds and oestrogen similarly altered the expression of multiple microRNAs in MCF-7 cells, including miR-21, differential patterns of microRNA expression were induced by DDT and BPA compared to oestrogen. This study shows the oestrogenic potential of BPA and the DDT in vitro.

Vo TTB, An B-S, Yang H, Jung E-M, Hwang I, and Jeung E-B, 2012. Calbindin-D9k as a sensitive molecular biomarker for evaluating the synergistic impact of estrogenic chemicals on GH3 rat pituitary cells. International Journal of Molecular Medicine, 30, 1233-1240.

The authors studied the effect of 10^{-7}, 10^{-6} and 10^{-5} M BPA in combination with equal concentrations of 4-nonylphenol (NP), 4-tert octylphenol (OP) and isobutylparabene (IBP) on the concentration and expression of the cytosolic calcium-binding protein calbindin, which was used as indictor of endocrine activation. Mixtures of BPA+NP, BPA+NP+OP and BPA+NP+IBP increased the gene and protein expression of calbindin significantly, compared to incubations with single substances. These effects were lower after preincubation with fulvestrant, an anti-estrogen compound.

The expression of the progesterone receptor (PR) significantly increased after incubation with mixtures of BPA+NP+OP or BPA+NP+IBP (10^{-5}-10^{-7} M), compared to the exposure of each chemical alone.

Wang J, Sun B, Hou M, Pan X and Li X, 2013. The environmental obesogen bisphenol A promotes adipogenesis by increasing the amount of 11β-hydroxysteroid dehydrogenase type 1 in the adipose tissue of children. International Journal of Obesity, 37, 999-1005.

See study description in Appendix B under section "Metabolic effects - In vitro studies".

Wu S, Wei X, Jiang J, Shang L and Hao W, 2012. Effects of bisphenol A on the proliferation and cell cycle of HBL-100 cells. Food and Chemical Toxicology, 50, 3100-3105.

Proliferation, progression through cell cycle and cyclin D1 expression were studied in normal human breast cells (HBL-100). In these cells BPA induced growth at a 100-fold lower concentration, i.e. at 10^{-10} M, than E2. The BPA effect could not be completely blocked by the ER antagonist ICI 182780. Additionally BPA induced cyclinD1 expression but no ERα expression. According to these data the proliferation of HBL-100 cells is a sensitive endpoint to BPA.

(i) Toxicokinetic/metabolism issues

Coughlin JL, Thomas PE and Buckley B, 2012. Inhibition of genistein glucuronidation by bisphenol A in human and rat liver microsomes. Drug Metabolism and Disposition, 40, 481-485.

The authors addressed the influence of BPA on the *in vitro* metabolism (microsomal glucuronidation) of an endocrine-active substance, *i.e.* genistein. This issue may be particularly relevant for risk assessment of mixtures of endocrine disrupters. The BPA-induced inhibition of glucuronidation of genistein was studied in human liver microsomes (pooled from 50 donors, mixed gender) and rat liver microsomes (pooled from 100 female and 100 male Wistar rats). Non-competitive and competitive inhibition was observed in human and rat liver microsomes, respectively. However, for these experiments only one high BPA concentration (25 µM) was used. Additionally, a concentration range of 5 to 250 µM BPA was used to establish an IC_{50} value of 37 µM BPA for the inhibition of genistein (100 µM) metabolism. In conclusion, these findings refer to high in vitro BPA concentrations.

Trdan Lusin T, Roskar R and Mrhar A, 2012. Evaluation of bisphenol A glucuronidation according to UGT1A1*28 polymorphism by a new LC-MS/MS assay. Toxicology, 292, 33-41.
The paper describes a novel method for biomonitoring BPA exposure using an internal standard ($BPAG_{d16}$) and a LC-MS/MS method for simultaneous determination of BPA and its metabolite.* Using this analytical approach the authors confirmed the high metabolic capacity of human liver microsomes, *i.e.* 400-fold higher compared to intestinal microsomes. No metabolic activity was detected in lung microsomes. Therefore, it can be assumed that BPA intake by inhalation (which is not known to have a relevant contribution to human BPA exposure) would result in "unconjugated BPA" in the blood. In addition the authors addressed the impact of UGT1A1*28 polymorphism on BPA metabolism using genotyped human liver microsomes (wild-type homozygotes, heterozygotes and polymorphic homozygotes). Based on differences in the glucuronidation efficiency (V_{max}) this polymorphism could contribute to a minor extent to differences in BPA elimination which is more actively triggered by UGT2B15 isoforms (Hanioka et al., 2008).

Overall, this paper does not change the view on the toxicokinetics of BPA expressed by the CEF Panel in its Opinions (EFSA 2006, 2008, 2010).

(ii) Gene expression

Humans

Hanna CW, Bloom MS, Robinson WP, Kim D, Parsons PJ, Vom Saal FS, Taylor JA, Steuerwald AJ, Fujimoto VY, 2012. DNA methylation changes in whole blood is associated with exposure to the environmental contaminants, mercury, lead, cadmium and bisphenol A, in women undergoing ovarian stimulation for IVF. Human Reproduction, 27, 1401-1410.

Blood concentrations of mercury, lead, cadmium and unconjugated BPA (uBPA) were examined in relation to DNA methylation in 43 women undergoing ovarian stimulation for IVF. Blood and urine were collected on the day of oocyte retrieval. Unconjugated BPA was quantified in serum of 35 women with median values of 2.4 µg/l (0.0-67). This is in contrast to values reported by Teeguarden et al. (2011) for persons with high BPA exposure via canned food (intake = urinary excretion/24 hrs: <0.3 µg/kg bw per day): the peak serum concentrations of unconjugated BPA were between 0.001 and 0.11 nM corresponding to 0.23 – 25 ng/l. Tayler et al. (2011) reported a serum concentration of unconjugated BPA in monkeys of 0.5 µg/l after oral intake of 400 µg/kg bw per day. Candidate CpG sites were identified using a Diff score >|13| (p=0.005) and an absolute difference of 10% which were confirmed using bisulfite pyrosequencing. BPA exposure was divided into higher and lower exposure groups by median concentrations. Women with higher BPA exposure had significantly lower methylation of promotor CpG site at the TSP50 gene, and BPA exposure was inversely correlated to methylation (r=-0.51, p=0.001). The negative correlation suggests that increased BPA exposure may be associated with increased expression of TSP50. The TSP50 gene encodes "testis specific protease 50" expressed in the testis. In vitro studies showed that TSP50 is related to cell proliferation. Knock-down of TSP50 resulted in a decreased cell proliferation (Zhou et al. 2010) and overexpression increased cell proliferation (Song et al. 2011). Increased TSP50 has also been observed in female breast cancer tissue.

No confounding factors were considered. BPA values for samples measured <LOD were generated by extrapolation from the standard curve, and where no evidence for the presence of BPA at any concentrations existed, BPA values were assigned a value of zero. This could influence the results. The authors themselves discuss the limitations of their study due to its pilot nature. In addition to the issues above they mention: no correction for multiple comparisons, heterogeneity of the sample with regard to infertility diagnosis, no adjustment for factors that might potentially alter methylation as well as body burdens of Hg, Pb, Cd (unmeasured confounding), assessment in whole peripheral blood (varying cell types).

Melzer D, Harries L, Cipelli R, Henley W, Money C, McCormack P, Young A, Guralnik J, Ferruci L, Bandinelli S, Corsi AM, Galloway T, 2011. Bisphenol A Exposure is Associated with In-Vivo Estrogenic Gene Expression in Adults. Environmental Health Perspectives, 119, 1788-1793.

The study investigated associations between urinary BPA exposure and in vivo expression of six estrogen receptor, estrogen-related receptor, and androgen receptor genes in peripheral blood leukocytes in 96 adult men in an Italian population based cohort. Urinary BPA concentration (mean 3.65 ng/ml) was in the normal range (NHANES 2003/4: 2.7 ng/ml). Positive associations were found between urinary BPA and ESR2 (estrogen receptor type beta) and ESRRA (estrogen related receptor alpha) expression.

The study is sound despite the relatively low sample size and this study presents new results. Considering that the BPA induced pattern of gene expression is cell-specific (EFSA Opinion, 2010, p73) the relevance for ERS2 (ERβ) expression in blood leukocytes is unclear. While BPA exposure increases ERβ in breast cancer cells, it was unaffected by BPA (1 nM) in prostate cancer cells with wild-type androgen receptor (AR) and was down-regulated by 1 nM BPA in AR-mutant prostate cancer cells (Hess-Wilson et al., 2007). Considering also the fast metabolism of BPA the time-dependence of the expression changes in ERβ would be interesting. Finally, the question has to be answered whether or not such small changes (i.e. 65% higher mean ESR2 expression in upper-tertile BPA excretors) could be biologically meaningful. Although the clinical significance of these results is

unknown, the activation in humans suggests that BPA could be a xenoestrogen in this population representative sample. The results need to be replicated and expanded in a larger sample.

Singh S, Li SS, 2012. Bisphenol A and phthalates exhibit similar toxicogenomics and health effects. Gene, 494, 85-91.

This paper compares toxicogenomics and adverse effects on human health of BPA exposure with those of phthalates by using the Comparative Toxicogenomics Database (CTD) in order to find biomarkers of toxicity. The CTD include data on a huge set of interactions between chemicals and genes/proteins from several species, gene/protein-disease direct relationships, and chemical-disease direct relationships. The authors identified 1932 –BPA-gene/protein interactions, and among them estrogen receptor 1 and 2 appeared most frequently.

The comparative toxicogenomics and health effects between BPA and the five most frequently curated phthalates revealed 89 common genes/proteins that may serve as biomarkers to assess their toxicity. It is noted however, that most phthalates (e.g. DEHP) have very poor or no estrogenic activity but act via other modes of action (e.g. Borch et al., 2006). Therefore, the relevance of a common mode of action of phthalates and BPA particularly on estrogen receptors is questionable.

Animals

Doshi T, Mehta SS, Dighe V, Balasinor N, Vanage G, 2011. Hypermethylation of estrogen receptor promoter region in adult testis of rats exposed neonatally to bisphenol A. Toxicology, 289, 74-82. (AUG-11)

A single BPA dose (2.4 µg/pup/day which the authors state corresponds roughly to 300 µg/kg bw per day), was administered by subcutaneous injection to male Holtzman rats on the first 5 days postnatally. The testes were collected on day 125 (adult) and increased Dnmt expression and Esr1 and Esr2 promoter region hypermethylation were observed.

This limited but well performed study of epigenetic effects on the rat testis suggests that BPA exposure during fetal/neonatal life could disrupt estrogen receptor mediated signalling at least in part by Esr hypermethylation and might be a mechanism contributing to disrupted testis development in rodents caused by BPA. The acute nature of the exposure (and the fact that adverse effects on the testes do not appear to have been checked) renders extrapolation to the human difficult.

Horstman et al. 2012. Effects of transplacental 17-α-ethynyl estradiol or bisphenol A on the developmental profile of steroidogenic acute regulatory protein in the rat testis. Birth Defects Research (Part B) 00:1–8

In this study pregnant Sprague Dawley rats were dosed from gestational day 11 with either oestradiol or BPA by the subcutaneous route. Doses of oestradiol were 0.001, 0.1 or 10 µg/kg/day or BPA at 0.02, 0.5, 400 mg/kg/day. Fetal testes were harvested on gestational days 16, 18 or 20. They were studied using quantitative reverse transcriptase PCR for changes in steroidogenic acute regulatory (StAR) protein transcript levels and immunocytochemistry for StAR protein. Neither oestradiol nor BPA exposure caused morphological changes in the developing seminiferous tubules or the interstitial region at gestational days16–20. However, BPA and oestradiol slightly reduced StAR mRNA and protein levels at gestational day 18 and 20 but only the highest doses of 10 µg/kg/day oestradiol or 400 mg/kg/day BPA. Immunohistochemistry also demonstrated decreases in StAR protein levels but again only at the highest doses. Whilst this study demonstrates the potential effects of neonatal exposure to BPA on testicular function of offspring, this seems to be limited to high exposures which are probably not relevant to human exposures.

(iii) Epigenetics

In vivo studies on epigenetic effects of BPA

Anderson OS, Nahar MS, Faulk C, Jones TR, Liao C, Kannan K, Weinhouse C, Rozek LS and Dolinoy DC, 2012. Epigenetic responses following maternal dietary exposure to physiologically relevant levels of bisphenol A. Environmental and Molecular Mutagenesis, 53, 334-342.

Anderson et al. (2012) tested the effect of 3 doses of BPA given via the diet (50 ng BPA/kg feed, 50 µg BPA/kg feed, 50 mg BPA/kg feed) before and during pregnancy until lactation day 22 using the viable yellow agouti (A^{vy}) mouse model. Virgin *a/a* dams (aged 6 weeks) were treated orally with BPA via a phytoestrogen-free diet for 2 weeks, before mating with A^{vy}/a males. All animals were housed in polycarbonate-free cages and provided ad libitum access to diet and BPA-free water. Dams remained on the assigned diet throughout pregnancy and lactation The A^{vy}/a offspring were examined on PND 22. Nine to 14 litters were used per each experimental group, and therefore the sample size was acceptable. Analysis of coat color phenotype replicated previous results showing that the distribution of 50 mg BPA/kg A^{vy}/a offspring shifts toward yellow by decreasing DNA methylation in the retrotransposon upstream of the Agouti gene. Maternal exposure to 50 µg or 50 ng BPA/kg, however, resulted in altered coat color distributions in comparison with control, but no DNA methylation effects at the Agouti gene were noted. DNA methylation at the CDK5 activator-binding protein ($Cabp^{IAP}$) metastable epiallele shows hypermethylation in the 50 µg BPA/kg - offspring, compared with controls. Free and conjugated BPA concentrations were analyzed
in a subset of d22 mouse liver samples (1 pup per litter) from each BPA exposure and control group as well as in 51 human fetal liver samples. Total BPA concentrations in human fetal liver ranged from below LOQ to 96.8 ng/g. Comparison of exposed mouse liver BPA levels to human fetal liver BPA levels led the authors to state that the three experimental exposures were physiologically relevant. The authors concluded that perinatal BPA exposure affects offspring phenotype and epigenetic regulation across multiple doses.

Bromer JG, Zhou Y, Taylor MB, Doherty L and Taylor HS, 2010. Bisphenol-A exposure in utero leads to epigenetic alterations in the developmental programming of uterine estrogen response. FASEB Journal, 24, 2273-2280.

Bromer at al. (2010) studied whether an epigenetic mechanism underlies BPA-mediated alterations in *Hoxa10* expression. Pregnant CD-1 mice (housed in polypropylene cages) were treated with BPA (5 mg/kg bw, intraperitoneal) or vehicle control on days 9–16 of pregnancy. Food (Purina chow) and water were given ad libitum (no other information were given as to the estrogenicity of the diet or the type of water bottles). For statistical analysis a Student's t-test not corrected for multiple comparisons was used. *Hoxa10* mRNA and protein expression were increased by 25% in the reproductive tract of mice exposed *in utero*. Bisulfite sequencing revealed that cytosine-guanine dinucleotide methylation was decreased from 67 to 14% in the promoter and from 71 to 3% in the intron of *Hoxa10* after *in utero* BPA exposure. Decreased DNA methylation led to an increase in binding of ER-alpha to the *Hoxa10* ERE both *in vitro* as and *in vivo* as determined by EMSA and chromatin immunoprecipitation, respectively. Diminished methylation of the ERE-containing promoter sequence resulted in an increase in EREdriven gene expression in reporter assays. The authors concluded that altered methylation is a mechanism of BPA-induced altered developmental programming and that permanent epigenetic alteration of ERE sensitivity to estrogen may be a general mechanism through which endocrine disruptors exert their action.

Chao HH, Zhang XF, Chen B, Pan B, Zhang LJ, Li L, Sun XF, Shi QH and Shen W, 2012. Bisphenol A exposure modifies methylation of imprinted genes in mouse oocytes via the estrogen receptor signaling pathway. Histochemistry and Cell Biology, 137, 249-259.

BPA-induced effects on DNA methylation and oocyte maturation were studied in postnatally exposed (s.c. injection of 0, 20 and 40 µg/kg) female CD-1 mice. No information on housing condition, diet

and water bottles were given. A hypomethylation of 3 gene (insulin like growth factor 2 receptor, Peg3 and H19) was observed along with a dose-dependent reduction in the mRNA expression of 4 methyltransferases. BPA-induced hypomethylation was abolished by an ER inhibitor. ER-alpha but not ER-betha mRNA and protein expression were significantly up-regulated in BPA-treated cells. Additionally, BPA induced an abnormal ratio of spindle assembling in meiosis I, which was not increased by dose.

Dolinoy DC, Huang D and Jirtle RL, 2007. Maternal nutrient supplementation counteracts bisphenol A-induced DNA hypomethylation in early development. Proceedings of the National Academy of Sciences of the United States of America, 104, 13056-13061.

Dolinoy et al. (2007) analysed the effect of maternal nutrient supplementation on bisphenol A-induced DNA hypomethylation using the agouti (A^{vy}) mouse model. Virgin *a*/*a* females, 8–10 weeks of age, were assigned to receive one of four diets: (*a*) modified AIN-93G diet (control), (*b)* modified AIN-93G containing 50 mg BPA/kg diet; (*c*) modified AIN-93G diet containing 50 mg BPA/kg diet and supplemented 250 mg genistein/kg diet (*d*) modified AIN-93G diet with 50 mg BPA/kg diet and supplemented with methyl donor compounds (4.3 mg folic acid/kg diet, 0.53 mg vitamin B12/kg diet, 5 g betaine/kg diet, 7.97 g choline chloride/kg diet). As doses were given as BPA-concentration in the diet, BPA-intake was not adjusted to body weight. Diets were provided 2 weeks before mating with A^{vy}/a males and throughout pregnancy and lactation. No information on cage and drinking bottle material was provided. The offspring was examined on PND 22. The sample size was acceptable. Maternal exposure to BPA shifted the coat color distribution of viable yellow agouti (A^{vy}) mouse offspring toward yellow by decreasing CpG methylation in an intracisternal A particle retrotransposon upstream of the *Agouti* gene. CpG methylation was also decreased at another metastable locus, the CDK5 activator-binding protein (*CabpIAP*). DNA methylation at the A^{vy} locus was similar in tissues from the three germ layers, supporting that epigenetic patterning during early stem cell development is sensitive to BPA exposure. Maternal dietary supplementation with either methyl donors like folic acid or the phytoestrogen genistein counteracted BPA-induced hypomethylation. The authors concluded that early developmental exposure to BPA can change offspring phenotype by stably altering the epigenome, an effect that can be counteracted by maternal dietary supplements.

Doherty LF, Bromer JG, Zhou Y, Aldad TS and Taylor HS, 2010. In utero exposure to diethylstilbestrol (DES) or bisphenol-A (BPA) increases EZH2 expression in the mammary gland: an epigenetic mechanism linking endocrine disruptors to breast cancer. Horm Cancer, 1, 146-155.

Doherty et al. (2010) studied the effect of BPA on expression and function of Enhancer of Zeste Homolog 2 (EZH2), a histone methyltransferase that has been linked to breast cancer risk and epigenetic regulation of tumorigenesis, in MCF-7 cells and in mammary glands of mice exposed in utero. DES served as positive control. Treatment of MCF-7 cells with BPA (2.5×10E−4, 2.5×10E−5, 2.5×10E−6, 2.5×10E−7, 2.5×10E−8M) or DES (5×10E−6, 5×10E−7, 5×10E−8, 5×10E−9, 5×10E−10 M) led to a 3- and 2-fold increase in EZH2 mRNA expression, respectively as well as increased EZH2 protein expression. Histone H3 trimethylation was increased in MCF-7 cells treated with BPA or DES. Student's t-test was not corrected for multiple comparisons. For the in in vivo studies, environmental contamination was controlled by means of the use of standard polypropylene cages but no information was given on drinking bottles. Mice exposed to DES in utero (maternal dose: 10 µg DES/kg bw, intraperitoneal on gestation day 9-26) showed a >2-fold increase in EZH2 expression in adult mammary tissue compared with controls. EZH2 protein was elevated in mammary tissue of mice exposed to DES or BPA (maternal dose: 5 mg BPA/kg, i.p. on gestation day 9-26). Mice exposed to BPA or DES in utero also showed increased mammary histone H3 trimethylation. The authors suggested that developmental programming of EZH2 is a novel mechanism by which in utero exposure to BPA and DES leads to epigenetic regulation of the mammary gland.

Doshi T, D'Souza C, Dighe V and Vanage G, 2012. Effect of neonatal exposure on male rats to bisphenol A on the expression of DNA methylation machinery in the postimplantation embryo. Journal of Biochemical and Molecular Toxicology, 26, 337-343.

Doshi et al. (2012) addressed the mechanism involved in resorption of rat embryos (postimplantation loss; POL) as a result of BPA treatment (PND 1-5). Neonatal male pups received 5 subcutaneous injections of BPA (400 µg/kg bw) for the first 5 days of life (PND 1-5) or sesame oil (vehicle). On , PND 75 BPA-treated and control males (12/group) were mated with normal cycling female (n=24). Animals were fed a diet of soy-free, in-house-prepared rat pellets, and water ad libitum throughout the study. No information was provided on cages and drinking water bottle material. Pregnant rats were sacrificed on GD 20. qPCR expression data were measured on "BPA resorbed embryos". The authors reported that neonatal exposure of male rats to BPA downregulated the gene expression of Dnmts and related transcription factors in resorbed embryos as compared with the viable embryo, and suggested that BPA may have altered the sperm epigenome, which might have affected the embryo development leading to an increase in the postimplantation loss. The expression levels were calculated in relation to endogenous control ribosomal L19 gene. However, the authors neither provided data on endogenous L19 expression levels in controls as compared to BPA-treated, nor a comment on the degree of resorption/ tissue lysis, preventing the validity of the relative expression values from being estimated.

Ho S-M, Tang W-Y , Belmonte de Frausto J and Prins GS, 2006. Developmental exposure to estradiol and bisphenol A increases susceptibility to prostate carcinogenesis and epigenetically regulates phosphodiesterase type 4 variant 4. Cancer Research 66, 5624-5632.

Ho et al. (2006) studied the effect of neonatal exposure of rats to BPA (0.1 g/pup; 10 g/kg bw) on the occurrence of prostate intraepithelial neoplasia (PIN) and DNA methylation pattern. 17ß-oestradiol-3-benzoate (high dose: 25 µg EB/pup=2500g/kg bw; low dose: 0.1 g/pup=10 µg/kg bw) served as positive control. The compounds were administered subcutaneously on PND 1, 3 and 5. The sample size was acceptable and environmental contamination was controlled (new polysulfone cages, water in glass bottles, low exposure to phytoestrogens (12 ppm), one feed batch for whole experiment). At PND 90, an increased in the incidence and score of prostate intraepithelial hyperplasia (PIN), associated with an increased prostatic cell turnover was observed in BPA treated rats. For phosphodiesterase type 4 variant 4 (PDE4D4), an enzyme responsible for cyclic AMP breakdown, a specific methylation cluster was reported in the 5-flanking CpG island. In normal prostate, this site gradually was hypermethylated with aging, resulting in loss of gene expression. Neonatal exposure to BPA resulted in continued, elevated PDE4D4 expression. Studies with a normal prostatic epithelial cell line (NbE-1) and a rat cancer cell line (AIT) confirmed that site-specific methylation is involved in transcriptional silencing of the PDE4D4 gene and showed hypomethylation of this gene in prostate cancer cells. The PDE4D4 alterations in BPA-exposed prostates were distinguishable before histopathologic changes of the gland. The authors concluded that low-dose exposures to BPA affect the prostate epigenome during development and thereby promote prostate disease with aging.

Rosenfeld CS, Sieli PT, Warzak DA, Ellersieck MR, Pennington KA and Roberts RM, 2013. Maternal exposure to bisphenol A and genistein has minimal effect on Avy/a offspring coat color but favors birth of agouti over nonagouti mice. Proceedings of the National Academy of Sciences of the United States of America, 110, 537-542.

Rosenfeld et al. (2013) fed groups of C57/B6 *a/a* females, which are nonagouti, either a phytoestrogen-free control diet or one of six experimental diets: diets 1–3 contained BPA (50 mg, 5 mg, and 50 µg BPA/kg food, respectively); diet 4 contained genistein (G; 250 mg/kg food); diet 5 contained G plus BPA (250 and 50 mg/kg food, respectively); and diet 6 contained 0.1 µg of ethinyl oestradiol (EE)/kg food. Mice were bred to A^{vy}/a males over multiple parities. In all, 2,824 pups from 426 litters were born. None of the diets provided any significant differences in relative numbers of brown, yellow, or intermediate coat color A^{vy}/a offspring. However, BPA plus G ($P < 0.0001$) and EE diets ($P = 0.005$), but not the four others, decreased the percentage of black (a/a) to A^{vy}/a offspring from the expected Mendelian ratio of 1:1. The authors concluded that – in contrast to Anderson et al.

2012, Dolinoy et al. 2006 (genistein), 2007(BPA)- the present study indicates that exposure of A^{vy}/a conceptuses to genistein and BPA through maternal diet did not cause any consistent shift in offspring coat color relative to controls. However, Rosenfeld noted that two diets likely to promote an enriched estrogenic environment (BPA plus genistein; ethinyloestradiol) distorted the anticipated 1:1 ratio of agouti A^{vy}/a to nonagouti *a/a* offspring in $a/a \times A^{vy}/a$ crosses in favor of the latter. This effect became more pronounced with parity and according to the authors, possibly because the expression of the paracrine agouti-regulated protein (AGRP; synom. agouti signaling protein (ASIP)) provides a short-term, competitive advantage *in utero*.

Tang WY, Morey LM, Cheung YY, Birch L, Prins GS and Ho SM, 2012. Neonatal exposure to estradiol/bisphenol A alters promoter methylation and expression of Nsbp1 and Hpcal1 genes and transcriptional programs of Dnmt3a/b and Mbd2/4 in the rat prostate gland throughout life. Endocrinology, 153, 42-55.

Tang et al. (2012) studied the effects of neonatal BPA treatment (10 µg/kg bw, subcutaneous injection on PND 1, 3 and 5) at PND 10, 90 and 200 in male Sprague-Dawley rats. Animals were housed in new polysulfone cages, and given deionized water from glass bottles, and fed with a single feed lot of soy-free, phytoestrogen-reduced diet containing 12 ppm phytoestrogens as determined by high performance liquid chromatography. Litter effects were properly avoided. The promoter of nucleosome binding protein-1 (Nsbp1) was found to be hypomethylated. Hippocalcin-like 1 (Hpcal1) was reported to be progressively demethylated during aging but this age-related process was found to be blocked by neonatal BPA exposure, resulting in silencing of RNA-expression. Early and persistent overexpression were reported for DNA methyltransferases (Dnmt 3a/b) and methyl CpG binding protein (Mbd2/4), which was not a function of DNA methylation at their promoters. The authors suggested that their lifelong aberrant expression implicates them in early-life reprogramming and prostate carcinogenesis during adulthood.

Yaoi T, Itoh K, Nakamura K, Ogi H, Fujiwara Y and Fushiki S, 2008. Genome-wide analysis of epigenomic alterations in fetal mouse forebrain after exposure to low doses of bisphenol A. Biochem Biophys Res Commun, 376, 563-567.

Yaoi et al. (2008) studied the effect of maternal exposure to BPA (20 µg BPA/kg of body weight , subcutaneous injection once daily from E0; dams sacrificed at E12.5 or E14.5) on the epigenome in mouse forebrain. The CpG methylation status was scanned in 2500 NotI loci, representing 48 (de)methylated unique loci. Methylation status in most of them was primarily developmental stage-dependent. Each of almost all cloned NotI loci was located in a CpG island (CGI) adjacent to 5′ end of the transcriptional unit. The mRNA expression of two functionally related genes changed with development as well as the exposure to BPA, namely: 1. Vps52, encoding a protein constituting a protein complex involved in the Golgi-associated retrograde transport system; 2. LOC72325, encoding a hypothetical protein with a functional domain (Vps9) that catalyzes nucleotide exchange on a small GTPase, Rab5. In both genes, changes at the transcriptional level correlated with the changes in NotI methylation status. The authors concluded that epigenetic alterations in promoter-associated CGIs after exposure to BPA may underlie some effects on brain development.

Zhang XF, Zhang LJ, Feng YN, Chen B, Feng YM, Liang GJ, Li L and Shen W, 2012b. Bisphenol A exposure modifies DNA methylation of imprint genes in mouse fetal germ cells. Molecular Biology Reports, 39, 8621-8628.

Zhang et al. treated pregnant mice from 0.5 day post coitum with BPA at doses of 0, 40, 80 and 160 µg BPA/kg body weight/day (orally via (Eppendorf pipette) until 12dpc. DNA methylation of imprinting genes, *Igf2r, Peg3* and *H19*, was decreased with the increase of BPA concentration in fetal mouse germ cells. The relative mRNA levels of Nobox were lower in BPA-treated group compared to control (BPA free) in female fetal germ cells, but in male fetal germ cells, a significant higher in Nobox expression was observed in BPA-treated group compared to control. Decreased mRNA

expression of specific meiotic genes including *Stra8* and *Dazl* were obtained in the female fetal germ cells. The authors concluded that BPA exposure can affect the DNA methylation of imprinting genes in fetal mouse germ cells.

Cell culture studies on epigenetic effects of BPA

Avissar-Whiting M, Veiga KR, Uhl KM, Maccani MA, Gagne LA, Moen EL and Marsit CJ, 2010. Bisphenol A exposure leads to specific microRNA alterations in placental cells. Reprod Toxicol, 29, 401-406.

Avissar-Whiting et al. (2010) investigated the effect of BPA (0,25 to 25 ng/µL of BPA for six days (medium refreshed on day 2 and 4) on microRNAs (miRNAs) in human placental cells. miRNA microarray was performed following BPA treatment in three immortalized cytotrophoblast cell lines (3A, first-trimester villous cells; TCl-1, third trimester extravillous cells; HTR-8, first trimester extravillous cells) and the results validated using quantitative real-time PCR. For functional analysis, overexpression constructs were stably transfected into cells that were then assayed for changes in proliferation and response to toxicants. Microarray analysis revealed several miRNAs to be significantly altered in response to BPA treatment in two cell lines (3A and HR-8). Real-time PCR results confirmed that *miR-146a* was particularly strongly induced and its overexpression in cells led to slower proliferation as well as higher sensitivity to the DNA damaging agent, bleomycin. The authors concluded that BPA can alter miRNA expression in placental cells, a potentially novel mode of BPA toxicity.

Fernandez SV and Russo J, 2010. Estrogen and xenoestrogens in breast cancer. Toxicology and Pathology, 38, 110-122.

Fernandez previously demonstrated that BPA was able to induce the transformation in vitro of human breast epithelial cells. While the normal-like human breast epithelial cell line, MCF-10F, formed tubules in collagen (3-D cultures), treatment with BPA (10E-5 M and 10E-6 M BPA) reduced the cells tubules production (73% and 80%, respectively) and produced some spherical masses (27% and 20%, respectively). In the present study, expression and DNA methylation analyses were performed in these cells after exposure to BPA. These cells showed an increased expression of *BRCA1*, *BRCA2*, *BARD1*, *CtIP*, *RAD51* and *BRCC3*, all of which are genes involved in DNA repair, as well as the downregulation of *PDCD5* and *BCL2L11 (BIM*), both of which are involved in apoptosis. Furthermore, DNA methylation analysis showed that the BPA exposure induced the hypermethylation of *BCL2L11*, *PARD6G*, *FOXP1* and *SFRS11*, as well as the hypomethylation of *NUP98* and *CtIP (RBBP8).* The authors concluded that normal human breast epithelial cells exposed to BPA have increased expressions of genes involved in DNA repair in order to overcome the DNA damage induced by this chemical.

Hashimoto S, Shiomoto K, Okada K and Imaoka S, 2012. The binding site of bisphenol A to protein disulphide isomerase. Journal of Biochemistry, 151, 35-45.

In this mechanistic study, protein disulphide isomerase (PDI) was isolated as a binding protein of BPA in the rat brain. The authors determined and characterized the binding sites of BPA to PDI. The BPA-binding domain was identified with ab, b'a'c, a, b, b' and a'c fragment peptides of PDI by surface plasmon resonance spectroscopy. BPA interacted with ab, b'a 'c, a and b', suggesting that a and b' domains are important in their interaction. Second, ab, b'a'c, a,b,b',a', abb'a', abb', b'a', Δb' and a'c fragment peptides were used for their isomerase activity with RNase as a substrate. BPA could inhibit the activity of peptide fragments including b', suggesting that b' domain contributes to inhibition of catalytic activity of PDI by BPA. The authors investigated the BPA-binding capacity of PDI by amino acid substitution. PDI lost the BPA-binding activity by the mutation of H258 and mutation of Q245 and N300 also decreased its activity. Furthermore, acidic condition increased the BPA-binding activity of PDI. Based on their findings, the authors concluded that the charge of these amino acid especially, H258, is important for the BPA binding to PDI.

Qin X-Y , Fukuda T , Yang L, Zaha H, Akanuma H, Zeng Q, Yoshinaga J and Sone H, 2012b. Effects of bisphenol A exposure on the proliferation and senescence of normal human mammary epithelial cells. Cancer Biology Therapy 13, 1-11.

See study description in Appendix B under section "Carcinogenicity – In vitro studies/Mechanisms of action".

Weng YI, Hsu PY, Liyanarachchi S, Liu J, Deatherage DE, Huang YW, Zuo T, Rodriguez B, Lin CH, Cheng AL and Huang TH, 2010. Epigenetic influences of low-dose bisphenol A in primary human breast epithelial cells. Toxicology and Applied Pharmacology, 248, 111-121.

Weng et al. (2010) examined the effect of BPA epigenetic changes in breast epithelial cells using mammospheres as a model. Breast progenitor cells from noncancerous human mammary tissues were enzymatically dissociated and grown into floating spherical colonies so called mammospheres. These mammospheres enriched in breast progenitor cells were exposed to BPA (4 nM), or DMSO for 3 weeks. DES (70 nM) served as positive control. The differentiated cells were studied using immunofluorescence with anti-ERα antibody, gene expression microarrays and reverse transcription-quantitative PCR. The effect of BPA on the ERα signaling pathway and global gene expression profiles was investigated. Compared to control cells, nuclear internalization of ERα was observed in epithelial cells pre-exposed to BPA. The authors identified 170 genes with expression changes in response to BPA. Functional analysis confirmed that gene suppression was mediated in part through an ERα-dependent pathway. As a result of exposure to BPA or other oestrogen-like chemicals, the expression of lysosomal-associated membrane protein 3 (LAMP3) became epigenetically silenced in breast epithelial cells. Furthermore, increased DNA methylation in the LAMP3 CpG island positively correleated with ERα-positive breast tumours. This study shows potential epigenetic alterations to progenitor mammary cells in response to BPA in vitro.

(iv) Excluded studies

Excluded in vivo mixture studies

The following animal studies in which BPA was tested as part of a mixture of chemicals were excluded a priori from the evaluation.

- Christiansen S, Kortenkamp A, Axelstad M, Boberg J, Scholze M, Jacobsen PR, Faust M, Lichtensteiger W, Schlumpf M, Burdorf A and Hass U, 2012. Mixtures of endocrine disrupting contaminants modelled on human high end exposures: an exploratory study in rats. International Journal of Andrology, 35, 303-316.

- Manikkam M, Tracey R, Guerrero-Bosagna C and Skinner MK, 2013. Plastics Derived Endocrine Disruptors (BPA, DEHP and DBP) Induce Epigenetic Transgenerational Inheritance of Obesity, Reproductive Disease and Sperm Epimutations. PLoS One, 8, e55387.

- Xi W, Wan HT, Zhao YG, Wong MH, Giesy JP, Wong CK, 2011. Effects of perinatal exposure to bisphenol A and di(2-ethylhexyl)-phthalate on gonadal development of male mice. Environmental Science and Pollution Research International, 19, 2515-2527.

Excluded in vitro studies

Studies using high concentrations of BPA ($\geq 10^{-6}$ M) which were not considered by the CEF Panel as relevant for risk assessment and therefore excluded from this review.

- Aoki T and Takada T, 2012. Bisphenol A modulates germ cell differentiation and retinoic acid signaling in mouse ES cells. Reproductive Toxicology, 34, 463-470.

- Bulzomi P, Bolli A, Galluzzo P, Acconcia F, Ascenzi P and Marino M, 2012. The naringenin-induced proapoptotic effect in breast cancer cell lines holds out against a high bisphenol a background. IUBMB Life, 64, 690-696.

- Huang H, Tan W, Wang CC and Leung LK, 2012. Bisphenol A induces corticotropin-releasing hormone expression in the placental cells JEG-3. Reproductive Toxicology, 34, 317-322.

- Kang NH, Hwang KA, Kim TH, Hyun SH, Jeung EB and Choi KC, 2012. Induced growth of BG-1 ovarian cancer cells by 17β-oestradiol or various endocrine disrupting chemicals was reversed by resveratrol via downregulation of cell cycle progression. Molecular Medicine Reports, 6, 151-156.

- Li Z, Zhang H, Gibson M and Li J, 2012. An evaluation on combination effects of phenolic endocrine disruptors by estrogen receptor binding assay. Toxicology in Vitro, 26, 769-774.

- Lee MS, Lee YS, Lee HH and Song HY, 2012c. Human endometrial cell coculture reduces the endocrine disruptor toxicity on mouse embryo development. Journal of Occupational Medicine and Toxicology 7, 7.

- Taxvig C, Dreisig K, Boberg J, Nellemann C, Blicher Schelde A, Pedersen D, Børgesen M, Mandrup S and Vinggaard AM, 2012. Differential effects of environmental chemicals and food contaminants on adipogenesis, biomarker release and PPARγ activation. Molecular and Cellular Endocrinology, 361, 106-115.

Excluded studies (Jan 2012 - Sept 2012) from the list submitted by "Réseau Environnement Santé" (RES, 2012)

The compilation of published scientific studies on BPA submitted by Réseau Environnement Santé (RES, 2012) to the European Commission was compared with EFSA's comprehensive literature database. The few publications identified as missing were screened against the relevance criteria defined in Appendix I. As a result of this screening the following studies were excluded from this review for the motivations indicated.

- Maserejian NN, Trachtenberg FL, Hauser R, McKinlay S, Shrader P, Tavares M and Bellinger DC, 2012. Dental Composite Restorations and Psychosocial Function in Children. Pediatrics, 130, E328-E338.

  Reason: bisGMA-based dental composite restorations, not directly BPA.

- Kuan YH, Huang FM, Li YC and Chang YC, 2012a. Proinflammatory activation of macrophages by bisphenol A-glycidyl-methacrylate involved NF kappa B activation via PI3K/Akt pathway. Food and chemical toxicology, 50, 4003-4009.

  Reason: bisGMA-based dental composite restorations, not directly BPA.

- Lee S, Kim YK, Shin TY and Kim SH, 2013c. Neurotoxic Effects of Bisphenol AF on Calcium-Induced ROS and MAPKs. Neurotoxicity Research, 23, 249-259.

  Reason: BPAF, not directly BPA.

- O'Boyle NM, Delaine T, Luthman K, Natsch A and Karlberg A-T, 2012. Analogues of the Epoxy Resin Monomer Diglycidyl Ether of Article Bisphenol F: Effects on Contact Allergenic Potency and Cytotoxicity. Chemical Research in Toxicology, 25, 2469-2478.

Reason: DGEBF, not directly BPA.

- Chevalier N, Vega A, Bouskine A, Siddeek B, Michiels J-F, Chevallier D and Fenichel P, 2012. GPR30, the Non-Classical Membrane G Protein Related Estrogen Receptor, Is Overexpressed in Human Seminoma and Promotes Seminoma Cell Proliferation. PLoS One, 7.

  Reason: Not dealing with BPA.

- Feng Y, Yin J, Jiao Z, Shi J, Li M and Shao B, 2012. Bisphenol AF may cause testosterone reduction by directly affecting testis function in adult male rats. Toxicology Letters, 211, 201-209.

  Reason: BPAF, not directly BPA.

- Liao C, Liu F and Kannan K, 2012. Bisphenol S, a New Bisphenol Analogue, in Paper Products and Currency Bills and Its Association with Bisphenol A Residues. Environmental Science & Technology, 46, 6515-6522.

  Reason: BPS, not directly BPA.

- Trentham-Dietz A, Sprague BL, Wang J, Hampton JM, Buist DSM, Bowles AE, Sisney G, Burnside E, Hemming J and Hedman C, 2012. Phenol Xenoestrogens and Mammographic Breast Density. Cancer Epidemiology Biomarkers & Prevention, 21, 561-562.

  Reason: Only a Congress Abstract

- Liu X, Matsushima A, Nakamura M, Costa T, Nose T and Shimohigashi Y, 2012. Fine spatial assembly for construction of the phenol-binding pocket to capture bisphenol A in the human nuclear receptor estrogen-related receptor gamma. Journal of Biochemistry, 151, 403-415.

  Reason: Structural biology and biophysics, no toxicity

- Blasiak J, Synowiec E, Tarnawska J, Czarny P, Poplawski T and Reiter RJ, 2012. Dental methacrylates may exert genotoxic effects via the oxidative induction of DNA double strand breaks and the inhibition of their repair. Molecular Biology Reports, 39, 7487-7496.

  Reason: Bis-GMA, not directly BPA.

- Li YC, Kuan YH, Huang FM and Chang YC, 2012d. The role of DNA damage and caspase activation in cytotoxicity and genotoxicity of macrophages induced by bisphenol-A-glycidyldimethacrylate. International Endodontic Journal, 45, 499-507.

  Reason: Bis-GMA, not directly BPA.

- Kuan YH, Li YC, Huang FM and Chang YC, 2012b. The upregulation of tumour necrosis factor-α and surface antigens expression on macrophages by bisphenol A-glycidyl-methacrylate. International Endodontic Journal, 45, 619-626.

  Reason: Bis-GMA, not directly BPA.

- Rowas SA, Haddad R, Gawri R, Al Ma'awi AA, Chalifour LE, Antoniou J and Mwale F, 2012. Effect of in utero exposure to diethylstilbestrol on lumbar and femoral bone, articular cartilage, and the intervertebral disc in male and female adult mice progeny with and without swimming exercise. Arthritis Res Ther, 14.

Reason: Not dealing with BPA.

- Michelsen VB, Kopperud HB, Lygre GB, Bjorkman L, Jensen E, Kleven IS, Svahn J and Lygre H, 2012. Detection and quantification of monomers in unstimulated whole saliva after treatment with resin-based composite fillings in vivo. European Journal of Oral Sciences, 120, 89-95.

  Reason: Bis-GMA, not directly BPA.

- Hsu WY, Wang VS, Lai CC and Tsai FJ, 2012. Simultaneous determination of components released from dental composite resins in human saliva by liquid chromatography/multiple-stage ion trap mass spectrometry. Electrophoresis, 33, 719-725.

  Reason: No directly BPA

Appendix C. WoE approach to hazard identification

A detailed description of the approach taken to the hazard identification is given in the methodological section (Appendix A). After being grouped by macro-areas of interest, e.g. reproductive and developmental effects, and relative study type, i.e. human, animal or in vitro study the relevant studies were appraised on their strengths and weaknesses and included in a structured WoE approach to hazard identification to identify the critical toxicological effects ("likely" or "very likely" effects) for BPA. A tabular format was developed to facilitate the consistent treatment of the whole body of human and animal evidence and transparently document the WoE analysis.

For each toxicological endpoint, different *questions* (Q1, Q2, etc.) were formulated to address the association between BPA and the endpoint (e.g. "does BPA cause ... (type of effect)?". These were placed in the first column on the top left handside of the WoE table. Under each question (first column) the conclusions from the 2006 and/or 2010 EFSA opinions on BPA were taken as a *"starting point"* for an answer. The new studies relevant to each question (see Appendices B and C) were organised into a number of "*lines of evidence*" in order to address the different findings that relate to the question concerned. Some lines of evidence referred to a single study, whereas others referred to a group of studies addressing the same issue. In the same first column the CEF Panel also summarised the strengths and weaknesses of each study and/or line of evidence, and those of the pre-2010 EFSA assessments.

The second column of the WoE tables reports the *"answer to the question as reported by the study authors"* (e.g. a positive, negative or uncertain answer to the question), irrespectively of the CEF Panel's consideration of the study strengths and weaknesses.

The third column of the tables gives the CEF Panel's assessment of the *"reliability"*of each line of evidence, expressed qualitatively on a scale of low, medium or high. The CEF Panel considered strengths and weaknesses when judging the reliability of each line of evidence. Notably, there was not a pre-defined outcome of a study reliability based on its number of strengths and weaknesses, given their different relative weight. The reliability of the evidence was agreed based on collective expert judgement (starting from a proposal made by the study's rapporteur and co-rapporteur, that was discussed at least at one but more commonly at many WG meetings and that then was reviewed by the CEF Panel during Plenary meetings).

In the fourth column the weight or influence of each line of evidence on the "*likelihood*" of a positive answer to each question is expressed using a defined set of symbols (see table 30) when considered independently of the other lines of evidence. The number (from one to three) of upward and downward arrows indicates the degree (small, medium, high) of the impact of the new evidence to increase or decrease, respectively, the likelihood of a positive answer to the question. The "dot" is used when the reliability of the new line of evidence is considered as insufficient as to have an impact on the likelihood of a positive answer to a question. Pairs of symbols indicate uncertainty about the influence, e.g. ●/↑ indicate between negligible and minor positive influence on likelihood.

Table 30: Definition of symbols used for expressing the influence on likelihood of each line of evidence in the WoE tables

Symbols	Interpretation
↑	minor contribution to increasing likelihood
↑↑	moderate contribution to increasing likelihood
↑↑↑	major contribution to increasing likelihood
↓	minor contribution to decreasing likelihood
↓↓	moderate contribution to decreasing likelihood
↓↓↓	major contribution to decreasing likelihood
●	negligible influence on likelihood

A conclusion on the overall likelihood that BPA exposure was associated with a particular effect in humans and/or animals was expressed in the bottom row both as a narrative statement and using a seven level-scale of likelihood terms, ranging from "very unlikely" to "very likely" (see box below). Note that, on this scale, "As likely as not" means a level of likelihood between "Unlikely" and "Likely", where it is about equally likely that BPA causes, or does not cause, the effect.
Such conclusion was drawn after considering the individual influences of all the lines of evidence and the starting point. As for the judgement of the reliability of each line of evidence, also the assessment of the overall likelihood resulted from from a complex and collective expert judgement, as opposed to the application of a pre-defined formula.

Set of standard terms used for expressing the overall likelihood in the WoE tables (adapted from Mastrandrea et al., 2010)

Likelihood
Very likely
Likely
From -as likely as not- to likely
As likely as not
From unlikely to -as likely as not-
Unlikely
Very unlikely

.

WoE of reproductive and developmental effects

Whether BPA has the potential to cause developmental and reproductive effects in humans and animals was considered using a tabular format for weighing different lines of evidence (WoE evaluation). The WoE evaluation tables for these endpoints are presented in full below.

Human studies

Table 31: Assessment of the likelihood of associations between BPA exposure and developmental and reproductive effects in humans.

Q1: Is there an association between BPA exposure and reproductive and health effects in humans?	**Answer to the question as reported by the study authors (Positive, Negative or Uncertain)**	**Reliability of evidence (Low, Medium or High)**	**Influence on Likelihood (see Table 28)**
Starting point based on previous assessments (EFSA CEF Panel, 2010). Eight studies investigating the association between BPA exposure and reproductive disorders in human adults (Itoh et al., 2007; Braun et al., 2009; Cobellis et al., 2009; Yang et al., 2009; Li et al., 2010a, b; Meeker et al., 2010; Mendiola et al., 2010; Mok-Lin et al., 2010). *Weakness:* The CEF Panel noted that the studies were limited by their mostly cross sectional design	Positive	Low	●
Line of Evidence 1: Associations with embryo quality and implantation success during IVF Several studies reported inverse associations between increasing BPA levels in serum or urine and one or more parameters of embryo quality and implantation (Fujimoto et al., 2010; Bloom et al., 2011a; 2011b; Ehrlich et al., 2012a; 2012b). *Strengths*: – Prospective study design (Ehrlich et al., 2012a; 2012b) – Urine, contained specified (Ehrlich et al., 2012a; 2012b) – Repeated measurements (≥ 2) (Ehrlich et al., 2012a; 2012b) – Standardised samples (specific gravity) (Ehrlich et al., 2012a; 2012b) – Analytical method (LC-MS-MS) (Ehrlich et al., 2012a; 2012b) – Quality controls, including blanks (all studies) *Weaknesses:* – Cross-sectional study design (Fujimoto et al., 2010; Bloom et al., 2011a, b)	Positive	Low	●

– Short time frame (only days) (Ehrlich et al., 2012a; 2012b) – Small sample size (Fujimoto et al., 2010; Bloom et al., 2011a; b) – Serum BPA measurement (Fujimoto et al., 2010; Bloom et al., 2011a, b) – Single exposure measurements (Fujimoto et al., 2010; Bloom et al., 2011a; b) – No distinction between unconjugated and conjugated BPA (Ehrlich et al., 2012a; 2012b) – Potential BPA exposure by diet or by concurring exposure factors (contamination through medical treatment during IVF) not reported (all studies) – Poor generalisability for the population other than IVF couples (all studies)			
Line of Evidence 2: Associations with semen quality. One study showed association with semen quality in occupationally and environmentally exposed workers (Li et al., 2011) *Comment:* Confounding by multiple chemical exposures was evaluated *Strengths:* – Standardised samples (urinary creatinine or specific gravity) *Weaknesses:* – Cross-sectional study design – Selection bias of the study population (58 % participation rate, without explanation) – Single spot urine BPA measurement (for men without occupational exposure) – No quality control and quality assurance procedures – No distinction between unconjugated and conjugated BPA – Confounding by diet not considered – Occupational exposure	Positive	Low	●
Line of Evidence 3: Associations with sex hormones. One study showed weak association with testosterone in men only, no associations with other sex hormones examined and no associations with sex hormones in women (Galloway et al., 2010). One study showed associations with sex hormones in men (Zhou et al., 2013) *Strenghts:* – Standardised samples (24-h urine collection, urinary creatinine) (Galloway et al., 2010) – Analytical method (SPE LC-MS-MS) (Galloway et al., 2010) – Quality control, including blanks (all studies) *Weaknesses:* – Cross-sectional study design (all studies) – Small sample size (Zhou et al., 2013) – Serum BPA measurement (Zhou et al., 2013) – Single exposure measurements (Zhou et al., 2013)	Positive/Negative	Low	● (men) ↓ (women)

– Confounding by diet or by concurring exposure factors not reported (all studies) – Unclear clinical relevance due to small effect size in men (Galloway et al., 2010) – Inconsistency in the results, significant association between BPA exposure and testosterone but no association for other hormones (Galloway et al., 2010) – Occupational exposure (Zhou et al., 2013)			
Line of Evidence 4: Associations with age of menarche. One study showed no association (Buttke et al., 2012) *Comment:* Confounding by multiple chemical exposures was evaluated *Strengths*: – Standardised samples (urinary creatinine) – Analytical method (SPE LC-MS-MS) *Weaknesses*: – Cross-sectional study design – Single spot urine BPA measurement – No distinction between unconjugated and conjugated BPA – Confounding by diet not considered	Negative	Low	↓
Line of Evidence 5: Associations with hormones and metabolic parameters in women with polycystic ovary syndrome (PCOS). Two studies reported associations (Kandaraki et al., 2010; Tarantino et al., 2012). *Weaknesses:* – Cross-sectional study design (all studies) – Small sample size (Tarantino et al., 2012) – Serum BPA measurement (all studies) – Single exposure measurements (all studies) – Analytical method (ELISA) (all studies) – No quality control and quality assurance procedures (all studies) – No distinction between unconjugated and conjugated BPA (all studies) – Statistics (unjustified use of non-parametric and parametric models) (Tarantino et al., 2012) – Generalisability to the overall population (other than women with PCOS) (all studies)	Positive	Low	•
Overall conclusion on Likelihood: An association between BPA and embryo quality and implantation success during IVF, semen quality, sex hormones or age of menarche in humans is considered unlikely.			**Unlikely**

Q2: Is there an association between BPA exposure and gestational /birth outcomes?	Answer to the question as reported by the study authors (Positive, Negative or Uncertain)	Reliability of evidence (Low, Medium or High)	Influence on Likelihood (see Table 28)
Starting point based on previous assessments (EFSA CEF Panel, 2010). Two studies investigated the association between BPA exposure and birth outcomes (Padmanabhan et al., 2008; Wolff et al., 2008), both were limited by having cross-sectional design.	Positive	Low	●
Line of Evidence 1: Associations with preterm delivery. The only study identified on this issue showed association with urinary BPA (Cantonwine et al., 2010) *Strenghts:* – Standardised samples (specific gravity and creatinine) – Analytical method (SPE LC-MS-MS) – Quality controls, including blanks *Weaknesses:* – Cross-sectional study design – Small sample size – Single spot urine BPA measurements – No distinction between unconjugated and conjugated BPA – Invalid/imprecise outcome assessment – Confounding by diet and concurring exposure factors not considered	Positive	Low	●
Line of Evidence 2: Associations with fetal growth. Three studies showed associations with growth restriction (Miao et al., 2011a; Snijder et al., 2013; Chou et al., 2011) and two studies showed associations with increased growth (Lee et al., 2013a; Philippat et al., 2012). *Strengths:* – Prospective study design (Miao et al., 2011a; Snijder et al., 2013; Lee et al., 2013a) – Repeated measurements (Snijder et al., 2013) – Container specified (Miao et al., 2011a; Snijder et al., 2013; Chou et al., 2011) – Standardised samples (urinary creatinine) (Miao et al., 2011a; Snijder et al., 2013; Lee et al., 2013a; Philippat et al., 2012) – Analytical method (LC-MS-MS) (Snijder et al., 2013; Lee et al., 2013a)	Positive	From Low to Medium	●/↑

– Quality controls, including blanks (Lee et al., 2013a; Chou et al., 2011) – Repeated growth measurement (Snijder et al., 2013) *Weaknesses*: – Cross-sectional study design (Chou et al., 2011) or case-control study (Philippat et al., 2012) – Long recall period (Miao et al., 2011a) – Blood/plasma and cord blood BPA measurement (Chou et al., 2011) – Single exposure measurements (Miao et al., 2011a; Lee et al., 2013a; Chou et al., 2011; Philippat et al., 2012) – No distinction between unconjugated and conjugated BPA (all studies) – Confounding by diet and concurring exposure factors not considered (all studies) – Unclear clinical relevance (small sample effect size) (Philippat et al., 2012) – Inconsistent results, some showed growth restriction (Miao et al., 2011a; Snijder et al., 2013; Chou et al., 2011) some showed increased growth (Lee et al., 2013a; Philippat et al., 2012) – Occupational exposure (Miao et al., 2011a)			
Line of Evidence 3: Associations with cryptorchidism. The only study identified on this issue showed no association (Fénichel et al., 2012) *Comment:* Sound statistical modelling *Strengths:* – Container specified (BPA-free) – Quality control, including blanks – Consistency in results among different studies *Weaknesses:* – Cross-sectional study design – Single exposure measurement – Analytical method (RIA, no correlation with GC-MS data for values in the low range) – Confounding by diet and concurring exposure factors not considered	Negative	Low	●
Line of Evidence 4: Associations with anogenital distance, congenital hypothyroidism and hypospadia. One study showed association with anogenital distance (Miao et al., 2011b), one study showed no association with congenital hypothyroidism (Jung et al., 2013) and one showed inconsistent associations with hypospadias (Choi et al., 2012) *Strengths:* – Container specified (BPA-free) (Choi et al., 2012; Miao et al, 2011b) – Analytical method (GC-MS) (Jung et al., 2013; Choi et al., 2012)	Positive (anogenital distance) Negative (congenital hypothyroidism) Inconsistent (hypospadias)	Low	●

Weaknesses: – Case-control study design (Choi et al., 2012; Jung et al., 2013; Miao et al., 2011b) – Small sample size (Miao at al., 2011b) – Invalid/imprecise BPA exposure assessment combination of paternal and maternal occupational exposure through inhalation (Miao at al., 2011b) – Plasma PBA measurement (Choi et al., 2012; Jung et al., 2013) – Single spot urine BPA measurement (Choi et al., 2012) – No distinction between conjugated and unconjugated BPA (Choi et al., 2012; Jung et al., 2013) – Confounding by diet and concurring exposure factors not considered (all studies) – Insufficient study reporting (Choi et al., 2012) – Statistics (Miao at al., 2011b; Jung et al., 2013; Choi et al., 2012) – Occupational exposure (Miao at al., 2011b)			
Line of Evidence 5: Associations with maternal and infant thyroid function. The only study identified on this issue showed associations with reduced TSH in neonates and reduced T4 in mothers (Chevrier et al., 2012) *Comment:* Iodine status (nutritional) was taken into account *Strengths:* – Prospective study design – Urine, container specified (BPA-free) – Repeated measurements – Standardised samples (creatinine) – Analytical method (SPE LC-MS-MS) – Quality controls, including blanks *Weaknesses:* – No distinction between unconjugated and conjugated BPA – Confounding by diet (except nutrition iodine) and concurring exposure factors not considered – Unclear clinical relevance (association between BPA and T4 observed in urine samples taken during the second half of pregnancy only).	Positive	Low	●/↑
Overall conclusion on Likelihood: There are indications from prospective studies that BPA exposure during pregnancy may be associated with fetal growth, and weak indications that BPA exposure during pregnancy may be associated with maternal and infant thyroid function. However, it cannot be ruled out that the results are confounded by diet or concurrent exposure factors. For fetal growth, two studies showed reduced fetal growth, while one study reported increased fetal growth with increasing maternal BPA exposure. The associations do not provide sufficient evidence to infer a causal link between BPA exposure and reproductive effects in humans.			**As likely as not**

Animal studies

Table 32: Assessment of the likelihood that BPA causes developmental and reproductive toxicity in animals when exposed during their adult life (post-pubertal) only.

NOTE: The cut-off of 5 mg/kg/day from Tyl et al. (2002) is used below as a rodent NOAEL. This figure has been translated into an HED of 3.6 mg/kg bw per day. All monkey, mouse and rat exposures have been converted into an HED using the values in Table 3 and studies with an effect ≤3.6 mg BPA/kg bw per day have been included below. The equivalent data for sheep are not available and the BPA doses for those studies have been used at equivalence for HED.

Q1: Does adult exposure to BPA at doses equal to, or below the HED NOAEL equivalent of 3.6 mg/kg/bw per day disturb reproductive capacity? (Dobrzynska and Radzikowska , 2013; Castro et al. 2013; Qiu et al. 2013; Jin et al. 2013; Liu et al., 2013; Tiwari and Vanage 2013; Lee et al., 2013b; Tan et al. 2013; El Ghazzawy et al. 2011)	**Answer to the question as reported by the study authors (Positive, Negative or Uncertain)**	**Reliability of evidence (Low, Medium or High)**	**Influence on Likelihood (see Table 28)**
Starting point based on previous assessments (EFSA CEF Panel, 2010): Conclusion on developmental and reproductive toxicity **Tyl et al. (2002)** CD Sprague-Dawley rats (n= 20 pregnant females) were exposed to dietary BPA in a three generation study at 0, 0.015, 0.3, 4.5, 75, 750, 7500 ppm (giving doses of approximately 0, 0.02, 0.3, 5, 50 and 500 mg/kg bw per day). Adult systemic toxicity at 750 and 7500 ppm in all generations included: reduced body weights and body weight gains, reduced absolute and increased relative weanling and adult organ weight (liver, kidney, adrenals, spleen, pituitary and brain), and female mild renal and hepatic pathology. Reproductive organ histology and function were unaffected, except for reduced ovarian weights, a significantly reduced number of implantation sites and decreased number of pups/litter on PND 0 at 7500 ppm. Adult oral NOAEL was 5 mg/kg bw per day. **Tyl et al. (2008)** In a two-generation study dietary BPA was given to CD-1 mice (n=28) at 0, 0.018, 0.18, 1.8, 30, 300, or 3500 ppm in feed (giving 0, 0.003, 0.03, 0.3, 5, 50, or 600 mg BPA/kg bw per day). 17β-oestradiol (0.5 ppm) was used as positive control. The oral NOAEL was 30 ppm (5 mg/kg bw per day) based on liver effects.	Negative	High	↓↓↓
Line of Evidence 1: new evidence on the effects of BPA on the adult testis (1) Dobrzynska and Radzikowska, 2013; (2) Qiu et al., 2013; (3) Jin et al., 2013; (4) Liu et al., 2013; (5) Tiwari and Vanage, 2013; (6) El Ghazzawy et al., 2011. *Comment*: Six studies, all in the rat: some effects on sperm counts *Strengths*:	Positive	Low-Medium	●/↑

- Number of doses (≥3) (Liu et al., 2013, Qiu et al., 2013, Dobrzynska and Radzikowska, 2013) - Adequate positive controls included (Liu et al., 2013, Jin et al., 2013) - Oral administration via gavage (El Ghazzawy et al., 2011, Liu et al., 2013, Qiu et al., 2013, Jin et al., 2013) - Use of non-PC cages (El Ghazzawy et al., 2011, Jin et al., 2013) - Use of glass bottle (Liu et al., 2013, Jin et al., 2013) - Phytoestrogen-free diet (e.g. soy-free diet) (Tiwari and Vanage, 2013) *Weaknesses:* - Single dose level study (El Ghazzawy et al., 2011, Jin et al., 2013) - No vehicle controls were tested (Dobrzynska and Radzikowska, 2013) - Drinking water consumption (containing BPA) not measured (Dobrzynska and Radzikowska, 2013) - Animal diet poorly described (El Ghazzawy et al., 2011, and Liu et al., 2013: animals were provided with a rodent experimental diet in which no phytoestrogens could be detected – this was not checked in the study, Qiu et al., 2013, Jin et al., 2013, Dobrzynska and Radzikowska, 2013) - Study design not appropriate to the scope (Qiu et al., 2013: control rats appeared to receive corn oil only rather than ethanol further diluted in corn oil as was the case for the BPA-exposed groups, Liu at al., 2013: description of the study design was poor and confusing in terms of exactly what groups received what and which were compared with what controls) - Inappropriate statistics (El Ghazzawy et al., 2011: no multiple comparisons statistics, Qiu et al., 2013: basic statistical analysis, Liu at al., 2013: statistics not adequate) - Insufficient study reporting (Jin et al., 2013: data presentation is confusing)			
Line of Evidence 2: new evidence on the effects of BPA on the adult prostate gland (7) Castro et al., 2013: The changes described in the rat, especially the skewing of the T/E2 ratio and increased aromatase is considered symptomatic of prostate disease. *Comment*: Dose-response to some BPA effects *Comment:* Data presented do not prove prostate disease *Comment*: Very short exposure (4 days) – acute response *Strengths:* - Number of doses (≥3) - Use of non-PC cages and of glass bottles *Weaknesses:* - Insufficient study reporting (animal diet poorly described)	Positive	Medium	•

Line of Evidence 3: new evidence on the effects of BPA on increased incidence of early delivery and disturbed endocrine and placental signaling. (8) Tan et al., 2013: Study in mice: increased plasma T, E2, CRH placental CREB and PKC. *Comment:* Majority of effects reported >3.6 mg/kg bw per day *Comment:* Assessment of early pregnancy loss used a good number of animals (>15 mice/dose) *Comment:* Effect on early delivery only significant when analysing all BPA groups and including group >3.6 mg/kg bw per day *Comment:* early delivery assessed in different group to signalling indices *Strengths:* - Number of doses (≥3), - Oral administration via gavage *Weaknesses:* - Animal diet poorly described Small sample size (small group size (3-5) for most measures other than pregnancy loss) - Animal diet and phytoestrogen content not reported	Positive	Low	•
Line of Evidence 4: new evidence on the effects of BPA on the adult ovary (9) Lee et al., 2013b: Study in rat: decreased circulating E2 and T associated with increased LH and increased ovarian cell apoptosis and decreased theca cell steroidogenesis. *Strengths:* - Large sample size - Adequate positive controls included - Oral administration by gavage *Weaknesses:* - Animal diet and phyoestrogen content not reported	Positive	High	↑↑
Overall conclusion on Likelihood that BPA causes reproductive toxicity in animals when exposed during their adult life (post-pubertal) only As more studies emerge with doses ≤3.6 mg BPA/kg bw per day HED, there are increasing indications of some negative effects of adult exposure to BPA on gonadal or reproductive tract physiology. Very few studies have assessed the effects of long term exposure of adults to such doses of BPA (human scenario) on the reproductive gold standard – fertility and reproductive ageing. Since only single studies on ovary and prostate gland fit the methodological criteria used in this opinion, no conclusion can be drawn on BPA-related effects on these organs. Taken together the studies assessed here suggest that BPA at a HED of ≤3.6 mg/kg bw per day may have adverse effects on testis function, especially various measures of spermatogenesis. There is much less evidence to support a conclusion that BPA will significantly impair testis morphology or reproductive endocrinology, especially in the longer term. **Note: Alteration of reproductive capacity are likely at high doses (above an HED of 3.6 mg/kg bw per day)**			**As likely as not**

Table 33: Assessment of the likelihood that BPA causes developmental and reproductive toxicity in animals exposed during pre- and post-natal (during lactation) development.

NOTE: the NOAEL HED of 3.6 mg/kg bw per day refers to the dose administered to the MOTHER if fetus or neonate is exposed through the mother. If the neonate is exposed separately post-natally prior to tissue harvesting, then the dose will be higher as a calculated HED than if the neonate is treated only via lactation through the dam.

Q2: Does developmental (fetal and/or prepubertal period) exposure to BPA at oral doses equal to or below the NOAEL of 5 mg/kg bw per day (HED equivalent 3.6 mg/kg bw per day) impair reproductive development and/or function in adulthood? (Ferguson et al., 2011, Hunt et al., 2012, Kobayashi et al., 2012, Larocca et al., 2011, Lopez-Casas et al., 2012, Nanjappa et al., 2012, US FDA/NCTR, 2013 and Delclos et al., 2014, Christiansen et al., 2014, Horstman et al., 2012, Veiga-Lopez et al., 2013, Zhang et al., 2012a, 2013, de Catanzaro et al., 2013, Nah et al., 2011, Pelch et al., 2012, Xiao et al., 2011; Signorile et al., 2012, Nakagami et al., 2009, Mendoza-Rodriguez et al., 2011, Newbold et al., 2007, Newbold et al., 2009, Signorile et al., 2010, Berger et al., 2007, Cabaton et al., 2011)	**Answer to the question as reported by the study authors (Positive, Negative or Uncertain)**	**Reliability of evidence (Low, Medium or High)**	**Influence on Likelihood (see Table 28)**
Starting point based on previous assessments (EFSA, 2006; EFSA CEF Panel, 2010): Conclusion on developmental and reproductive toxicity (including anogenital distance, fertility, endometrial hyperplasia and ovarian cysts).			
Tyl et al., 2002 CD Sprague-Dawley rats (n= 20 pregnant females) were exposed to dietary BPA in a three generation study at 0, 0.015, 0.3, 4.5, 75, 750, 7500 ppm (giving doses of approximately 0, 0.02, 0.3, 5, 50 and 500 mg/kg bw per day). Adult systemic toxicity at 750 and 7500 ppm in all generations included: reduced body weights and body weight gains, reduced absolute and increased relative weanling and adult organ weight (liver, kidney, adrenals, spleen, pituitary and brain), and female mild renal and hepatic pathology. Reproductive organ histology and function were unaffected, except for reduced ovarian weights, a significantly reduced number of implantation sites and decreased number of pups/litter on PND 0 at 7500 ppm. Adult oral NOAEL were 5 mg/kg bw per day. **Tyl et al., 2008** Dietary BPA in CD-1 mice (n=28) two-generation study at 0, 0.018, 0.18, 1.8, 30, 300, or 3500 ppm in feed (giving 0, 0.003, 0.03, 0.3, 5, 50, or 600 mg BPA/kg bw per day). 17β-oestradiol (0.5 ppm) was used as positive control. Reproductive/developmental NOAEL in the offspring was 300 ppm (50 mg/kg bw per day) based on the effect in the testes of F1/F2 offspring.	Negative	High	↓↓↓
Rubin et al., 2001 Exposure of Sprague-Dawley female rats to BPA at 100, 1,200 µg BPA/kg bw per day from day GD 6 throughout lactation was associated with increased body weight of the offspring from PND 4-11 and a dose-dependent	Positive	Low/medium	↑

reduction in the percentage of females with regular cycles and in the mean number of regular 4 or 5-day ooestrous cycles per animal. There were no significant effects of BPA on litter size, sex ratios, day of vaginal opening, AGD or macroscopic abnormalities of genital tracts. *Strengths:* - Use of glass water bottles - Cages and bedding content of oestrogens negligible at E-screen *Weaknesses:* - Animal diet and phytoestrogen content poorly described. - Study design not appropriate to the scope (small number of litters (n=6) - Litter effect not taken into account - Drinking water consumption (containing BPA) not measured - Insufficient study reporting			
Salian et al., 2009 A 3 generation-study where pregnant rats were gavaged with 1.2 or 2.4 µg BPA/kg bw per day, resulting in a significant increase in post implantation loss in the F3 offspring and a decrease in litter size in F1, F2 and F3 offspring Sperm count and motility were significantly reduced in the F1, F2 and F3 male offspring, with a dose related reduction in sperm counts. *Strengths:* - Oral administration via gavage - Adequate positive control included (DES) *Weaknesses:* - Type of cages not reported - Insufficient study reporting (the nature of the diet is unclear: prepared "in house" although stated as soya-free) - Study design not appropriate to the scope (small numbers of mated F0 dams per group (n = 8) - Litter effect not taken into account	Positive	Low	↑
Howdeshell et al., 2008 Long Evans rats were dosed with BPA at 2, 20, 200 µg/kg bw per day by oral gavage from GD7 to PND18. Apart from two minor exceptions BPA had no significant effect on AGD, birth or weaning weights, age or weight at weaning, growth rates, external genitalia, endoc rine measures, reproductive tract size and morphology or any other indices of reproductive development or function measured. *Strengths:* - ≥3 dose levels of BPA - Oral administration via gavage	Negative	Low/Medium	↓

- Large sample size (9-38 litters/group) - Adequate positive controls included (EE) *Weaknesses:* - Use of polycarbonate cages (although new) - Animal diet and phytoestrogen content poorly described			
Ryan et al., 2010a Long Evans rats were dosed with BPA at 2, 20, 200 µg/kg bw per day by oral gavage from GD7 to PND18. BPA had no significant effect on AGD, birth or weaning weights, age or weight at vaginal opening, lordosis quotient, external genitalia, number of pups or fertility/fecundity rates and also had no effects on sex-specific behaviour. *Strengths:* - ≥3 dose levels of BPA - Oral administration via gavage - Large sample size (9-38 litters/group) - Adequate positive control included (EE) *Weaknesses:* - Use of polycarbonate cages - Animal diet and phytoestrogen content poorly described	Negative	Low/Medium	↓
Tinwell et al., 2002 Two strains of rat (Sprague-Dawlery and Alderly Park) were exposed by oral gavage to BPA at 20, 100, 50,000 µg/kg bw per day from GD6-21. BPA had no significant effect on litter size or sex ratio, body weights, AGD in either sex. In females BPA had no significant effect on, age or body weight at vaginal opening or age at first estrus or any organ weights. In males there was no significant effect of BPA on age or body weight at preputial separation, or any organ weights or on histology of the testis or on sperm production. *Strengths:* - Oral administration via gavage - ≤3 BPA dose levels - Large sample size (6-7 litters/21-33 individuals/group) *Weaknesses:* - Use of polycarbonate cages (PC)	Negative	Medium	↓↓
Markey et al., 2005 Pregnant CD-1 mouse dams were implanted, from GD9 to PND4, with subcutaneous osmotic pumps containing BPA at 25, 250 ng/kg bw/ The highest BPA dose significantly reduced absolute and relative vagina, but not uterus, weight and decreased the absolute volume of the uterine lamina propria. Uterine but not vaginal DNA synsthesis	Positive	Low/Medium	↑

was significantly reduced by the higher BPA dose in the glandular epithelium although apoptosis was not altered. ESR1 and PGR staining and proportion of high intensity staining were increased in the uterine luminal epithelium (and stroma, ESR1). *Strengths:* - Feed, cages and bedding content of oestrogens negligible at E-screen - Use of glass water bottles *Weaknesses:* - Study design not appropriate to the scope (small sample size:, 6/group in some cases)			
Honma et al., 2002 Pregnant ICR-Jci mice were administered BPA at 2,20 µg BPA/kg bw per day from GD11-17. BPA had no significant effect on gestation, numbers of live pups or pup sex ration. In females BPA reduced body weight at PND 22 and 60 and increased AGD at PND22 although this effect was no longer seen at PND60. BPA significantly advanced the day of vaginal opening, reduced body weight/age at vaginal opening and increased oestrous cycle length, but had no significant effects on mating, pups/litter or pup sex ratio. In males BPA significantly reduced body weight at PND60 and while AGD was not affected at PND22, at PND60 BPA was associated with significantly increased AGD. *Strengths:* - Adequate positive controls included (3 doses of DES) - Large sample size (9-10 and 41-51 females, 24-35 males respectively per group) *Weaknesses:* - Inappropriate statistics (not clear if data normality established, use of Student's t-test, analysis of dose-effects not stated) - Animal diet and phytoestrogens content not reported Type of cages and drinking bottles not reported	Negative/Positive	Low	↑/↓
Toyama and Yuasa 2004. Neonatal male Wistar rats and ICR mice were injected subcutaneously with BPA on PND1, 3, 5, 7, 9, 11(Mice: 0.014, 0.140, 0.700, 1.401 mg/kg bw per day, Rats: 0.046, 0.461, 4.611, 27.666 µg/kg bw per day) [All rat BPA doses were higher than the 3.6 mg/kg bw per day HED cut-off dose and therefore the findings ≤3.6 BPA mg/kg bw per day are limited to the mouse. BPA at the lowest dose had no significant effect on any measure. BPA at 3.48 mg/kg bw per day, approaching the cut-off of 3.6 mg/kg bw per day HED had no significant effects on body, testis or seminal vesicle weights and no significant effect on litter size, pup birthweights, gestation length or pup sex ratio. *Strengths:* - ≥3 BPA dose levels	Negative	Low	↓

- Adequate positive controls included (oestradiol & oestradiol benzoate) *Weaknesses:* - Small sample size ((n=5), especially for the controls (n=3-4) - Inappropriate statistics (litter effect not considered and statistical analysis not described) - Type of cages and drinking bottles not reported - Animal diet poorly described - Insufficient study reporting			
Kato et al., 2006 Neonatal Sprague-Dawley male were rats administered BPA at 2, 11, 56, 277, 97,000 µg/kg bw per day by daily subcutaneous injection from PND0 (birth) to PND9. At all BPA doses, there were no significant effects on any parameter measured other than a reduction in the proportional of abnormal sperm, not adverse outcome. The litters sired by the BPA treated males were no different from controls. *Strengths:* - ≥3 dose levels of BPA - Adequate positive controls included (oestradiol) *Weaknesses:* - Type of cages and drinking bottles not reported	Negative	Low/Medium	↓
Rubin et al., 2006 CD-1 mice were treated from D8-PND16 via osmotic minipumps with 25, 250 ng BPA/kg bw per day. BPA had no significant effect on pups/litter or sex ratio. Sexual dimorphism of the rostral periventricular preoptic area was significantly reduced by changes in the female but, contradictorally, behavioural studies reported that male offspring behaved more like female offspring. *Strengths:* - Use of glass water bottles, diet tested negative at E-screen - Cages and bedding content of oestrogens negligible at E-screen - Good sample size (10-17 offspring/group) used for behavioural studies *Weaknesses:* - Study design not appropriate to the scope (sometimes small number of offspring (n=4-8/sex/group) for morphological analyses) - Litter effect not taken into account - Insufficient study reporting (not clear how many dams were used per group)	Negative/Positive	Low	↑/↓
Line of Evidence 1: new evidence on the effect of BPA on testis development and/or function (e.g. sperm count and sperm motility) and masculinisation (e.g. nipple-retention, anogenital distance, androgens)	Uncertain	From Low to High	↑/↓

(1) US FDA/NCTR, 2013 and Delclos et al., 2014, (2) Christiansen et al., 2014, (3) Kobayashi et al., 2012, (4) Ferguson et al., 2011, (5) Lopez-Casas et al., 2012, (6) Nanjappa et al., 2012, (7) Larocca et al., 2011, (8) Horstman et al., 2012, (9) deCatanzaro et al., 2013, (10) Zhang et al., 2013. *Comment*: Of the 10 studies included, four found no significant effect of BPA ≤3.6 mg/kg bw per day HED on male reproductive development: Larocca et al., 2011, Lopez-Casas et al., 2012, Ferguson et al., 2012, Horstman et al., 2012. Three found limited negative effects of BPA ≤3.6 mg/kg bw per day HED on male reproductive development: US FDA/NCTR, 2013 and Delclos et al., 2014 (slightly delayed testis descent, seminiferous tubule giant cells, each at different single doses), Kobayashi et al., 2012 (reduced epididymis weights), Nanjappa et al., 2012 (increased Leydig cell numbers but no change in testosterone). Three found clear negative effects of BPA ≤ 3.6 mg/kg bw per day HED on male reproductive development: deCatanzaro et al., 2013 (only in conjunction with high phytoestrogen diet: reduced vascular-coagulating gland weight and increased latency to inseminate), Christiansen et al., 2014 (decreased AGD, dose-dependent increase in nipple retention, latter not significant ≤3.6 mg/kg bw per day) while four of the other studies showed no significant reduction in male AGD, Zhang et al., 2013 (reduced sperm number, survival and viability). *Strengths:* - Large sample size (US FDA/NCTR, 2013 and Delclos et al., 2014, Ferguson et al., 2011, Christiansen et al., 2014, Nanjappa et al., 2012, LaRocca et al., 2011 Horstman et al., 2012, deCatanzaro et al., 2013) - Number of doses (≥3) (US FDA/NCTR, 2013 and Delclos et al., 2014: especially in the low dose range, Kobayashi et al., 2012, Christiansen et al., 2014, Lopez-Casas et al., 2012, Horstman et al., 2012, deCatanzaro et al., 2013) - Both naïve and vehicle controls available (US FDA/NCTR, 2013 and Delclos et al., 2014) - Adequate positive controls included (Ferguson et al., 2011, LaRocca et al., 2011, US FDA/NCTR, 2013 and Delclos et al., 2014) - Oral administration via gavage (US FDA/NCTR, 2013 and Delclos et al., 2014, Ferguson et al., 2011, Christiansen et al., 2014, Nanjappa et al., 2012, LaRocca et al., 2011) - Phytoestrogen-free diet (US FDA/NCTR, 2013 and Delclos et al., 2014, Ferguson et al., 2011, Christiansen et al., 2014, Nanjappa et al., 2012) - Use of non-PC cages (US FDA/NCTR, 2013 and Delclos et al., 2014, Ferguson et al., 2011, Christiansen et al., 2014, Nanjappa et al., 2012, Horstman et al., 2012, deCatanzaro et al., 2013) - Study/analysis performed under OECD guideline, under GLP (US FDA/NCTR, 2013 and Delclos et al., 2014) *Weaknesses*: - Feed consumption (BPA given by the diet) not measured (Kobayashi et al., 2012) - BPA concentration and homogeneity in the feed mixture not guaranteed analytically (Kobayashi et al., 2012)			

- Drinking water consumption (containing BPA) not measured (Lopez-Casas et al., 2012) - Small sample size (Lopez-Casas et al., 2012) - Insufficient study reporting and/or inappropriate statistics (Lopez-Casas et al., 2012, Kobayashi et al. 2012, Horstman et al., 2012, Zhang et al., 2013) - Animal diet and phytoestrogen content not reported (Kobayashi et al., 2012, Lopez-Casas et al., 2012, LaRocca et al., 2011, Horstman et al., 2012, Zhang et al., 2013) - Use of polycarbonate cages or cage type not reported (LaRocca et al., 2011) - Dietary confounder in the study – e.g. BPA effects seen with high phytoestrogen diet (deCatanzaro et al., 2013) - Unintended exposure of the control groups (US FDA/NCTR, 2013 and Delclos et al., 2014, see Churchwell et al, 2014)			
Line of Evidence 2: new evidence on the effect of BPA on male reproductive development observed to lead to impaired fertility and offspring neonatal growth. (10) Zhang et al. 2013. *Comment*: Fewer offspring, heavier at birth with poorer growth trajectories and increased dystocia (Zhang et al., 2013). *Strengths:* - Prolonged treatment duration (Zhang et al., 2013) *Weaknesses:* - Insufficient study reporting (lack of experimental details and/or statistical analyses), (Zhang et al., 2013). - Study design not appropriate to the scope (lack of a positive control), (Zhang et al., 2013) - Animal diet and phytoestrogen content not reported (Zhang et al., 2013)	Uncertain	Low	↓/↑
Line of Evidence 3: new evidence on the effect of BPA on ovary development (e.g. follicle and oocyte number) and female morphology/function (e.g. oestrogens, endometrial hyperplasia, cycstic ovaries, anogenital distance) (1) US FDA/NCTR, 2013 and Delclos et al., 2014, (2) Christiansen et al., 2014, (3) Kobayashi et al., 2012, (4) Ferguson et al., 2011, (11) Veiga-Lopez et al., 2013, (12) Hunt et al., 2012, (13) Zhang et al., 2012a , (14) Nah et al., 2011, (15) Signorile et al., 2012, (16) Mendoza-Rodriguez et al., 2011, (17) Newbold et al., 2007, (18) Newbold et al., 2009, (19) Signorile et al., 2010. *Comment*: Of the 13 studies included, one found no significant effect of BPA ≤3.6 mg/kg bw per day HED on female reproductive development: Ferguson et al. 2012. Seven found limited negative effects of BPA ≤3.6 mg/kg bw per day HED on female reproductive development: US. FDA/NCTR, 2013 and Delclos et al., 2014 (oestrous cyclicity abnormalities at two BPA doses), Hunt et al., 2012 (increased proportion of multi-oocyte follicles), Veiga-Lopez et al., 2013 (changes in some ovarian transcripts and miRNA, more in younger than older fetuses), Kobayashi	Negative/Uncertain/Positive	From low to high	↓/↑↑

et al., 2012 (reduced female AGD, normalised at adulthood), Signorile et al., 2010 (increased incidence of ovarian cysts) Signorile et al., 2012 (reduced numbers of follicles, increased numbers of atretic follicles), Newbold et al., 2009 (2 BPA doses associated with endometrial abnormalities). Five found clear negative effects of BPA ≤3.6 mg/kg bw per day HED on female reproductive development: Christiansen et al., 2014 (reduced AGD at all doses), Zhang et al., 2012a (increased retention of oocye nests, reduced numbers of primordial follicles, delayed meiotic progression), Nah et al., 2011 (reduced ovary weights and delayed puberty), Newbold et al., 2007 (increased incidence of ovarian cycts and endometrial hyperplasia at a single BPA dose), Mendoza-Rodruigez et al., 2011 (abnormalities in oestrous cyclicity and increased evidence of endometrial hyperplasia). *Comment:* Significance of reduced AGD is not clear – i.e. suggests and effect but whether adverse is not currently known (Christiansen et al., 2014, Kobayashi et al., 2012). 3 of the other studies found no significant effect on female AGD and in the case of Kobayashi et al., 2012 the effect (reduced AGD) was lost as the animals matured. *Comment*: Signs of adaptation/loss of BPA effects in adulthood (Nah et al., 2011, Kobayashi et al., 2012) *Comment*: In Hunt et al., 2012 only the results for the oral route were considered for evaluation because of the inadequate number of animals dosed via the subcutaneous route (only 2 monkeys in the control group) *Comment*: In Nah et al., 2011 the administration of BPA on one single day (then followed) reduced confidence in the absence of a repeat study. *Comment:* Most of the significant effects of BPA ≤3.6 mg/kg body weight/day HED were from the studies with the strengths/weaknesses ratios skewed more heavily towards weaknesses. *Comment:* With regard to endometrial hyperplasia/abnormalities some studies showed a statistically significant increase (Mendoza-Rodriguez et al., 2011, Newbold et al., 2009) but findings were not statistically significant in Signorile et al. (2010). Signorile et al (2010; 2012) report unconvincing "endometriosis-like" features. *Comment:* An increased incidence of ovarian cysts were observed by Signorile et al., 2010 and Newbold et al., 2007 but not significantly in Newbold 2009 and not in Signorile et al. (2012) which reported changes in follicle types. *Comment:* Aberrations in oestrous/ovarian cyclicity were reported (Mendoza-Rodriguez et al., 2011, Newbold et al., 2007, US FDA/NCTR, 2013 and Delclos et al., 2014). Other studies found no significant effects in cyclicity (Newbold et al., 2009) *Strengths:* - Large sample size (US FDA/NCTR, 2013 and Delclos et al., 2014, Ferguson et al. 2012, Christiansen et al., 2014, Mendoza-Rodriguez et al., 2011, Newbold et al., 2007, Newbold et al., 2009, Signorile et al., 2010, Signorile et al., 2012) - Number of doses (≥3) (US FDA/NCTR, 2013 and Delclos et al., 2014: especially in the low dose range, Kobayashi et al., 2012, Christainsen et al., 2013, Zhang et al., 2012a, Nah et al., 2011) - Both naïve and vehicle controls available (US FDA/NCTR, 2013 and Delclos et al., 2014) - Adequate positive controls included (Ferguson et al., 2011) - BPA measurement in serum (Hunt et al., 2012, Veiga-Lopez et al., 2013) - Oral administration via gavage (US FDA/NCTR, 2013 and Delclos et al., 2014, Ferguson et al., 2011,			

Christiansen et al., 2014) - Phytoestrogen-free diet (US FDA/NCTR, 2013 and Delclos et al., 2014, Ferguson et al., 2011, Christiansen et al., 2014) - Cages and diet/water tested by E-screen (Signorile et al., 2010, Signorile et al., 2012) - Use of non-PC cages (US FDA/NCTR, 2013 and Delclos et al., 2014, Ferguson et al., 2011, Christiansen et al., 2014, Hunt et al., 2012) - Study/analysis performed under OECD guideline (US FDA/NCTR, 2013 and Delclos et al., 2014) - Study/analysis performed under GLP (US FDA/NCTR, 2013 and Delclos et al., 2014) *Weaknesses*: - Animal species and strains not reported (Zhang et al., 2012a) - Animal age and body weight not given (Zhang et al., 2012a, Signorile et al., 2010, Signorile et al., 2012) - Small sample size **(**Hunt et al., 2012), small litter number (Signorile et al., 2010, Signorile et al., 2012) - Plastic cages used or tyope not reported (Newbold et al., 2007, Newbold et al., 2009) - Feed/water consumption (BPA given by the diet/water) not measured (Kobayashi et al., 2012, Mendoza-Rodriguez et al., 2011) - BPA concentration and homogeneity in the feed mixture not guaranteed analytically (Kobayashi et al., 2012) - Single dose level study (Hunt et al., 2012, Veiga-Lopez et al., 2013, Mendoza-Rodriguez et al., 2011) - Insufficient study reporting/inappropriate statistics (Kobayashi et al. 2012, Zhang et al., 2012a, Nah et al., 2011, Signorile et al., 2012, Mendoza-Rodriguez et al., 2011, Newbold et al., 2007, Newbold et al., 2009) - Internal inconsistencies in data (Signorile et al., 2010, 2012). - Animal diet and phytoestrogen content not reported (Kobayashi et al., 2012, Nah et al., 2011, Zhang et al., 2012a, Mendoza-Rodriguez et al., 2011) - BPA concentration and homogeneity not guaranteed analytically (Hunt et al., 2012) - Diet phytoestrogen content not reported (Hunt et al., 2012, Veiga-Lopez et al., 2013) - Unintended exposure of the control groups (US FDA/NCTR, 2013 and Delclos et al., 2014, see Churchwell et al, 2014)			
Line of Evidence 4: new evidence on the effect of BPA on implantation and early development or survival of the conceptus or female fertility. (21) Xiao et al., 2011, (22) Berger et al., 2007, (23) Nakagami et al., 2009: No effects on implantation, development/survival or uterine PGR expression at ≤3.6 mg BPA/kg bw per day. (24) Cabaton et al., 2011: significantly reduced cumulative number of pups. *Strengths:* - Number of doses (5, but only one ≤3.6 mg/kg bw per day, Xiao et al., 2011, Berger et al., 2007) - Positive controls included (Xiao et al., 2011, Cabaton et al., 2011) - Use of non-PC cages/E-screen checked (Xiao et al., 2011, Cabaton et al., 2011, Nakagami et al., 2009)	Uncertain	Low/Medium	↓/↑

- Good sample size (Nakagami et al., 2009) *Weaknesses:* - Animal diet and phytoestrogen content not given (Xiao et al., 2011, Nakagami et al., 2009) - Small sample size (n=4 animals, Xiao et al., 2011, n<8 animals, Berger et al., 2007) - Drinking bottle type not reported (Berger et al., 2007, Nakagami et al., 2009) - Some statistical flaws (Cabaton et al., 2011) - Single dose level (Nakagami et al., 2009)			
Line of Evidence 5: new evidence on the effect of BPA on bone in subsequent adulthood. (25 Pelch et al., 2012: extremely small effect on male femur length and larger reduction in energy to failure (males and females) but not torsional strength or collagen content *Strengths:* - Positive control included - Use of non-PC cages and of non plastic water bottles *Weaknesses:* - Animal age and body weight not given - Single dose level study - Animal diet phytoestrogen content not reported	Uncertain	Low	•
Overall conclusion on Likelihood: Taken overall there are some data suggesting negative effects of doses of BPA ≤ an HED of 3.6 mg/kg bw per day on reproductive development in animals. However, the disagreement between studies on whether there is an effect, how extensive the effect is and whether the effect is lost as the animal matures into adulthood renders a high degree of uncertainty (e.g. disturbed oestrous cyclicity without any effect on ovarian morphology of circulating hormones). The most consistent findings are generally of small magnitude and often not accompanied by associated changes (e.g. reduced AGD in males would be expected to be associated with reduced testosterone). In addition, a number of studies present molecular findings without accompanying morphological data. Given the difficulty in determining whether such molecular changes are due to adaptation, causal or just a result of modest morphological changes, weight given to such studies must be reduced. There was only a single non-human primate study included and this was hampered by inadequate numbers of animals per group and reported only a single sex. The most convincing evidence for adverse effects are reduced (by 7%) male AGD in Christiansen et al., 2014 but four other studes found no effect. Therefore our overall conclusion reflects the contradictory nature and highly variable quality of the data and the high levels of uncertainty.			**As likely as not**

Table 34: Summary of the WoE assessment of the likelihood that BPA causes reproductive and developmental effects

Humans	
Overall conclusion on Likelihood of reproductive effects of BPA in humans: An association between BPA and embryo quality and implantation success during IVF, semen quality, sex hormones or age of menarche is considered unlikely.	**Unlikely**
Overall conclusion on Likelihood of gestational /birth outcomes of BPA in humans: There are indications from prospective studies that BPA exposure during pregnancy may be associated with effects on fetal growth, and weak indications that BPA exposure during pregnancy may be associated with effects on maternal and infant thyroid function. However, it cannot be ruled out that the results are confounded by diet or concurrent exposure factors. For fetal growth, two studies showed reduced fetal growth, while one study reported increased fetal growth with increasing maternal BPA exposure. The associations do not provide sufficient evidence to infer a causal link between BPA exposure and reproductive effects in humans. Potential effects are considered to be as likely as not."	**As likely as not**
Animals	
Overall conclusion on Likelihood for reproductive and developmental effects in animals when exposed during their adult life (post-pubertal) only at doses ≤ HED of 3.6 mg/kg bw per day: As more studies emerge with doses ≤3.6 mg BPA/kg bw per day HED, there are increasing indications of some negative effects of adult exposure to BPA on gonadal or reproductive tract physiology. Very few studies have assessed the effects of long term exposure of adults to such doses of BPA (human scenario) on fertility/fecundity and reproductive ageing. Since only single studies on ovary and prostate gland fit the methodological criteria used in this opinion, no firm conclusion can be drawn on BPA-related effects on these organs. Taken together the studies assessed here suggest that BPA at an HED of ≤3.6 mg/kg bw per day may have adverse effects on testis function, especially various measures of spermatogenesis. There is much less evidence to support a conclusion that BPA will significantly impair testis morphology or reproductive endocrinology, especially in the longer term. **Note: Alterations of reproductive capacity are likely at high doses (above an HED of 3.6 mg/kg bw per day)**	**As likely as not**
Overall conclusion on Likelihood for reproductive and developmental effects in animals when exposed during development (prenatally and pre-pubertally) ≤ HED of 3.6 mg/kg bw per day: Taken overall, there are some data suggesting negative effects of doses of BPA ≤ an HED of 3.6 mg/kg bw per day on reproductive development in animals. However, the lack of agreement between studies and the highly variable quality of the studies renders a high degree of uncertainty. The most consistent findings are generally of small magnitude (e.g. reduced male AGD) and often not accompanied by associated changes (e.g. abnormal oestrous cyclicity in females would be expected to associate with ovarian or endocrine effects of BPA in the same groups). Given difficulties in determining whether molecular changes are causal or due to adaptation or morphological changes, the weight given to studies presenting molecular findings without accompanying morphological data is low. The single non-human primate study of the effects of BPA on the fetal ovary included was hampered by inadequate numbers of animals per group. The likelihood rating of the studies reflects the variability of the data. **Note: Alterations of reproductive development are likely at high doses (above an HED of 3.6 mg/kg bw per day)**	**As likely as not**

WoE of neurological, neurodevelopmental and neuroendocrine effects

Whether BPA has the potential to cause neurological, neurodevelopmental and neuroendocrine effects in humans and animals was considered using a tabular format for weighing different lines of evidence (WoE evaluation). The WoE evaluation tables for these endpoints are presented in full below.

Human studies

Table 35: Assessment of the likelihood of associations between BPA exposure and neurological, neurodevelopmental or neuroendocrine effects in humans.

Q1: Is there an association between prenatal BPA exposure and neurodevelopmental effects?	**Answer to the question as reported by the study authors (Positive, Negative or Uncertain)**	**Reliability of evidence (Low, Medium or High)**	**Influence on Likelihood (see Table 28)**
Starting point based on previous assessments (EFSA CEF Panel, 2010). The only study identified on this issue showed associations between BPA exposure and externalizing behaviour in 2 year old girls (Braun et al., 2009). *Weaknesses:* Although the study provided some indication of possible effects on neurodevelopment in girls, EFSA concluded that the study had methodological limitations and that it was not possible to draw a conclusion for risk assessment from the study.	Positive	Low	●
Line of Evidence 1: Prenatal BPA exposure and neurodevelopmental effects. In total five prospective studies. One study showed significant associations in boys only (Harley et al., 2013a), two studies showed no associations in boys or girls (Miodovnik et al., 2011, Yolton et al., 2011), one study showed associations in girls only (Braun et al., 2011) and one study showed significant but different/conflicting associations in boys and girls, i.e. in boys higher prenatal BPA was associated with increased behavioural problems while in girls higher BPA was associated with decreased problems in girls (Perera et al., 2012). *Comment:* Adjustment for other environmental chemicals (Yolton et al., 2011; Braun et al., 2011; Miodovnik et al., 2011; Harley et al., 2013a) *Strengths:* – Prospective study design (all studies) – Urine, container specified (Braun et al., 2011; Harley et al., 2013a)	Positive	Medium	↑

– Repeated measurements for maternal (Yolton et al., 2011; Braun et al., 2011; Harley et al., 2013a) and children urine (Braun et al., 2011) – Standardised samples (all studies) – Analytical method (LC-MS-MS) (all studies) – Quality controls, including blanks (Harley et al., 2013a) – Multiple outcome assessment (maternal and teacher-report at age 7 and direct observation at age 9) (Harley et al., 2013a) *Weaknesses:* – Small sample size (all studies) – Single spot urine BPA measurement (Miodovnik et al., 2011; Perera et al., 2012) – No distinction between unconjugated and conjugated BPA (all studies) – Confounding by diet (all studies) or other chemicals (Perera et al., 2012) not considered – Unclear clinical relevance (small effect size, conflicting results in boys and girls) (Braun et al., 2011; Perera et al., 2012; Harley et al., 2013a) – Imprecise/unreliable outcome (parent-reported but validated methods only) (Miodovnik et al., 2011; Braun et al., 2011; Perera et al., 2012) – Generalisability to the overall population (Perera et al., 2012; Harley et al., 2013a) – Inconsistent results amongst different studies (all studies)			
Overall conclusion on Likelihood of neurodevelopmental effects of BPA in humans: There are indications from prospective studies that prenatal BPA exposure (BPA exposure during pregnancy) may be associated with child behaviour in a sex-dependent manner. However, the associations were not consistent across the studies. It cannot be ruled out that the results are confounded by diet or concurrent exposure factors. The associations are not sufficient evidence to infer a causal link between BPA exposure during pregnancy and neurodevelopmental effects in humans. Potential effects are considered to be as likely as not.			**As likely as not**
Q2: Is there an association between childhood BPA exposure and neurological/behavioural effects?	**Answer to the question as reported by the study authors (Positive, Negative or Uncertain)**	**Reliability of evidence (Low, Medium or High)**	**Influence on Likelihood (see Table 28)**
Line of Evidence 1: Childhood BPA exposure and neurological effects. Three prospective studies, of which one found no associations between urinary BPA levels in children and behavioural effects (Braun et al., 2011) one found an association with 1 of 7 outcomes (Perera et al., 2012), and one found associations in both boys and girls (Harley et al., 2013a). A cross-sectional study with boys and girls analysed together found associations between childhood urinary BPA and behaviour and learning (Hong et al., 2013) *Comment:*	Both positive and negative	Medium	Prospective studies:↑/● Cross-sectional study:↑

– Adjustment for other environmental chemicals (Braun et al., 2011; Harley et al., 2013a). *Strengths*: – Prospective study design (Braun et al., 2011; Perera et al., 2012; Harley et al., 2013a) – Urine, container specified (Braun et al., 2011; Harley et al., 2013a) – Repeated measurements for maternal (Braun et al., 2011; Harley et al., 2013a) and children urine (Braun et al., 2011) – Standardised samples (all studies) – Analytical method (LC-MS-MS) (all studies) – Quality controls, including blanks (Harley et al., 2013a) – Multiple outcome assessment (maternal and teacher-report at age 7 and direct observation at age 9) (Harley et al., 2013a) *Weaknesses*: – Cross-sectional study design (Hong et al., 2013) – Small sample size (Braun et al., 2011; Perera et al., 2012; Harley et al., 2013a) – Single spot urine BPA measurement (Perera et al., 2012; Hong et al., 2013) – No distinction between unconjugated and conjugated BPA (all studies) – Confounding by diet not considered (all studies) – Imprecise/unreliable outcome (parent-reported but validated methods) (Braun et al., 2011; Perera et al., 2012) – Generalisability to the overall population (Perera et al., 2012; Harley et al., 2013a) – Inconsistent results amongst different studies (all studies)			
Overall conclusion on Likelihood of neurological/behavioural effects of BPA in humans: There are indications from one prospective study that childhood BPA exposure may be associated with behavioural problems in both girls and boys. It cannot be ruled out that the results are confounded by diet or concurrent exposure factors. The associations do not provide sufficient evidence to infer a causal link between childhood BPA exposure and neurological effects/behavior in humans. Potential effects are considered to be as likely as not.			**As likely as not**

Animal studies

Table 36: Assessment of the likelihood that BPA produces neurobehavioural changes in laboratory rodents after pre- and/or postnatal exposure to BPA.

Q1: Is there any evidence that BPA exposure changes response in tests for anxiety-like behaviour in rodents?	**Answer to the question as reported by the study authors (Positive, Negative or Uncertain)**	**Reliability of evidence (Low, Medium or High)**	**Influence on Likelihood (see Table 28)**
Starting point based on previous assessments (EFSA, 2006; EFSA CEF Panel, 2010[19]) Overall, no consistent pattern in the data on anxiety-like behaviour across species and gender. Uncertainties include study design limitations, inclusion of only one sex, age at examination (EFSA, 2006). In 2010, the CEF Panel concluded that currently available data addressing neurobehavioural endpoints (e.g. learning and memory behaviour, anxiety-related behaviour and gender-specific behaviour) does not provide convincing evidence of neurobehavioural toxicity of BPA (EFSA CEF Panel, 2010).	Some Positive	Low	●
Line of evidence 1: New studies on Anxiety-like behaviour (Diaz Weinstein et al., 2013; Ferguson et al., 2012; Fujimoto et al., 2013; Gioiosa et al., 2013; Inagaki et al., 2012; Jasarevic et al. 2013; Jones and Watson, 2012; Kundakovic et al., 2013; Matsuda et al. 2012; Nakamura et al., 2012; Patisaul et al., 2012; Viberg et al., 2011; Wolstenholme et al., 2011a; Wolstenholme et al., 2012; Wolstenholme et al., 2013; Xu et al. (2011); Xu et al. 2012; Xu et al., 2013a):			
Diaz-Weinstein et al., 2013 *Comment:* Adolescent rats exposed daily by subcutaneous injection to BPA for 12 days *Comment:* anxiety-like behaviour tested in open field and elevated plus maze *Strengths:* - Use of glass water bottles - Blind evaluation of the observed behaviour *Weaknesses:* - Single dose level study - Study design not appropriate to the scope (behavioural tests performed only once with limitation to one trial, subsequent testing in two different tests on the same day)	Positive	Low	●

[19] The WOE refers to the studies evaluated in the EFSA opinion

- Insufficient study reporting (body weights not measured daily in conjunction with treatment; no information on sexual maturation and insufficient information on recording of behavioural testing; animal diet and phytoestrogen content not reported) - Inapproriate statistics (repeated measures for the same animal are not taken into account, cycling is not adjusted for in the analysis; no multiple comparison statistics - Fisher's protected LSD test applied which does not adequately protect from Type 1 Error increase due to multiple comparisons)			
Ferguson et al., 2012 (see Q2 and Q4 for other endpoints) *Comment*: Pre- and postnatal exposure by maternal administration of BPA (gavage), plus direct postnatal exposure to pups (gavage) *Comment*: Motor activity of naïve controls similar to that of BPA –treated groups *Comment*: Multiple tests performed (Novelty preference test (PND 29), open field test (PND 40-42), motor coordination (PND 43-44), Barnes maze (PND 47-50), acoustic startle response (PND 54), and Morris water maze (PND 75-79). Here open field test is addressed. *Strengths:* - Large sample size - Both naïve and vehicle controls available (to account for the handling of the animal) - Adequate positive controls included - Use of non-PC cages and of glass water bottles *Weaknesses:* - Study design not appropriate to the scope (only low doses of BPA used)	Negative/uncertain (males only)	Low	•
Fujimoto et al., 2013 *Comment:* lactational BPA-exposure (maternal dosing via drinking water) *Comment:* Two different tests used to investigate anxiety-like behaviour (open field at 6 weeks of age, Elevated plus maze at 7 weeks of age) *Strengths:* - Use of glass water bottles *Weaknesses:* - Single dose level study - Animal diet and phytoestrogen content not reported - Study design not appropriate to the scope (small sample size, N = 5 dams and litters per treatment group, littermates treated as independent observations) - Insufficient BPA-exposure assessment (drinking water consumption (containing BPA) not measured) - Insufficient study reporting (insufficient information on recording of behavioural testing) - Inappropriate statistics (litter effect not considered; no multiple comparison statistics - Fisher's protected	Positive	Low	•

LSD test applied which does not adequately protect from Type 1 Error increase due to multiple comparisons)			
Gioiosa et al., 2013 *Comment*: Crossfostering design, pre- or postnatal exposure only (maternal dosing by spontaneous consumption of oil-based solution from a modified syringe) *Comment:* Three different tests used to investigate anxiety-like behaviour (novelty test in juveniles, open-field and EPM at adulthood) *Strengths:* - Blind collection and analyses of behavioural data *Weaknesses:* - Single dose level study - Animal diet and phytoestrogen content not reported (soy-based standard diet used) - Use of polycarbonate cages and bottles (new ones) - Insufficient BPA-exposure assessment (BPA dose adjusted to body weight seemingly not on a daily basis) - Inappropriate statistics (no correction for multiple comparisons applied; comparison between the two exposure windows is not appropriate since the same dose is used for either gestational or lactational exposure – resulting in very different internal dose)	Positive/Uncertain	Low	•
Inagaki et al., 2012 (see also Q2) *Comment*: Single subcutaneous administration to adult cycling female rats *Comment*: Parallel assessment of learning/memory and relevant neurobiological marker (spinogenesis) in two different brain areas *Comment:* Elevated plus maze (EPM) was used to examine whether BPA indirectly may impair recognition memory by increasing anxiety levels of the rats *Strengths:* - BPA measurement in biological samples (serum) *Weaknesses:* - Single acute dose administration - Test performed in one sex only - Insufficient study reporting (study design not properly described, doses and number of animals for the various tests is unclear; except from feed content, no information of environmental contamination of	Negative	Low	•

BPA is given, i.e. cages, water bottles, or bedding) - Inappropriate statistics (considerations of repeated measures of the same animal not included in the analyses, nor multiple endpoint within a test)			
Jasarevic et al., 2013 (see also Q2) *Comment*: Deer mice with pre- and postnatal exposure (maternal dosing via feed 2 week prior to mating) *Comment:* Indication of weak dose-response reaching a plateau at the 2 top doses (≥5 mg/kg bw per day). *Comment*: Anxiety-like behaviour evaluated by elevated plus maze (EPM) *Strengths:* - Adequate positive controls included - Number of BPA doses (3) - Use of non-PC cages and glass water bottles - BPA exposure measurement in animal samples (serum) *Weaknesses:* - Study design not appropriate to the scope (multiple breeding; use of littermates in testing; study not controlled for reproductive cycling at testing time; unclear exposure time of the respective dams or if the controls were concurrent with the treated animals; possible additional dietary exposure of the offspring during late lactation) - Feed consumption (BPA given by the diet) not measured - Insufficient study reporting (no information on normalization for dams' body weight; maternal amount of feed consumed daily not specified; unclear whether offspring may have been exposed directly through feed during late lactation when incisors have appeared; total number of dams and their general reproductive outcome not given) - Inappropriate statistics (litter effect not considered; no multiple comparison statistics - Fisher's protected LSD test applied which does not adequately protect from Type 1 Error increase due to multiple comparisons)	Positive (males)	Low	●/↑
Jones and Watson, 2012 (see also Q2) *Comments*: Pre- and postnatal exposure (maternal oral dosing by licking oil drops) *Comment:* three behavioural tests performed (Morris Water Maze, Forced Swim Test (FST) and for anxiety-like behaviour in the Elevated Plus Maze, EPM) *Strengths:* - Number of BPA doses (4) - Use of non-PC cages, and of BPA-free water sacks (for dams) *Weaknesses:* - Study design not appropriate to the scope (small number (2-3) of dams per group; littermates treated as	Positive	Low	●

independent observations; females not controlled for cycling at the time of testing) - Insufficient study reporting (unclear reports for the statistical findings in the figures/tables presented, especially for FST) - Inappropriate statistics (because of the limited number of litters tested (2-3) per dose group, the samples size is considered underpowered; litter effect not considered, lack of analysis of the two way interaction between treatment and sex; no adjustment of cycling included)			
Kundakovic et al. 2013 (see Q3 for another endpoint) *Comment*: Gestational exposure (maternal oral administration of BPA during pregnancy only) *Comment:* Parallel assessment of relevant molecular markers (oestrogen receptors and DNA methylation for ER genes) *Comment:* The large scatter within the dose-groups for the behavioural endpoints does not allow to conclude on the shape of the dose-response curve and even a conclusion that there is no dose-response would be compatible with the data *Comment:* Two different tests for social behaviour used (home cage social behaviour in juveniles and dyadic interaction with a same-sex stimulus mouse at PND 70) *Comment:* Anxiety-like behaviour tested in open field (OF) area at PND 60 (video recording) *Strengths:* - Number of BPA doses (3) - BPA measured in biological sample (urine) - Use of non-polycarbonate (non-PC) cages *Weaknesses:* - Insufficient study reporting (general reproductive information like maternal body weight, litter size and sex- ratio is not given; frequency of various postpartum maternal behaviours given without the litter size; the administration to dams is not specified except that it is oral; urine collection and analysis not described; the sacrificing and brain sampling procedures are not detailed; insufficient information concerning the scoring of social/aggressive behaviour; poor description of the social behaviour testing) - Inappropriate statistics (lack of accounting for multiple observations within each litter [pseudo-replication] in the ANOVA; the application of the multilevel regression model showed a large scatter of data within dose-groups [only four] and thus a large number of different dose-response curves can be fitted to the data so that it is not possible to conclude on the biological relevance for any of them) - Animal diet and phytoestrogen content not reported - Type of drinking bottles not reported	Positive (females only)	Low	•

Matsuda et al., 2012 *Comment*: Pre- and postnatal exposure (maternal dosing via subcutanous route) *Comment:* Parallel examination of neurobiological (dopaminergic markers) and functional end points (anxiety-like behaviour by open field) *Strengths:* - None *Weaknesses:* - Single dose level study - Study design not appropriate to the scope (dosing not daily adjusted to body weight; limited sample size (n=4-6) for neurochemical assessment) - Insufficient study reporting (number of dams lacking, general reproductive outcome and information on check for cycling in female offspring not given; type of cages and drinking bottles not reported; animal diet and phytoestrogen content not reported) - Insufficient BPA-exposure assessment (constant during pregnancy and lactation and not adjusted to body weight)	Positive (males only)	From Low to Medium	●
Nakamura et al., 2012 (see also Q2) *Comment:* Pre- and postnatal exposure by maternal administration of BPA (subcutaneously) *Comment:* Use of open field, Elevated plus maze and Morris water maze *Strengths:* - None *Weaknesses:* - Single dose level study - Insufficient study reporting (maternal body weight not given; lack of information on check for cycling in female offspring; no information of environmental BPA-control, i.e. animal diet and phytoestrogen content, type of cages, bedding and drinking bottles not reported) - Insufficient BPA-exposure assessment (constant during pregnancy and lactation and not adjusted to body weight) - Inappropriate statistics (litter effect not considered)	Negative	Low	●
Patisaul et al., 2012 *Comment*: Pre- and postnatal exposure (maternal dosing via drinking water, plus direct exposure of offspring via drinking water until PND 40) *Comment:* BPA-exposed dams were given soy-free or soy-based diets *Comment:* cycling taken into account; including only females in the same estral phase to avoid variability *Comment:* Parallel assessment of molecular markers (ER-beta and Kisspeptin1) and functional end points	Positive	Low	●

Comment: anxiety-like behaviour evaluated by light/dark box and Elevated plus maze *Strengths:* - Positive control included (estrogenic) - BPA measurement in animal samples (dams' serum) - Phytoestrogen-free diet (one group) *Weaknesses:* - Single dose level study - Insufficient BPA-exposure assessment (estimated based on maternal water intake and not normalized to body weight) - Lack of constant levels of exposure in time (lactational exposure is much lower than the gestational or juvenile exposure). - Insufficient study reporting (effects of animal breeding schedule not well described, mating was split in four cohorts with no information on distribution of dose groups, insufficient reporting of number of dams, unclear whether parallel behavioural testing of different dose groups of offspring was performed, duration of testing in EMP not given; type of cages and drinking bottles not reported) - Inappropriate statistics (unclear if litter effect was properly considered).			
Viberg et al., 2011 (also see Q2) *Comment*: single oral administration by gavage on PND 10 to males only *Comment*: anxiety-like behaviour (Elevated plus maze) was not affected by BPA treatment (tested at 3 month) *Strengths:* - Number of dose groups (3) *Weaknesses* - Single acute dose administration - Small sample size (3-4 litters/group) - Test performed in one sex only - Study design not appropriate to the scope (pup body weight recorded only four times during the 6 months study; only male pups behaviourally tested and it appears as the same 12-15 males representing 3-4 litters were used in all four tests; littermates treated as independent observations) - Insufficient study reporting (number of dams and general reproductive outcome lacking; litters said to be standardised by size (12-14 pups) but sex ratio is not given; type of cages, bedding and drinking bottles not reported) - Inappropriate statistics (because of the limited number of litters tested (3-4), the samples size is considered underpowered; the litter effect is not properly considered in the statistics)	Negative	Low	•

- Phytoestrogen content in animal diet not given			
Wolstenholme et al., 2011a *Comment*: Prenatal exposure (maternal exposure via feed prior to mating and through gestation) *Comment:* To ensure prenatal exposure only and exclude BPA-induced differences of maternal care, foster-dams were used implying mixed litters and tail clipping of pups, which might both be a strength and a weakness *Comment:* Parallel assessment of relevant neurobiological (oxytocin) and functional endpoints *Comment:* anxiety-like behaviour evaluated by elevated plus maze (EPM) *Strengths:* - BPA measurement in animal samples (serum) - Phytoestrogen-free diet - Scoring blind to treatment *Weaknesses:* - Single dose level study - Feed consumption (BPA given by the diet) not measured - Type of cages and drinking bottles not reported - Insufficient study reporting (information of the content of the mixed litters, like number of pups and sex ratio is missing) - Inappropriate statistics (litter effect not considered)	Negative	Low	•/↓
Wolstenholme et al., 2012 *Comment:* The study addressed association of BPA behavioural effects with expression of genes implicated in regulation of social behaviour and related sex dimorphism (ERs, oxytocin and vasopressin) in embryos. *Comment*: transgenerational effects (F2 and F4) were addressed *Comment:* anxiety-like behaviour evaluated by elevated plus maze (EPM) *Strengths:* - BPA measurement in animal samples (F0 dams, serum) - Phytoestrogen-free diet (F0-generation only) *Weaknesses:* - Single dose level study - Study design not appropriate to the scope (the number of dams in F0 generation was limited) - Insufficient study reporting (animal age and body weight not given; no normalization of food consumption on body weight, potential variability of exposure; lack of information of control of environmental contamination of BPA, i.e. cages, bedding and water bottles; lack of information of mixed litters e.g. number of pups and sex ratio; reproductive outcome of the breeding brother-sister pairs done to obtain F2 to F4 generation is not given) - Insufficient BPA-exposure assessment (estimated on the basis of the daily quantity of food ingested, see	Negative	Low	•

line above) - Inappropriate statistics (litter effect not considered)			
Wolstenholme et al., 2013 (see also Q3 and Q4) *Comment:* Transgenerational study (F0-F3) where F0 dams were exposed to BPA via feed *Comment:* Cross-fostering design (F0 to F1) to ensure prenatal BPA exposure to F1 generation *Comment:* At postnatal day (PND) 24, F1 and F3 mice were tested in an open field *Strengths:* - Phytoestrogen-free diet (F0 dams) *Weaknesses:* - Single dose level study - Insufficient study reporting (information on sex ratio in the F1 litters is missing, as well as reproductive outcome of the sibling mating performed to obtain F2 and F3 generations, unclear whether the authors actually measured the daily amount of feed consumed for each dam or averaged the amount based on previous knowledge; lack of information of control of environmental contamination of BPA, i.e. cages, bedding and water bottles) - F1-F3 fed chow containing phytoestrogens)	Positive	Low to Medium	●
Xu et al., 2011 (see also Q2) *Comment:* Adolescent mice orally exposed to BPA for 8 weeks; behavioural testing started at day 91 *Comment:* four behavioural tests used (open field, Elevated plus maze, Morris water maze, passive avoidance (step down)) *Comment*: index of anxiety-like behaviour given; unclear abolished (or induced) sex difference of BPA *Strengths:* - Phytoestrogen-free diet *Weaknesses:* - Inappropriate statistics (multiple comparison statistics not considered) - Type of cages and drinking bottles not reported	Positive	Low	●
Xu et al., 2012 *Comment*: Prenatal or postnatal exposure (maternal oral dosing presumably by gavage) *Comment*: Degree of effects are similar irrespectively of exposure period was gestational only or lactational only (exposure differs by orders of magnitude) *Comment:* Multiple functional test PND 56-60: Open field, Dark/light transition, Mirrored maze, Elevated plus maze, Forced swim task, and assessment of neurobiological end points (AMPA and NMDA receptors) *Comment:* Multiple tests performed to address the same endpoint and results consistent in 5 different tests for females and 3 different tests for males *Comment:* Use of ovariectomized mice which underwent surgery 1 week before testing for anxiety-like behaviour	Positive	From Low to Medium	●/↑

Strengths: - Phytoestrogen-free diet *Weaknesses:* - Study design not appropriate to the scope (the sequence of testing was not randomized) - Insufficient study reporting (two doses of BPA (4 and 0.4 mg/kg bw per day) were administered through the oral route without specifying how, presumably by gavage - Type of cages and drinking bottles not reported - Inappropriate statistics (multiple comparison statistics not considered)			
Xu et al., 2013a (see Q2 for other endpoint) *Comment:* Adult exposure by oral administration (gavage) for 12 weeks *Comment:* the highest BPA dose 40mg/kg per day is high *Comment*: anxiety-like behaviour evaluated by open field. Dose dependency for medium and high dose males in the measures of grooming *Comment:* Parallel measurement of synaptic morphology (neural plasticity index) and functional endoints *Strengths:* - Number of BPA doses (3) - Phytoestrogen-free diet - Use of non-PC cages and of non-plastic water bottles - Blinded evaluation of data from the open field test, not specified for the MWM or for passive avoidance *Weaknesses:* - Insufficient study reporting (body weight data collected during treatment not shown)	Positive	Low	•
Overall conclusion on Likelihood on Anxiety-like behaviour in animals after pre- and/or postnatal exposure to BPA: Several studies report on increased anxiety-like behaviour in laboratory rodents after exposure to BPA. Due to the limitation in study design and statistics, and the inconsistency in the reported results, potential effects are considered to be as likely as not.			**As likely as not**
Q2: Is there any evidence that BPA exposure affects learning and memory?	**Answer to the question as reported by the study authors (Positive, Negative or Uncertain)**	**Reliability of evidence (Low, Medium or High)**	**Influence on Likelihood (see Table 28)**

Starting point based on previous assessments (EFSA, 2006; EFSA CEF Panel, 2010) Overall the CEF Panel concluded that currently available data addressing neurobehavioural endpoints (e.g. learning and memory behaviour, anxiety-related behaviour and gender-specific behaviour) does not provide convincing evidence of neurobehavioural toxicity of BPA (EFSA CEF Panel, 2010). The Stump (2010) study was inconclusive with respect to the learning and memory endpoint. The Panel considered that the biochemical changes in Xu et al., 2010a (in rats) were not sufficient to revise the TDI in the absence of a clear link to an adverse functional outcome, also in consideration of the shortcomings identified.	Uncertain	Low [20]	●
Line of evidence 1: new studies on impairment of learning and memory following BPA exposure (Diaz-Weinstein et al., 2013; Eilam- Stock et al., 2012; Ferguson et al., 2012; Inagaki et al., 2012; Jang et al., 2012; Jasarevic et al. 2012; Jones and Watson, 2012; Kim et al., 2011; Matsuda et al., 2013, Nakamura et al., 2012; Viberg et al., 2011; Xu et al., 2010b; Xu et al., 2011; Xu et al., 2013a)			
Diaz-Weinstein et al., 2013 (also see Q1) *Comment:* Adolescent rats exposed daily by subcutaneous injection to BPA for 12 days *Comment:* spatial memory was tested (object placement task) *Strengths:* - Use of glass water bottles - Blind evaluation of the observed behaviour *Weaknesses:* - Single dose level study - not appropriate to the scope not appropriate to the scope (behavioural tests performed only once with limitation to one trial, subsequent testing in two different tests on the same day) - Insufficient study reporting (body weights not measured daily in conjunction with treatment; no information on sexual maturation and insufficient information on recording of behavioural testing; animal diet and phytoestrogen content not reported) - Inappropriate statistics (repeated measures for the same animal are not taken into account, cycling is not adjusted for in the analysis; Fisher's protected LSD test applied which does not adequately protect from Type 1 Error increase due to multiple comparisons)	Positive	Low	●
Eilam-Stock et al., 2012 *Comment:* Single subcutaneous administration in adult male rats *Comment:* Parallel assessment of neurobiological markers (decreased spinogenesis and PSD95) in two different brain areas and functional effects	Positive	Low	●

[20] Refers to the evaluated studies on neurobehavioural toxicity and not to the review by EFSA

Strengths: - None *Weaknesses:* - Small sample size (n = 6) - Single acute dose administration - Single dose study level - Study design not appropriate to the scope (all test animals were exposed to BPA, no control group) - Test performed in one sex only (male) - Animal diet and phytoestrogen content not reported			
Ferguson et al., 2012 (see Q1 and Q4 for other endpoint) *Comment*: Pre- and postnatal exposure by maternal administration of BPA (gavage), plus direct postnatal exposure to pups (gavage) *Comment*: Motor activity of naïve controls similar to that of BPA –treated groups *Comment*: Multiple tests performed (Novelty preference test (PND 29), Open field test (PND 40-42), Motor coordination (PND 43-44), Barnes maze (PND 47-50), Acoustic startle response (PND 54), and Morris water maze (PND 75-79). Here Barnes maze and Morris water maze is addressed *Strengths:* - Large sample size - Both naïve and vehicle controls available (to account for the handling of the animal) - Adequate positive controls included - Use of non-PC cages and of glass water bottles *Weaknesses:* - Study design not appropriate to the scope (only low doses of BPA used)	Negative	High	↓
Inagaki et al., 2012 (see also Q1) *Comment*: Single subcutaneous administration to adult cycling female rats *Comment*: parallel assessment of learning/memory and relevant neurobiological marker (spinogenesis) in two different brain areas *Strengths:* - BPA measurement in biological samples (serum) *Weaknesses:*	Positive	Low	•

- Single acute dose administration - Test performed in one sex only - Insufficient study reporting (study design not properly described, doses and number of animals for the various tests is unclear; except from feed content, no information of environmental contamination of BPA is given, i.e. cages, water bottles, or bedding) - Inappropriate statistics (considerations of repeated measures of the same animal not included in the analyses, nor multiple endpoint within a test)			
Jang et al., 2012 *Comment*: Gestational exposure of the F0 dams by intraperitoneal injection (i.p.) in a multigeneration study Comment: highest dose 10 mg/kg bw i.p. *Comment:* use of Morris water maze (no effect) and Passive Avoidance (with effect) *Comment*: Parallel assessment of neurobiological (CREB expression) and neuroanatomical (neurogenesis) markers *Strengths:* - Number of BPA doses (3) *Weaknesses:* - Small sample size - Maternal administration via ip injection during pregnancy - Test performed in one sex only - Low number of animals tested - Insufficient study reporting (number of females in the F0 generation was not given; animal body weight not given; animal diet and phytoestrogen content not reported; no information of environmental contamination of BPA is given, i.e. cages, water bottles, or bedding) - Inappropriate statistics (litter effect not considered, no correction for multiple comparisons)	Positive	Low	•
Jasarevic et al., 2013 (also see Q1) *Comment*: Deer mice with pre- and postnatal exposure (maternal dosing via feed 2 week prior to mating) *Comment:* Indication of weak dose-response reaching a plateau at the 2 top doses (≥5 mg/kg bw per day). *Comment*: anxiety-like behaviour evaluated by elevated plus maze (EPM) and spatial learning capabilities in a modified Barnes Maze as adults *Strengths:* - Adequate positive controls included - Number of BPA doses (3)	Positive (male)	Low	●/↑

- Use of non-PC cages and glass water bottles - BPA exposure measurement in animal samples (serum) *Weaknesses:* - Study design not appropriate to the scope (exposure pre mating may influence on reproductive outcome; multiple breeding; use of littermates in testing; study not controlled for reproductive cycling at testing time; unclear exposure time of the respective dams or if the controls were concurrent with the treated animals; possible additional dietary exposure of the offspring during late lactation) - Insufficient study reporting (no information on normalization for dams' body weight and feed consumption described; maternal amount of feed consumed daily not specified; unclear whether offspring may have been exposed directly through feed during late lactation when incisors have appeared; total number of dams and their general reproductive outcome not given) - Inappropriate statistics (the litter effect was not adequately considered; no multiple comparison statistics; Fisher's protected LSD test applied which does not adequately protect from Type 1 Error increase due to multiple comparisons)			
Jones and Watson, 2012 (see also Q1) *Comments*: Pre- and postnatal exposure (maternal oral dosing by licking oil drops) *Comment:* three behavioural tests performed (Morris Water Maze, Forced Swim Test (FST) and for anxiety-like behaviour in the Elevated Plus Maze, EPM) *Strengths:* - Number of BPA doses (4) - Use of non-PC cages, and of BPA-free water sacks (for dams) *Weaknesses:* - Study design not appropriate to the scope (small number (2-3) of dams per group; littermates treated as independent observations; females not controlled for cycling at the time of testing) - Insufficient study reporting (unclear reports for the statistical findings in the figures/tables presented, especially for FST) - Inappropriate statistics (because of the limited number of litters tested (2-3) per dose group, the samples size is considered underpowered; litter effect not considered, lack of analysis of the two way interaction between treatment and sex; no adjustment of cycling included)	Positive	Low	•
Kim et al., 2011 *Comment*: Two weeks exposure orally by gavage in young adult mice *Comment:* Parallel assessment of neuroanatomical markers and functional effects *Comment:* effects seen only at high dose (20mg/kg bw), not at 5mg /kg bw per day or the lower 1 mg/kg bw	Positive	Low	•

Strengths: - Number of doses (3) *Weaknesses:* - Small sample size (n = 5- 6 per group) - Test performed in one sex only (male) - Insufficient study reporting (unclear number of mice used and whether the investigation of newly generated cells was performed in separate groups of mice or not; animal diet and phytoestrogen content not reported - Inappropriate statistics (Fisher's protected LSD test applied does not adequately protect from Type 1 Error increase due to multiple comparisons)			
Matsuda et al., 2013 *Comment*: Pre- and postnatal exposure (maternal dosing via subcutanous route) *Comment:* Parallel assessment of molecular markers (5-HT and 5-HIAA) and functional end points (fear memory) *Strengths:* - None *Weaknesses:* - Single dose level (250 ng/kg bw per day BPA) - Insufficient study reporting (number of dams and general reproductive outcome not given; no information about check for cycling in adult female offspring) - Insufficient BPA-exposure assessment (constant during pregnancy and lactation and not adjusted daily to body weight; no information about environmental BPA contamination, e.g. feed and bedding is given) - Animal diet and phytoestrogen content not reported - Use of polycarbonate (PC) cages and PC water bottles	Positive (juvenile females only)	Low	●
Nakamura et al., 2012 (see also Q1) *Comment:* Pre- and postnatal exposure by maternal administration of BPA (subcutaneously) *Comment:* Use of open field, elevated plus maze and Morris water maze *Strength:* - None *Weaknesses:* - Single dose level study - Insufficient study reporting (maternal body weight not given; lack of information on check for cycling in female offspring; no information of environmental BPA-control, i.e. animal diet and phytoestrogen content, type of cages, bedding and drinking bottles not reported)	Negative	Low	●

- Insufficient BPA-exposure assessment (constant during pregnancy and lactation and not adjusted to body weight) - Inappropriate statistics (litter effect not considered)			
Viberg et al., 2011 (also see Q1) *Comment*: single oral administration by gavage on PND 10 to males only *Comment*: Spatial learning task (Morris water maze) performed at 6 months of age showed no BPA effect *Strengths:* - Number of dose groups (3) *Weaknesses* - Single acute dose administration - Small sample size (3-4 litters/group) - Test performed in one sex only - Study design not appropriate to the scope (pup body weight recorded only four times during the 6 months study; only male pups behaviourally tested and it appears as the same 12-15 males representing 3-4 litters were used in all four tests; littermates treated as independent observations) - Insufficient study reporting (number of dams and general reproductive outcome lacking; litters said to be standardised by size (12-14 pups) but sex ratio is not given; type of cages and drinking bottles not reported) - Inappropriate statistics (because of the limited number of litters tested (3-4), the samples size is considered underpowered; the litter effect is not properly considered in the statistics) - Phytoestrogen content in animal diet not given	Negative	Low	●
Xu et al., 2010b *Comment:* Pre- and postnatal exposure by oral maternal administration of BPA *Comment:* Two behavioural tests performed, Morris water maze and step-down passive avoidance test *Strengths:* - Number of doses (4) - Phytoestrogen-free diet *Weaknesses:* - Test performed in one sex only (male) - Insufficient study reporting (reproductive outcome not shown, e.g. maternal bw, no pre-weaning body weight data shown; information about type of bedding and water bottles missing) - Insufficient BPA-exposure assessment (unknown if constant during pregnancy and lactation or adjusted daily to body weight)	Positive (males only)	Low to Medium	●
Xu et al., 2011 (see also Q1) *Comment:* Adolescent mice orally exposed to BPA for 8 weeks; behavioural testing starts at day 91	Positive	Low	●

Comment: four behavioural tests used (Open field, Elevated plus maze, Morris water maze, Passive avoidance (step down)) *Comment*: unclear abolished (or induced) sex difference of BPA *Strengths* - Phytoestrogen-free diet *Weaknesses* - tics (multiple comparison statistics not considered) - Type of cages and drinking bottles not reported			
Xu et al., 2013a (see Q1 for other endpoint) *Comment:* Adult exposure by oral administration (gavage) for 12 weeks *Comment:* Parallel assessment of synaptic morphology (neural plasticity index) and functional end points. *Comment*: Two learning tasks used: the Morris Water Maze and the Passive Avoidance test (step down test). *Strengths:* - Number of BPA doses (3) - Phytoestrogen-free diet - Use of non-PC cages and of non plastic water bottles - Blinded evaluation of data from the open field test, not specified for the MWM or for passive avoidance *Weaknesses:* - Insufficient study reporting (body weight data collected during treatment not shown)	Positive (males only)	Medium to High	↑ (males)
Overall conclusion on Likelihood on Learning and memory in animals after pre- and/or postnatal exposure to BPA: The effects of BPA on learning and memory abilities of laboratory rodents are no fully consistent, as both positive and negative effects are reported in different papers. The papers have methodological shortcomings, such as underpowered sample size, lack of consideration of the litter effect, or not properly controlled variability of exposure through diet, and inadequate statistics. Potential effects are considered to be as likely as not.			**As likely as not**
Q3: Is there any evidence that BPA exposure affects social behaviour in the animal species tested?	**Answer to the question as reported by the study authors (Positive, Negative or Uncertain)**	**Reliability of evidence (Low, Medium or High)**	**Influence on Likelihood (see Table 28)**
Starting point based on previous assessments: EFSA opinion did not assess social behaviour separately from anxiety. Other reviews (FAO/WHO, 2011) Several studies reviewed suggest an effect of developmental exposure to BPA on social responses in rodents (increased aggression in males and defeminisation of social/affiliative behaviour in females). In general these specific end points have not been considered as relevant in the conclusions of the different previous reports.	Some positive/Some negative	Low	•

Line of evidence 1: New studies on social behaviour (Kundakovic et al., 2013; Wolstenholme et al., 2011a; Wolstenholme et al., 2012; Wolstenholme et al., 2013;):			
Kundakovic et al., 2013 (see also Q1) *Comment*: Gestational exposure (maternal oral administration of BPA during pregnancy only) *Comment:* Parallel assessment of relevant molecular markers (oestrogen receptors and DNA methylation for ER genes) *Comment:* The large scatter within the dose-groups for the behavioural endpoints does not allow concluding on the shape of the dose-response curve and even a conclusion that there is no dose-response would be compatible with the data *Comment:* Anxiety-like behaviour tested in Open field (OF) area at PND 60 (video recording) *Comment:* Two different tests for social behaviour used (home cage social behaviour in juveniles and dyadic interaction with a same-sex stimulus mouse at PND 70) *Strengths:* - Number of BPA doses (3) - BPA measured in biological sample (urine) - Use of non-polycarbonate (non-PC cages) *Weaknesses:* - Insufficient study reporting (type of bedding not given; general reproductive information like maternal body weight, litter size and sex- ratio is not given; frequency of various postpartum maternal behaviours given without the litter size; the administration to dams is not specified except that it is oral; urine collection and analysis not described; the sacrificing and brain sampling procedures are not detailed; insufficient information concerning the scoring of social/aggressive behaviour; poor description of the social behaviour testing) - Inappropriate statistics" (lack of accounting for multiple observations within each litter [pseudo-replication] in the ANOVA; the application of the multilevel regression model showed a large scatter of data within dose-groups [only four] and thus a large number of different dose-response curves can be fitted to the data so that it is not possible to conclude on the biological relevance for any of them) - Animal diet and phytoestrogen content not reported - Type of drinking bottles not given	Positive	Low	●/↑
Wolstenholme et al., 2011a. (also see Q1) *Comment*: Prenatal exposure (maternal exposure via feed prior to mating and through gestation); cross-fostering design *Comment:* To ensure prenatal exposure only and exclude BPA-induced differences of maternal care, foster-dams were used implying mixed litters and tail clipping of pups, which might both be a strength and a weakness *Comment:* Parallel assessment of relevant neurobiological (gene expression ER, oxytocin) and functional end points. *Comment:* Social interaction test on PND 20, social preference test on PND 24.	Positive	Low	↑/●

Strengths: - BPA measurement in animal samples (serum) - Phytoestrogen-free diet - Scoring blind to treatment *Weaknesses:* - Single dose level study - Feed consumption (BPA given by the diet) not measured - Type of cages and drinking bottles not reported - Insufficient study reporting (information of the content of the mixed litters, like number of pups and sex ratio is missing) - Inappropriate statistics (litter effect not properly considered)			
Wolstenholme et al., 2012 *Comment:* The study addressed association of BPA behavioural effects with expression of genes implicated in regulation of social behaviour and related sex dimorphism (ERs, oxytocin and vasopressin) in embryos. *Comment*: transgenerational effects (F2 and F4) were addressed. *Comment:* Social interaction on PND 20-21, social preference on PND 24. *Strengths:* - BPA measurement in animal samples (F0 dams, serum) - Phytoestrogen-free diet (F0-generation only) *Weaknesses:* - Single dose level study - Study design not appropriate to the scope (the number of dams in F0 generation was limited) - Insufficient study reporting (animal age and body weight not given; no normalization of food consumption on body weight, potential variability of exposure; lack of information of control of environmental contamination of BPA, i.e. cages, bedding and water bottles; lack of information of mixed litters e.g. number of pups and sex ratio; reproductive outcome of the breeding brother-sister pairs done to obtain F2 to F4 generation is not given) - Insufficient BPA-exposure assessment (estimated on the basis of the daily quantity of food ingested, see line above) - Inappropriate statistics (litter effect not considered)	Positive	Low	•
Wolstenholme et al., 2013 (see also Q1) *Comment:* Transgenerational study (F0-F3) where F0 dams were exposed to BPA via feed *Comment:* cross-fostering design F0 to F1 to ensure prenatal BPA exposure to F1 generation *Comment:* F1 and F3 tested at postnatal day (PND) 21 for social recognition *Strengths*	Positive	Low to Medium	•

- Phytoestrogen-free diet (F0 dams) *Weaknesses* - Single dose level study - Insufficient study reporting (information on sex ratio in the F1 litters is missing, as well as reproductive outcome of the sibling mating performed to obtain F2 and F3 generations, unclear whether the authors actually measured the daily amount of feed consumed for each dam or averaged the amount based on previous knowledge; lack of information of control of environmental contamination of BPA, i.e. cages, bedding and water bottles) - F1-F3 fed chow containing phytoestrogens			
The few new studies evaluating the effects of BPA on social behaviour as end point have some methodological shortcomings (e.g. litter effect not properly red, potential variability of exposure not controlled for). Due to the limitation in study design and statistics, and the inconsistency in the reported results, potential effects are considered to be as likely as not.			**As likely as not**
Q4: Is there any evidence that BPA exposure affects sensory-motor function?	**Answer to the question as reported by the study authors (Positive, Negative or Uncertain)**	**Reliability of evidence (Low, Medium or High)**	**Influence on Likelihood (see Table 28)**
Starting point based on previous assessments (EFSA, 2006; EFSA CEF Panel, 2010). Overall the Panel concluded that currently available data did not provide convincing evidence of sensory-motor function of BPA. The study by Stump (20) covered among other neurodevelopmental toxicity end points, the auditory startle response	Negative	Medium	↓
Line of evidence 1: new studies on changes in sensory-motor function (Ferguson et al., 2012; Wolstenholme et al., 2013):			
Ferguson et al., 2012 (see Q1 and Q2 for other endpoint tested) *Comment*: Pre- and postnatal exposure by maternal administration of BPA (gavage), plus direct postnatal exposure to pups (gavage) *Comment*: Motor activity of naïve controls similar to that of BPA –treated groups *Comment*: Multiple tests performed (Novelty preference test (PND 29), Open field test (PND 40-42), Motor coordination (PND 43-44), Barnes maze (PND 47-50), Acoustic startle response (PND 54), and Morris water maze (PND 75-79). Here acoustic startle response are addressed. *Strengths:* - Large sample size - Both naïve and vehicle controls available (to account for the handling of the animal) - Adequate positive controls included	Negative /uncertain (males)	Medium	•/↓

- Use of non-PC cages and of glass water bottles *Weaknesses:* - Study design not appropriate to the scope (only low doses of BPA used)			
Wolstenholme et al., 2013 (see also Q1 and Q3) *Comment:* Transgenerational study (F0-F3) where F0 dams were exposed to BPA via feed *Comment:* cross-fostering design F0 to F1 to ensure prenatal BPA exposure to F1 generation *Comment:* F3 mice of both sexes were tested for olfactory discrimination as adults *Strengths:* - Phytoestrogen-free diet (F0 dams) *Weaknesses:* - Single dose level study - Insufficient study reporting (information on sex ratio in the F1 litters is missing, as well as reproductive outcome of the sibling mating performed to obtain F2 and F3 generations, unclear whether the authors actually measured the daily amount of feed consumed for each dam or averaged the amount based on previous knowledge; lack of information of control of environmental contamination of BPA, i.e. cages, bedding and water bottles) - F1-F3 fed chow containing phytoestrogens	Negative	Low to Medium	●
Overall conclusion on Likelihood on Sensory-motor function in animals after pre- and/or postnatal exposure to BPA: One of the two studies considered reported some positive effects of BPA on sensory-motor function in adult male rats, not in females. The auditory startle response is not known as a sexually dimorphic behaviour and the study results were considered inconsistent. The potential effects are considered to be as likely as not.			**As likely as not**

Table 37: Summary of the WOE assessment of the likelihood that BPA causes neurodevelopmental or neurological/behavioural effects

Humans	
Overall conclusion on Likelihood of neurodevelopmental effects of BPA in humans: There are indications from prospective studies that prenatal BPA exposure (BPA exposure during pregnancy) may be associated with child behaviour in a sex-dependent manner. However, the associations were not consistent across the studies. It cannot be ruled out that the results are confounded by diet or concurrent exposure factors. The associations are not sufficient evidence to infer a causal link between BPA exposure during pregnancy and neurodevelopmental effects in humans. Potential effects are considered to be as likely as not.	**As likely as not**
Overall conclusion on Likelihood of neurological/behavioural effects of BPA in humans: There are indications from one prospective study that childhood BPA exposure may be associated with behavioural problems in both girls and boys. It cannot be ruled out that the results are confounded by diet or concurrent exposure factors. The associations are not sufficient evidence to infer a causal link between childhood BPA exposure and neurological effects/behavior in humans. Potential effects are considered to be as likely as not.	**As likely as not**
Animals	
Overall conclusion on Likelihood on Anxiety-like behaviour in animals after pre- and/or postnatal exposure to BPA: Several studies report on increased anxiety-like behaviour in laboratory rodents after exposure to BPA. Due to the limitation in study design and statistics, and the inconsistency in the reported results, potential effects are considered to be as likely as not.	**As likely as not**
Overall conclusion on Likelihood on Learning and memory in animals after pre- and/or postnatal exposure to BPA: The effects of BPA on learning and memory abilities of laboratory rodents are no fully consistent, as both positive and negative effects are reported in different papers. The papers have methodological shortcomings, such as underpowered sample size, lack of consideration of the litter effect, or not properly controlled variability of exposure through diet, and inadequate statistics. Potential effects are considered to be as likely as not.	**As likely as not**
Overall conclusion on Likelihood on Social behaviour in animals after pre- and/or postnatal exposure to BPA: The few new studies evaluating the effects of BPA on social behaviour as end point have some methodological shortcomings (e.g. litter effect not properly red, potential variability of exposure not controlled for). Due to the limitation in study design and statistics, and the inconsistency in the reported results, potential effects are considered to be as likely as not	**As likely as not**
Overall conclusion on Likelihood on Sensory-motor function in animals after pre- and/or postnatal exposure to BPA: One of the two studies considered reported some positive effects of BPA on sensory-motor function in adult male rats, not in females. The auditory startle response is not known as a sexually dimorphic behaviour and the study results were considered inconsistent. The potential effects are considered to be as likely as not.	**As likely as not**

WoE of immune effects

Whether BPA has the potential to cause immune effects in humans and animals was considered using a tabular format for weighing different lines of evidence (WoE evaluation). The WoE evaluation tables for this endpoints are presented in full below.

Human studies

Table 38: Assessment of the likelihood of associations between BPA exposure and developmental immunotoxic effects in humans

Q1: Is there an association between BPA exposure and developmental immunotoxic effects?	**Answer to the question as reported by the study authors (Positive, Negative or Uncertain)**	**Reliability of evidence (Low, Medium or High)**	**Influence on Likelihood (see Table 28)**
Starting point based on previous assessments (EFSA, 2006, 2010): No human studies on immune effects were available for evaluation.			
Line of Evidence 1: Associations with developmental immunotoxic effects, resistance to infection: the association with cytomegalovirus is positive in <18 years, and negative in >18 y (Clayton et al., 2011) *Strengths:* – Large sample size – Analytical method (LC-MS-MS) – Quality control and quality assurance procedures *Weaknesses:* – Cross-sectional study design – Single exposure measurements – Single spot urine BPA measurement – Confounding by diets or by concurring exposure factors not considered – Unclear clinical relevance (inconsistent results between groups stratified by age)	Positive	Low	●
Line of Evidence 2: Associations with developmental immunotoxic effects, allergy. Association with wheeze at 6 months of age (Spanier et al., 2012), and with asthma in females (Vaidya et al., 2012), no association with wheeze at other time points, no association with asthma in males, no association with sensitization (Savage et al., 2012). Urinary BPA in pregnant mothers associated with lower risk of wheeze, while urinary BPA in children was associated with higher risk of wheeze and asthma (Donohue et al., 2013)	Mainly Negative, some positive	Low to Medium	↓/↑

Strengths: – Longitudinal follow-up (Spanier et al., 2012; Donohue et al., 2013). – Large sample size (all studies) – Repeated measurements (Spanier et al., 2012; Donohue et al., 2013) – Analytical method (LC-MS-MS) (all studies) – Quality control and quality assurance procedures (all studies) – Multiple outcome assessment, for wheeze (Spanier et al., 2012), asthma (Donohue et al., 2013, Vaidya et al., 2012) and allergen sensitisation (Savage et al., 2012). *Weaknesses:* – Cross-sectional study design (Vaidya et al., 2012; Savage et al., 2012) – Single spot urine BPA measurements (all studies) – Not adjusted urine samples (Vaidya et al., 2012) – Confounding by diet or other exposures not considered (all studies) – Unclear clinical relevance: small effect size (Donohue et al., 2013), relevance of wheeze difficult to interpret in the absence of sensitization effects (Spanier et al., 2012), inconsistent results between groups (Vaidya et al., 2012; Donohue et al., 2013). – Inconsistent results amongst different studies (all studies)			
Overall conclusion on the likelihood of association between BPA exposure and developmental immunotoxic effects: There are indications that BPA may be linked to immunological outcomes in humans, although in view of the limitations of the studies only limited conclusions can be reached and it cannot be ruled out that the results are confounded by diet or concurrent exposure factors. The associations do not provide sufficient evidence to infer a causal link between BPA exposure during pregnancy or in childhood and immune effects in humans.			**As likely as not**

Animal studies

Table 39: Assessment of convincing associations between BPA exposure and developmental immunotoxic effects in animals.

Q1: Is BPA immunotoxic in animals?	**Answer to the question as reported by the study authors (Positive, Negative or Uncertain)**	**Reliability of evidence (Low, Medium or High)**	**Influence on Likelihood (see Table 28)**
Starting point based on previous assessments (EFSA CEF Panel, 2010): Based on the studies reviewed, the CEF Panel concludes that BPA showed indications of effects on immune parameters. *Weakness:* All studies suffered from shortcomings *Weakness*: Results were inconsistent	Some positive	Low	●/↑
Line of Evidence 1: new evidence on immunotoxic effects induction in adult life (Lee et al., 2012a; Kendziorski et al., 2012) Comment: no functional endpoints assessed (Lee et al., 2012a) *Strengths:* - Positive control included (Kendziorski et al., 2012) - Number of doses (≥3) (Kendziorski et al., 2012) - Phytoestrogen –free diet (Kendziorski et al., 2012) *Weaknesses:* - Animal body weight not given (Kendziorski et al., 2012) - Small sample size (Lee et al., 2012a; Kendziorski et al., 2012) - Single dose level study (to show effects on total IgE non-specific inflammatory mediators) (Lee et al., 2012a) - Test performed in one sex only (Lee et al., 2012a)	Positive	Low	●
Line of Evidence 2: new evidence on immunotoxic effects induction during pre- and post-natal (during lactation) development (Nakajima et al., 2012; Bauer et al., 2012) *Strengths:*	Positive	Medium	↑↑

- Phytoestrogen-free diet (Nakajima et al., 2012; Bauer et al., 2012) - Use of non-PC cages and of non plastic bottles Nakajima et al., 2012) - Oral administration by gavage (Bauer et al., 2012) - Number of doses (4 doses for the lung lavage) (Bauer et al., 2012) *Weaknesses:* - Animal age not given (Nakajima et al., 2012) - Animal body weight not given (Nakajima et al. 2012) - Small sample size for the endpoint studied (3 to 11 animals/group) (Nakajima et al., 2012; Bauer et al., 2012) - Single dose level study Nakajima et al., 2012 - Study design not appropriate to the scope (administration via drinking water, but water consumption not measured) (Nakajima et al., 2012)			
Overall conclusion on the likelihood of immunotoxic effects of BPA in animals: Evidence from the new studies adds to the indications of immunotoxicity of BPA in animals reported in previous reviews.			**ALAN to Likely**

WoE of cardiovascular effects

Whether BPA has the potential to cause cardiovascular effects in humans was considered using a tabular format for weighing different lines of evidence (WoE evaluation). The WoE evaluation tables for this endpoints are presented in full below.

Human studies

Table 40: Assessment of the likelihood of associations between BPA exposure and cardiovascular effects in human studies.

Q1: Question: Is there an association between BPA exposure and cardiovascular effects?	**Answer to the question as reported by the study authors (Positive, Negative or Uncertain)**	**Reliability of evidence (Low, Medium or High)**	**Influence on Likelihood (see Table 28)**
Starting point based on previous assessments (EFSA CEF PANEL, 2010): Two cross-sectional epidemiological studies showed statistically significant associations between BPA exposure and coronary heart disease (Lang et al., 2008, Melzer et al., 2010). *Weakness:* - Although the studies provided some indication of possible cardiovascular effects in humans, EFSA concluded that the cross-sectional design of the study limited the reliability and likelihood of a causal association.	Positive	Low	●
A prospective study showed that higher urinary BPA was associated with increased risk of developing myocardial infarction (Melzer et al., 2012a) and a prospective study showed that higher urinary BPA was associated with increased risk of coronary artery stenosis (Meltzer atl al., 2012b) *Comment:* Outcome definitions only included cases admitted to hospital (Melzer et al., 2012a). Urinary BPA values were normalized to blood urea creatinine ratio, while other studies use urinary creatinine level for normalization (Melzer et al., 2012b). *Strengths:* – Longitudinal follow up – Analytical method (SPE LC-MS-MS) – Quality control, including blanks and quality assurance procedures *Weaknesses:* – Small sample size (Meltzer atl al., 2012b)	Positive	Medium to low	●/↑

– Single spot urine BPA measurement – Confounding by diet or by concurring exposure factors not considered – Generalisability to the overall population			
Line of Evidence 2: Associations with coronary artery disease/heart attack in cross-sectional studies. Two studies found no association (Olsén et al., 2012; Lakind et al., 2012**)**, one showed no associations with two and significant associations with two outcome measures **(**Lind and Lind., 2011) *Comment:* A-priori defined inclusion and exclusion criteria, outcome definitions and confounders (Lakind et al., 2012) *Comment:* Total energy intake included among confounders (Lakind et al., 2012)*:* *Strengths:* – Large sample size (Lakind et al., 2012; Lind and Lind., 2011; Olsén et al., 2012) – Urine, container specified (Lakind et al., 2012) – Standardized samples (urinary creatinine included in the model as independent variable) (Lakind et al., 2012) – Analytical method (SPE LC-MS-MS) (all studies) – Quality control, including blanks and quality assurance procedures (all studies) *Weaknesses:* – Cross-sectional design (Lind and Lind., 2011; Olsén et al., 2012; Lakind et al., 2012) – Selection bias (Lind and Lind., 2011; Olsén et al., 2012) – Serum BPA measurement (invalid exposure measurement) (Lind and Lind., 2011; Olsén et al., 2012) – Single exposure measurements (all studies) – No distinction between unconjugated and conjugated BPA (Lind and Lind., 2011; Olsén et al., 2012; Lakind et al., 2012) – Handling of values below LOQ not reported (Lind and Lind., 2011; Olsén et al., 2012) – Confounding by diet or by concurring exposure factors not considered (all studies) – Generalisability to the total population (Lind and Lind., 2011; Olsén et al., 2012) – Inconsistency in results among different studies (all studies)	Positive	Low	●
Line of Evidence 3: Associations with metabolic syndrome. One study showed association with presence of metabolic syndrome (Teppala et al., 2012) *Strengths*: – Standardised samples (urinary creatinine included in the model as independent variable) – Analytical method (SPE LC-MS-MS) – Quality control, including blanks and quality assurance procedures *Weaknesses:*	Positive	Low	●

– Cross-sectional study – Single spot urine BPA measurement – No distinction between unconjugated and conjugated BPA – Confounding by diet or by concurring exposure not considered – Generalisability to the overall population			
Line of Evidence 4: Associations with hypertension and peripheral artery disease. Two studies found association with hypertension (Shankar and Teppala., 2012; Bae et al., 2012) and one study with peripheral arterial disease (Shankar et al., 2012). *Strengths*: – Urine, container specified (Bae et al., 2012) – Standardised samples (Bae et al., 2012) – Analytical method (SPE LC-MS-MS) (all studies) – Quality control, including blanks and quality assurance procedures (Shankar and Teppala., 2012; Shankar et al., 2012) *Weaknesses:* – Cross-sectional study design (all studies) – Selection bias (Bae et al., 2012) – Single spot urine BPA measurement (all studies) – No quality control (e.g., blanks) and quality assurance procedures (Bae et al., 2012) – No distinction between unconjugated and conjugated BPA (all studies) – Confounding by diet or by concurring exposure factors not considered (all studies) – Generalisability to the total population (all studies)	Positive	Low	●
Overall conclusion on Likelihood: There are indications from one prospective study that BPA exposure may be associated with cardiovascular effects, but it cannot be ruled out that the effect is confounded by diet or other concurrent exposure factors. This association does not provide sufficient evidence to infer a causal link between BPA exposure and cardiovascular effects in humans. Potential effects are considered to be as likely as not.			**As likely as not**

Table 41: Overall table on WoE evaluation on cardiovascular effects of BPA in humans

Overall conclusion on likelihood of cardiovascular effects of BPA in humans: There are indications from one prospective study that BPA exposure may be associated with cardiovascular effects, but it cannot be ruled out that the effect is confounded by diet or other concurrent exposure factors. This association does not provide sufficient evidence to infer a causal link between BPA exposure and cardiovascular effects in humans.	**As likely as not**

WoE of metabolic effects

Whether BPA has the potential to cause metabolic effects in humans and animals was considered using a tabular format for weighing different lines of evidence (WoE evaluation). The WoE evaluation tables for this endpoints are presented in full below.

Human studies

Table 42: Assessment of the likelihood of associations between BPA exposure and metabolic and hormonal effects in humans.

Q1: Is there an association between BPA exposure and obesity?	**Answer to the question as reported by the study authors (Positive, Negative or Uncertain)**	**Reliability of evidence (Low, Medium or High)**	**Influence on Likelihood (see Table 28)**
Starting point based on previous assessments (EFSA CEF PANEL, 2010): No association with obesity in one cross-sectional study (Lang et al., 2008). *Weakness:* cross-sectional design	Negative	Low	●/↓
Line of Evidence 1: Association with obesity in adults Four studies showed significant associations (Carwile and Michels, 2011; Shankar et al., 2012b; Wang et al., 2012a; Zhao et al., 2012), and one study showed no associations (Galloway et al., 2010) *Comment:* Study populations not only in the US (Galloway et al., 2010; Wang et al., 2012a; Zhao et al., 2012) *Comment:* Inconsistent modelling of BPA exposure across studies *Strengths:* – Large sample size (Galloway et al., 2010; Carwile and Michels, 2011; Shankar et al., 2012b; Wang et al., 2012a) – Standardised samples: 24-h urine collection, (Galloway et al., 2010), morning spot samples (Wang et al., 2012a) or second morning spot samples (Zhao et al., 2012) – Analytical method (SPE LC-MS-MS) (all studies) – Quality control and quality assurance procedures (Galloway et al., 2010; Carwile and Michels, 2011; Shankar et al., 2012b) *Weaknesses:* – Cross-sectional study design (all studies) – Small sample size (Zhao et al., 2012)	Mainly Positive	Low	●

– Single spot urine BPA measurement (Carwile and Michels, 2011; Shankar et al., 2012b; Wang et al., 2012a; Zhao et al., 2012) – Single exposure measurements (all studies) – Single spot urine BPA measurement (Carwile and Michels, 2011; Shankar et al., 2012b; Wang et al., 2012a; Zhao et al., 2012) – Not adjusted urine samples (Shankar et al., 2012b; Zhao et al., 2012) – No quality control and quality assurance procedures (Wang et al., 2012a; Zhao et al., 2012) – No distinction between unconjugated and conjugated BPA (Wang et al., 2012a; Zhao et al., 2012) – Handling of values below LOQ not reported (Galloway et al., 2010) – Confounding by diet and/or by concurring exposure factors not considered (Carwile and Michels, 2011; Shankar et al., 2012b; Wang et al., 2012a; Zhao et al., 2012) – Insufficient study reporting (urinary BPA stratified in quartiles, but no justification provided) (Shankar et al., 2012b) – Inconsistent results amongst different studies (all studies)			
Line of Evidence 2: Association with obesity in children and adolescents Cross-sectional studies showed that higher BPA was associated with increased obesity (Trasande et al., 2012; Wang et al., 2012b; Bhandari et al., 2013; Eng et al., 2013; Harley et al., 2013b; Li et al., 2013) while a longitudinal analysis showed that higher prenatal BPA was association with lower body mass in girls (Harley et al., 2013b) *Comments:* – Study populations not only in the US (Wang et al., 2012b; Li et al., 2013) – Evaluation of total caloric intake assessed (Trasande et al., 2012) – Evaluation of dietary behaviour (Li et al., 2013) *Strengths:* – Prospective study design (Harley et al., 2013b) – Large sample size (Trasande et al., 2012; Bhandari et al., 2013; Eng et al., 2013; Li et al., 2013) – Urine, container specified (Harley et al., 2013b; Li et al., 2013) – Repeated measurements (Harley et al., 2013b) – Standardised samples: first morning spot samples (Wang et al., 2012b) – Analytical method (LC-MS-MS) (Trasande et al., 2012; Wang et al., 2012b; Bhandari et al., 2013; Eng et al., 2013; Harley et al., 2013b) – Quality control and quality assurance procedures (Trasande et al., 2012; Bhandari et al., 2013; Eng et al., 2013; Harley et al., 2013b) *Weaknesses:* – Cross-sectional study design (Trasande et al., 2012; Wang et al., 2012b; Bhandari et al., 2013; Eng et al., 2013; Li et al., 2013) – Small sample size (Harley et al., 2013b)	Negative - Positive	Low	Cross-sectional: ●/↑ Longitudinal: ●/↓

– Single exposure measurements (Trasande et al., 2012; Wang et al., 2012b; Bhandari et al., 2013; Eng et al., 2013; Li et al., 2013) – Single spot urine BPA measurement (all studies) – Not adjusted urine samples (Li et al., 2013) – No quality control and quality assurance procedures (Wang et al., 2012b; Li et al., 2013) – No distinction between unconjugated and conjugated BPA (Wang et al., 2012b; Li et al., 2013) – Confounding by diets or by concurring exposure factors not considered (Wang et al., 2012b; Bhandari et al., 2013; Eng et al., 2013; Harley et al., 2013b) – Inconsistent results amongst different studies (different gender-related effects in cross-sectional studies) – Inconsistent results between cross sectional and longitudinal studies (higher BPA was associated with higher body mass in cross-sectional analyses, while the longitudinal analysis showed no associations in boys and that higher BPA was associated with lower body mass in girls)			
Overall conclusion on likelihood of associations between BPA and obesity in humans There are indications from a prospective study that prenatal BPA exposure (maternal urinary BPA concentrations) may be associated with reduced body mass in girls, while cross-sectional studies indicate associations between childhood BPA exposure and obesity. It cannot be ruled out that the results are confounded by diet or concurrent exposure factors. The associations do not provide evidence to infer a causal link between BPA exposure and obesity in humans. Potential effects are considered to be as likely as not."			**As likely as not**
Q2: Is there an association between BPA exposure and hormonal effects?	**Answer to the question as reported by the study authors (Positive, Negative or Uncertain)**	**Reliability of evidence (Low, Medium or High)**	**Influence on Likelihood (see Table 28)**
Starting point based on previous assessments (EFSA CEF PANEL, 2010): One cross-sectional study reported a significant association between urinary BPA and serum hormones in men recruited through an infertility clinic (Meeker et al., 2010). *Weakness:* cross-sectional design, small sample size and limited generalisability (only men from an infertility clinic).	Positive	Low	●
Line of Evidence 1: Associations with sex hormones The only study identified on this issue showed weak association with testosterone in men only, no associations with other hormones examined and no associations in women (Galloway et al., 2010) *Strengths:* – Large sample size – Standardised sample (24-h urine collection) – Analytical method (SPE LC-MS-MS)	Positive	Low	●

– Quality control, including blanks *Weaknesses:* – Cross-sectional study design – Single exposure measurements – Confounding by diet or by concurring exposure factors (drugs) not considered – Handling of values below LOD not reported – Unclear clinical relevance – Inconsistent results amongst different studies			
Line of Evidence 2: Associations with thyroid hormones One study showed no association with thyroid hormones (Mendez and Eftim, 2012). Two studies showed associations (Brucker-Davies et al., 2011; Wang et al., 2012c) *Strengths:* – Large sample size (Mendez and Eftim, 2012) – Analytical method (LC-MS-MS) (Mendez and Eftim, 2012; Wang et al., 2012c) – Quality control, including blanks (Mendez and Eftim, 2012) *Weaknesses:* – Cross-sectional study design (all studies) – Small sample size (Brucker-Davies et al., 2011; Wang et al., 2012c) – Cord blood BPA measurement (invalid exposure assessment) (Brucker-Davies et al., 2011) – Single exposure measurements (all studies) – Single spot urine BPA measurement (Mendez and Eftim, 2012; Wang et al., 2012c) – Analytical method (RIA) (Brucker-Davies et al., 2011) – No quality control and quality assurance procedures (Brucker-Davies et al., 2011; Wang et al., 2012c) – No distinction between unconjugated and conjugated BPA (Brucker-Davies et al., 2011; Wang et al., 2012c) – Handling of values below LOQ not reported (Brucker-Davies et al., 2011) – Confounding by diet (all studies) or by concurring exposures (Mendez and Eftim, 2012; Wang et al., 2012c) not considered – Generalisability to the overall population (Wang et al., 2012c) – Inconsistent results among st different studies (all studies) – Occupational exposure (Wang et al., 2012c)	Negative - Positive	Low	●
Line of Evidence 3: Associations with adipokine expression One cross-sectional study showed associations with adverse action of leptin and adiponectin (Chou et al., 2011), and one prospective study found that maternal urinary BPA was associated with higher plasma leptin in 9 year old boys and higher plasma adiponectin levels in 9 year old girls (Volberg et al., 2013).	Positive	Low	●/↑

Comment: – Pregnancy soda consumption and child soda, fast food and sweet snack consumption were evaluated among confounders (Volberg et al., 2013) *Strengths:* – Prospective study (Volberg et al., 2013) – Urine, container specified (Volberg et al., 2013) – Repeated measurements (n=2, maternal urine) (Volberg et al., 2013) – Analytical method (LC-MS-MS) (Volberg et al., 2013) – Quality controls, including blanks (all studies) *Weaknesses:* – Cross-sectional study design (Chou et al., 2011) – Limited sample size (all studies) – Blood and cord blood BPA measurement (invalid exposure assessment) (Chou et al., 2011) – Single exposure measurements (Chou et al., 2011) – Single spot urine measurement (Volberg et al., 2013) – No distinction between unconjugated and conjugated BPA (Chou et al., 2011) – Confounding by diet (Chou et al., 2011) or by concurring exposure factors (all studies) – Insufficient study reporting (inconsistency between abstract and text) (Chou et al., 2011) – Statistics (excessive categorisation) (Chou et al., 2011) – Unclear clinical relevance (all studies) – Generalisability to the overall population (Volberg et al., 2013)			
Overall conclusion on likelihood of associations between BPA and hormonal effects in humans There are indications from one prospective study that maternal BPA exposure may be associated with adipokine expression in 9 year old children, but it cannot be ruled out that the result is confounded by diet or concurrent exposure factors. The association does not provide sufficient evidence to infer a causal link between BPA exposure hormonal effects in humans. Potential effects are considered to be as likely as not."			**As likely as not**
Q3: Is there an association between BPA exposure and diabetes?	**Answer to the question as reported by the study authors (Positive, Negative or Uncertain)**	**Reliability of evidence (Low, Medium or High)**	**Influence on Likelihood (see Table 28)**
Starting point based on previous assessments (EFSA CEF PANEL, 2010): Two cross-sectional epidemiological studies showed statistically significant associations between BPA exposure and diabetes (Lang et al., 2008, Melzer et al. 2010).	Positive	Low	•

Weakness: Although the studies provided some indication of possible effects on diabetes incidence in humans, EFSA concluded that the cross-sectional design of the studies limited the reliability and likelihood of a causal association.			
Line of Evidence 1: Associations with diabetes or insulin resistance Three studies showed associations (Ning et al., 2011; Silver et al., 2011; Shankar et al., 2011) and two studies did not show associations with diabetes (Kim and Park, 2013; Lakind et al., 2012). One study showed association with insulin resistance (Wang et al., 2012a) *Comments:* – Study populations not only in the US (Ning et al., 2011; Wang et al., 2012a; Kim and Park, 2013) – A-priori defined inclusion and exclusion criteria, outcome definitions and confounders (Lakind et al., 2012) – Some studies relied on self-reported diabetes incidence (Shankar et al., 2011; Kim and Park, 2013) *Strengths:* – Large sample size (all studies) – Urine, container specified (Lakind et al., 2012) – Standardised samples: morning spot samples (Wang et al., 2012a) – Analytical method (GC-MS or LC-MS-MS) (all studies) – Quality controls, including blanks and quality assurance procedures (Silver et al., 2011; Shankar et al., 2011; Lakind et al., 2012; Wang et al., 2012a) *Weaknesses:* – Cross-sectional study design (all studies) – Single exposure measurements (all studies) – Single spot urine BPA measurement (all studies) – Not adjusted urine samples (Ning et al., 2011) – No quality controls and quality assurance procedures (Ning et al., 2011; Kim and Park, 2013) – No distinction between unconjugated and conjugated metabolites (Ning et al., 2011; Wang et al., 2012a; Kim and Park, 2013) – Confounding by diet or by concurring factors not considered (all studies) – Inconsistent results amongst different studies (all studies)	Positive	Low	●
Overall conclusion on likelihood of associations between BPA and diabetes effects in humans: The indications that BPA may be associated with diabetes in humans are unlikely.			**Unlikely**
Q4: Is there an association between BPA exposure and metabolic syndrome?	**Answer to the question as reported by the study authors (Positive, Negative or**	**Reliability of evidence (Low, Medium or High)**	**Influence on Likelihood (see Table 28)**

	Uncertain)		
Line of Evidence 1: Associations with metabolic syndrome. The only study identified on this issue showed association with presence of metabolic syndrome (Teppala et al., 2012) *Strengths:* – Large sample size – Analytical method (SPE LC-MS-MS) – Quality control, including blanks and quality assurance procedures *Weaknesses:* – Cross-sectional study – Single exposure measurements – Single spot urine BPA measurement – Confounding by diet or by concurring exposure factors not considered – Generalisability to the overall population	Positive	Low	●
Overall conclusion on likelihood of associations between BPA and metabolic syndrome in humans: The indication that BPA may be associated with metabolic syndrome in humans is unlikely.			**Unlikely**
Q5: Is there an association between BPA exposure and renal function?	**Answer to the question as reported by the study authors (Positive, Negative or Uncertain)**	**Reliability of evidence (Low, Medium or High)**	**Influence on Likelihood (see Table 28)**
Line of Evidence 1: Associations with renal function Two studies showed associations (Li et al., 2012a; You et al., 2011). *Strengths:* – Large sample size (all studies) – Urine, container specified (Li et al., 2012a) – Standardised sample: first morning spot samples (Li et al., 2012a) – Analytical method (LC-MS-MS) (all studies) – Quality control and quality assurance procedures (You et al., 2011) *Weaknesses:* – Cross-sectional study design (all studies) – Single exposure measurements (all studies) – Single spot urine BPA measurement (all studies) – No quality control, including blanks or quality assurance procedures reported (Li et al., 2012a)	Positive	Low	●

– Confounding by diet or by concurring exposure factors not considered (all studies) – Unclear clinical relevance (all studies)			
Overall conclusion on likelihood of associations between BPA and renal effects in humans: The indication that BPA may be associated with renal function in humans is unlikely.			**Unlikely**

Animal studies

Table 43: Assessment of the likelihood of associations between BPA exposure and metabolic effects in animals when exposed during their adult life (postnatally).

Q1: Does BPA affect metabolic function as evidenced by effects on glucose or insulin regulation in adult animals (exposed postnatally)	**Answer to the question as reported by the study authors (Positive, Negative or Uncertain)**	**Reliability of evidence (Low, Medium or High)**	**Influence on Likelihood (see Table 28)**
Starting point: EFSA CEF Panel, 2010, reported the study of Ropero et al., 2008 showing effects of BPA on insulin secretion in mice. *Weakness:* the study was not considered as reliable at that time	Positive	Low	●/↑
Line of Evidence 1: A number of recent studies show effects on glucose or insulin regulation and/or effects on pancreatic morphology and/or function **(**Batista et al. (2012 subcutaneous injection); D'Cruz et al., 2012a, b; Marmugi et al., 2012; Jayashree et al. 2013/ Indumathi et al., 2013; Bodin et al., 2013; US FDA/NCTR, 2013 and Delclos et al., 2014) *Comment*: the US FDA/NCTR, 2013 and Delclos et al., 2014 subchronic toxicity study showed no effect, but the animals were exposed both pre and post-natally *Comment*: The standard deviation is much smaller than normal variability (D'Cruz, 2012a,b) *Strengths:* - Large sample size (US FDA/NCTR, 2013 and Delclos et al., 2014) - Adequate positive controls included (D'Cruz et al., 2012a,b; US FDA/NCTR, 2013 and Delclos et al., 2014) - Both naïve and vehicle controls available (US FDA/NCTR, 2013 and Delclos et al., 2014) - Adequate positive controls included (US FDA/NCTR, 2013 and Delclos et al., 2014, D'Cruz et al.,	Positive/Negative	From low to high	↑/↓↓

2012a,b) - Number of doses (≥3) (D'Cruz et al., 2012a,b, Marmugi et al., 2012, US FDA/NCTR, 2013 and Delclos et al., 2014 – especially in the low dose range) - Oral administration by via gavage (D'Cruz et al., 2012a,b, Indumathi et al., 2013, Jayashree et al., 2013, US FDA/NCTR, 2013 and Delclos et al., 2014) - Phytoestrogen-free diet (Bodin et al., 2013, US FDA/NCTR, 2013 and Delclos et al., 2014) - Use of non-PC cages (D'Cruz et al., 2012b, Jayashree et al., 2013, Indumathi et al., 2013, Bodin et al., 2013, US FDA/NCTR, 2013 and Delclos et al., 2014) - Protocols according to EU guideline (Marmugi et al., 2012) - Study performed according to OECD guidelines, under GLP (US FDA/NCTR, 2013 and Delclos et al., 2014) *Weaknesses:* - Small sample size (D'Cruz et al., 2012a,b, Marmugi et al., 2012, Indumathi et al., 2013, Jayashree et al., 2013) - Test performed in one sex only (Batista et al., 2012, D'Cruz et al., 2012a,b, Marmugi et al., 2012, Indumathi et al., 2013, Jayashree et al., 2013) - Single dose level study (Batista et al., 2012) - Feed consumption (BPA given by the diet) not measured (Marmugi et al., 2012) - Inappropriate statstics (considering the small number of animals) (D'Cruz et al., 2012a,b) - Insufficient study reporting (Batista et al., 2012: number of animals tested is unclear for each endpoint) - Inappropriate statistics: insufficient studying reporting (no multiple comparisons statistics) (Marmugi et al., 2012; - Animal diet and phytoestrogen content not reported (Batista et al., 2012, D'Cruz et al., 2012a,b, Marmugi et al., 2012, Indumathi et al., 2013, Jayashree et al., 2013) - Unintended exposure of the control groups (US FDA/NCTR, 2013 and Delclos et al., 2014, see Churchwell et al, 2014)			
Overall conclusions: Although 5 studies with major weaknesses reported effects. One strong study (US FDA/NCTR, 2013 and Delclos et al., 2014) found no effect of BPA.			**As likely as not**
Q2: Does BPA affect metabolic function as evidenced by effects on adipose tissue in animals exposed in adult life?	**Answer to the question as reported by the study authors (Positive, Negative or Uncertain)**	**Reliability of evidence (Low, Medium or High)**	**Influence on Likelihood (see Table 28)**
Starting point: not addressed in previous EFSA opinions			

Line of Evidence 1 (Marmugi et al., 2012; Ronn et al., 2013; US FDA/NCTR, 2013 and Delclos et al., 2014~~)~~ There is some evidence of such effects in the studies of Marmugi et al., 2012, in which in the 50 µg/kg bw per day group perigonadic white adipose tissue was increased. Rönn et al. (2013) no changes in visceral fat and perirenal fat were observed but they described effects on lipids which are not considered adverse. They observed steatosis of the liver. *Comment:* the US FDA/NCTR, 2013 and Delclos et al., 2014 subchronic toxicity study,showed no effect up to 100 000 µg/kg bw per day, but the animals were exposed both pre and post-natally. *Strengths:* - Large sample size (US FDA/NCTR, 2013 and Delclos et al., 2014) - Adequate positive controls included (US FDA/NCTR, 2013 and Delclos et al., 2014) - Both naïve and vehicle controls available (US FDA/NCTR, 2013 and Delclos et al., 2014) - Number of doses (≥3) (Marmugi et al., 2012, US FDA/NCTR, 2013 and Delclos et al., 2014 – in the low dose range, Ronn et al., 2013) - Oral administration by via gavage (US FDA/NCTR, 2013 and Delclos et al., 2014) - Phytoestrogen-free diet (US FDA/NCTR, 2013 and Delclos et al., 2014, Ronn et al., 2013) - Use of non-PC cages (US FDA/NCTR, 2013 and Delclos et al., 2014, Ronn et al., 2013) - Protocols according to EU guideline (Marmugi et al., 2012) - Study performed according to OECD guidelines, under GLP (US FDA/NCTR, 2013 and Delclos et al., 2014) *Weaknesses:* - Animal body weight not given (Marmugi et al., 2012) - Small sample size (Marmugi et al., 2012) - Test performed in one sex only (Marmugi et al., 2012, Ronn et al., 2013) - Feed consumption (BPA given by the diet) not measured (Marmugi et al., 2012) - Inappropriate statistics: insufficient studying reporting (no multiple comparisons statistics) (Marmugi et al., 2012) - Study design not appropriate to the scope (Ronn et al., 2013: not fully appropriate for obtaining the substance specific data aimed at (BPA exposure combined with fructose) - Animal diet and phytoestrogen content not reported (Marmugi et al., 2012) - Unintended exposure of the control groups (US FDA/NCTR, 2013 and Delclos et al., 2014, see Churchwell et al, 2014)	Uncertain/ Negative	Medium to High	•/↓↓
Overall conclusions: Two new studies reporting effects have major weaknesses; one strong study reports no effects. therefore no reliable conclusions can be drawn.			**Unlikely**

Q3: Does BPA increase obesity in animals exposed postnatally?	Answer to the question as reported by the study authors (Positive, Negative or Uncertain)	Reliability of evidence (Low, Medium or High)	Influence on Likelihood (see Table 28)
Starting points*:* Long term regulatory studies on BPA (e.g. NTP, 1982; Tyl et al., 2002, 2008) have not shown obesity/excessive weight gain over the duration of the studies. There are no new studies showing long-term obesity.	Negative	High	↓↓↓
Line of Evidence 1: Associations with changes in body weight (Marmugi et al., 2012; Rönn et al., 2013; US FDA/NCTR, 2013 and Delclos et al., 2014) *Strengths:* - Large sample size (US FDA/NCTR, 2013 and Delclos et al., 2014) - Both naïve and vehicle controls available (US FDA/NCTR, 2013 and Delclos et al., 2014) - Adequate positive controls included (US FDA/NCTR, 2013 and Delclos et al., 2014) - Number of doses (≥3) (Marmugi et al., 2012, US FDA/NCTR, 2013 and Delclos et al., 20143 – in the low dose range, Ronn et al., 2013) - Oral administration by gavage (US FDA/NCTR, 2013 and Delclos et al., 2014) - Phytoestrogen-free diet (US FDA/NCTR, 2013 and Delclos et al., 2014, Ronn et al., 2013) - Use of non-PC cages (US FDA/NCTR, 2013 and Delclos et al., 2014, Ronn et al., 2013) - Protocols according to EU guideline (Marmugi et al., 2012) - Study performed according to OECD guidelines, under GLP (US FDA/NCTR, 2013 and Delclos et al., 2014) *Weaknesses:* - Animal body weight not given (Marmugi et al., 2012) - Small sample size (Marmugi et al., 2012) - Test performed in one sex only (Marmugi et al., 2012, Ronn et al., 2013) - Feed consumption (BPA given by the diet) not measured (Marmugi et al., 2012) - Inappropriate statistics: insufficient studying reporting (no multiple comparisons statistics) (Marmugi et al., 2012) - Study design not appropriate to the scope (Ronn et al., 2013: not fully appropriate for obtaining the substance specific data aimed at (BPA exposure combined with fructose) - Animal diet and phytoestrogen content not reported (Marmugi et al., 2012) - Unintended exposure of the control groups (US FDA/NCTR, 2013 and Delclos et al., 2014, see Churchwell et al, 2014)	Negative	Medium to High	↓↓

Overall conclusions: : There is no reliable evidence that BPA is obesogenic.	**Unlikely**
Overall conclusion on likelihood of metabolic effects in animals exposed postnatally Evidence for associations between BPA exposure and metabolic effects in animals exposed postnatally is inconsistent. There is reasonable evidence for effects on glucose or insulin regulation and/or effects on pancreatic morphology and/or function in shorter term studies, but no evidence that BPA is causing diabetes, insulin resistance and increases in weight (obesogenic) longer-term.	**As likely as not**

Table 44: Assessment of the likelihood of associations between BPA exposure and metabolic effects in animals when exposed prenatally.

Q1: Does BPA affect metabolic function as evidenced by effects on glucose or insulin regulation in animals exposed prenatally?	**Answer to the question as reported by the study authors (Positive, Negative or Uncertain)**	**Reliability of evidence (Low, Medium or High)**	**Influence on Likelihood (see Table 28)**
Starting point: EFSA CEF Panel, 2010 reported a study showing aggravated insulin resistance in mice during pregnancy (Alonso-Magdalena et al., 2010). In contrast the study of Ryan et al. (2010b) showed no indications of increased susceptibility to high-fat diet-induced obesity and glucose intolerance in adult mice exposed prenatally to BPA. *Comment*: Inconsistent results between the two studies *Weakness*: Ryan et al. was a single dose level study	Positive and Negative	Low to medium	↑/↓
Line of Evidence 1: Recent studies showing effects on glucose or insulin regulation and/or effects on pancreatic morphology and/or function. *Comment:* Two new studies have shown effects on several of these parameters (Wei et al., 2011, MacKay et al, 2013), whereas the results of Anderson et al. (2013) and the US FDA/NCTR, 2013 and Delclos et al., 2014 subchronic toxicity study (2013) did not show such effects. The study of Angle et al. (2013) reported several physiologically related effects which are inconsistent because changes in one parameter are not paralleled by expected changes in other physiological inter-related parameters measured. The US FDA/NCTR study (US FDA/NCTR, 2013 and Delclos et al., 2014) showed no effect, but the animals were exposed both pre and postnatally. *Strengths:* - Number of doses (≥3) (Anderson et al., 2013, Angle et al., 2013, Wei et al., 2011, US FDA/NCTR, 2013	Positive and Negative	Low to high	↑/↓↓

and Delclos et al., 2014 – especially in the low dose range) - Oral administration by gavage (Wei et al., 2011, US FDA/NCTR, 2013 and Delclos et al., 2014) - Adequate positive controls included (Angle et al., 2013, US FDA/NCTR, 2013 and Delclos et al., 2014MacKay et al., 2013) - Both naïve and vehicle controls available (US FDA/NCTR, 2013 and Delclos et al., 2014) - Phytoestrogen-free diet (Anderson et al., 2013, US FDA/NCTR, 2013 and Delclos et al., 2014) - Use of non-PC cages (Anderson et al., 2013, MacKay et al., 2013, Wei et al., 2011, US FDA/NCTR, 2013 and Delclos et al., 2014; Angle et al., 2013) - Study performed according to OECD guidelines, under GLP (US FDA/NCTR, 2013 and Delclos et al., 2014) *Weaknesses:* - Animal body weight not given (Andersen et al., 2013, Angle et al., 2013, MacKay et al., 2013) - Small sample size (MacKay et al., 2013, Wei et al., 2011) - Insufficient study reporting (Anderson et al., 2013: administration via diet but intakes of BPA not specifically calculated, MacKay et al., 2013: uncertainty about estimated intakes due to very low (< 1 ng/kg bw per day) dietary level and homogeneity of the diet not checked, Angle at al., 2013: insufficient study reporting, Wei et al., 2011: number of animals used for each end-point was variable and not always clear) - Inappropriate statistics (Andersen et al., 2013: validity of statistical analysis not clear, MacKay et al., 2013 and Wei et al., 2011: litter effect not completely controlled) - Study design not appropriate to the scope (Angle et al., 2013: only males tested for glucose and insulin tolerance tests) - Animal diet phytoestrogen content not reported (Angle et al., 2013, Wei et al., 2011) - Unintended exposure of the control groups (US FDA/NCTR, 2013 and Delclos et al., 2014, see Churchwell et al, 2014)			
Overall conclusions: Although three studies with major weaknesses reported effects, these were not consistent between the studies. One weak study (Anderson et al., 2013) and one strong study (US FDA/NCTR, 2013 and Delclos et al., 2014) found no effect of BPA.			**Unlikely to -as likely as not-**
Q2: Does BPA affect lipogenesis/adipogenesis in animals <u>exposed prenatally</u>?	**Direction of the reported evidence (Positive, Negative or Uncertain)**	**Reliability of evidence (Low, Medium or High)**	**Influence on Likelihood (see Table 28)**

Starting point: EFSA (2010) reported studies showing increased adipogenesis in the offspring of rats and mice exposed prenatally to BPA (Somm et al., 2009; Miyawaki et al, 2007). *Comment:* No dose-response in female (Miyawaki et al., 2007) *Comment:* Only effects in females (Somm et al., 2009) *Weakness*: single dose level study (Somm et al., 2009) *Weakness*: small sample size (Miyawaki et al., 2007) *Weakness*: litter effect not considered (Miyawaki et al.,, 2007) *Weakness*: diet not tested for phyto-estrogens (Miyawaki et al, 2007)	Positive	Low	↑/•
Line of Evidence 1: Two new studies have reported effects on fat weight in animals exposed prenatally to BPA only for one out of three doses (Wei et al., 2011, and only for high fat diet MacKay et al, 2013). One strong study (US FDA/NCTR, 2013 and Delclos et al., 2014) does not report effects up to 100 000 µg/kg bw per day. *Comment*: US FDA/NCTR toxicity study (2013) showed no effect, but the animals were exposed both pre and post-natally *Strengths:* - Number of doses (≥3) (Wei et al., 2011, US FDA/NCTR, 2013 and Delclos et al., 2014 – especially in the low dose range) - Oral administration by gavage (Wei et al., 2011, US FDA/NCTR, 2013 and Delclos et al., 2014) - Adequate positive controls included (US FDA/NCTR, 2013 and Delclos et al., 2014MacKay et al., 2013) - Both naïve and vehicle controls available (US FDA/NCTR, 2013 and Delclos et al., 2014) - Phytoestrogen-free diet (US FDA/NCTR, 2013 and Delclos et al., 2014) - Use of non-PC cages (MacKay et al., 2013, Wei et al., 2011, US FDA/NCTR, 2013 and Delclos et al., 2014) - Study performed according to OECD guidelines, under GLP (US FDA/NCTR, 2013 and Delclos et al., 2014) *Weaknesses:* - Animal body weight not given (MacKay et al., 2013) - Small sample size (MacKay et al., 2013, Wei et al., 2011) - Insufficient study reporting (MacKay et al., 2013: uncertainty about estimated intakes due to very low (< 1 ng/kg bw per day) dietary level and homogeneity of the diet not checked, Wei et al., 2011: number of animals used for each end-point was variable and not always clear) - Inappropriate statistics (MacKay et al., 2013 and Wei et al., 2011: litter effect not completely controlled) - Animal diet phytoestrogen content not reported (Wei et al., 2011)	Positive/Negative	Low to High	↑/↓↓

- Unintended exposure of the control groups (US FDA/NCTR, 2013 and Delclos et al., 2014, see Churchwell et al, 2014)			
Overall conclusions: Two new studies reporting effects have major weaknesses and one strong study does not report effects.			**As likely as not**
Q3: Does BPA affect metabolic function as evidenced by effects on energy expenditure?	**Answer to the question as reported by the study authors (Positive, Negative or Uncertain)**	**Reliability of evidence (Low, Medium or High)**	**Influence on Likelihood (see Table 28)**
Starting point: not addressed in previous EFSA opinions			
Line of Evidence 1: New evidence on energy expenditure. Anderson et al., (2013), MacKay et al., (2013) reported an effect on energy expenditure *Comment*: Only a specific genetic strain of mouse was used *Comment:* no underlying mechanistic explanation *Strengths:* - Number of doses (≥3) (Anderson et al., 2013) - Adequate positive controls included (MacKay et al., 2013) - Use of non-PC cages (Anderson et al., 2013, MacKay et al., 2013) - Phytoestrogen-free diet (Anderson et al., 2013) *Weaknesses:* - Animal body weight not given (Anderson et al., 2013, MacKay et al., 2013) - Small sample size (MacKay et al., 2013) - Insufficient study reporting (Anderson et al., 2013: administration via diet but intakes of BPA not specifically calculated, MacKay et al., 2013: uncertainty about estimated intakes due to very low (< 1 ng/kg bw per day) dietary level and homogeneity of the diet not checked) - Inappropriate statistics (Anderson et al., 2013: inappropriate statistical analysis, MacKay et al., 2013: litter effect not completely controlled)	Positive	Low	↑●
Overall conclusions: The studies report an effect which could be of interest. However, the studies have weaknesses. In addition, the paradigms used (special strain; high fat diet) render the interpretation of the findings with respect to human health difficult.			**As likely as not**

Q4: Does BPA cause obesity in animals exposed prenatally?	Answer to the question as reported by the study authors (Positive, Negative or Uncertain)	Reliability of evidence (Low, Medium or High)	Influence on Likelihood (see Table 28)
Starting point: Long term regulatory studies on BPA (Tyl et al. 2002, 2008) together with the study of Ryan et al. 2010, have not shown obesity/excessive weight gain over the duration of the studies. The study of Rubin et al. (2001) showed increased weight gain in BPA-exposed animals later in life *Weakness*: Ryan et al. was a single dose level study	Negative	High	↑/↓↓↓
Line of Evidence 1: There are no new long term studies. New studies have reported effects on body weight (e.g. MacKay et al., 2013; Wei et al., 2011; Xu et al., 2011b)), but conflicting results have been obtained in other studies (e.g. Anderson et al., 2013, US FDA/NCTR, 2013 and Delclos et al., 2014) *Comment:* High fat diets used in some studies cannot be considered as a good model for human health, also type of fat not specified *Strengths:* - Number of doses (≥3) (Anderson et al., 2013, Wei et al., 2011, US FDA/NCTR, 2013 and Delclos et al., 2014 – especially in the low dose range, Xu et al., 2011b) - Oral administration by gavage (Wei et al., 2011, US FDA/NCTR, 2013 and Delclos et al., 2014) - Adequate positive controls included (US FDA/NCTR, 2013 and Delclos et al., 2014MacKay et al., 2013) - Both naïve and vehicle controls available (US FDA/NCTR, 2013 and Delclos et al., 2014) - Phytoestrogen-free diet (Anderson et al., 2013, US FDA/NCTR, 2013 and Delclos et al., 2014) - Use of non-PC cages (Anderson et al., 2013, MacKay et al., 2013, Wei et al., 2011, US FDA/NCTR, 2013 and Delclos et al., 2014, Xu et al., 2011b) - Study performed according to OECD guidelines, under GLP (US FDA/NCTR, 2013 and Delclos et al., 2014) - Study performed under GLP (US FDA/NCTR, 2013 and Delclos et al., 2014) *Weaknesses:* - Small sample size (MacKay et al., 2013, Wei et al., 2011) - Insufficient study reporting (Anderson et al., 2013: administration via diet but intakes of BPA not specifically calculated, MacKay et al., 2013: uncertainty about estimated intakes due to very low (< 1 ng/kg bw per day) dietary level and homogeneity of the diet not checked, Wei et al., 2011: number of animals used for each end-point was variable and not always clear, Xu et al., 2011b: administration via drinking water but no information on consumption)	Negative and positive	Low to High	↑/↓↓

- Statistical analysis (Anderson et al., 2013: validity of statistical analysis not clear, MacKay et al., 2013, Wei et al., 2011 and Xu et al., 2011b: litter effect not completely controlled) - Animal diet phytoestrogen content not reported (Wei et al., 2011) - Study design not appropriate to the scope (only one BPA dose was assessed postnatally). - Unintended exposure of the control groups (US FDA/NCTR, 2013 and Delclos et al., 2014, see Churchwell et al, 2014)			
Overall conclusions: There are new studies on pre- and perinatal exposure towards BPA, some, not all of which indicate some effects on body weight. The studies have measured multiple endpoints at several time points and in the studies with positive outcome the procedure in which way the statistical adjustment was made is not clear, so that it is uncertain whether the results are chance findings. The positive studies used experimental paradigms (special strain, extremely high fat diet, sucrose) which do not represent the human situation.			**Unlikely to -as likely as not-**
Overall conclusion on likelihood of metabolic effects in animals exposed prenatally: Since the initial reports that BPA had potential effects on adiposity, glucose or insulin regulation, lipids and other end-points related to diabetes or metabolic syndrome in animals exposed prenatally, several new studies have been published. There is reasonable evidence for effects on glucose or insulin regulation and/or effects on pancreatic morphology and/or function in shorter term studies, but no evidence that BPA is causing diabetes, insulin resistance and increases in weight (obesogenic) longer-term.			**As likely as not**

Table 45: Summary of the WoE assessment of the likelihood that BPA causes metabolic effects

Humans	
Overall conclusion on Likelihood of associations between BPA and obesity in humans There are indications from a prospective study that prenatal BPA exposure (maternal urinary BPA concentrations) may be associated with reduced body mass in girls, while cross-sectional studies indicate associations between childhood BPA exposure and obesity. It cannot be ruled out that the results are confounded by diet or concurrent exposure factors. The associations do not provide sufficient evidence to infer a causal link between BPA exposure and obesity in humans. No firm conclusions can be drawn on the likelihood.	**As likely as not**
Overall conclusion on Likelihood of associations between BPA and hormonal effects in humans There are indications from one prospective study that maternal BPA exposure may be associated with adipokine expression in 9 year old children, but it cannot be ruled out that the result is confounded by diet or concurrent exposure factors. The association does not provide sufficient evidence to infer a causal link between BPA exposure hormonal effects in humans. No firm conclusions can be drawn on the likelihood.	**As likely as not**
Overall conclusion on Likelihood of associations between BPA and diabetes effects in humans: The indications that BPA may be associated with diabetes in humans are unlikely.	**Unlikely**
Overall conclusion on Likelihood of associations between BPA and metabolic syndrome in humans: The indication that BPA may be associated with metabolic syndrome in humans is unlikely.	**Unlikely**
Overall conclusion on Likelihood of associations between BPA and renal effects in humans: The indication that BPA may be associated with renal function in humans is unlikely.	**Unlikely**

Animals	
Overall conclusion on likelihood of metabolic effects in animals exposed postnatally Evidence for associations between BPA exposure and metabolic effects in animals exposed postnatally is inconsistent. There is reasonable evidence for effects on glucose or insulin regulation and/or effects on pancreatic morphology and/or function in shorter term studies, but no evidence that BPA is causing diabetes, insulin resistance and increases in weight (obesogenic) longer-term.	**As likely as not**
Overall conclusion on Likelihood for metabolic effects in animals exposed prenatally: Since the initial reports that BPA had potential effects on adiposity, glucose or insulin regulation, lipids and other end-points related to diabetes or metabolic syndrome in animals exposed prenatally, several new studies have been published. There is reasonable evidence for effects on glucose or insulin regulation and/or effects on pancreatic morphology and/or function in shorter term studies, but no evidence that BPA is causing diabetes, insulin resistance and increases in weight (obesogenic) longer-term.	**As likely as not**

WoE of genotoxicity

Whether BPA has the potential to cause genotoxicity in vitro and in vivo was considered using a tabular format for weighing different lines of evidence (WoE evaluation). The WoE evaluation tables for these endpoints are presented in full below.

In vitro studies

Table 46: Assessment of the likelihood that BPA exposure is genotoxic in vitro.

Q1: Is BPA genotoxic in vitro via a non-threshold mechanism?	**Answer to the question as reported by the study authors (Positive, Negative or Uncertain)**	**Reliability of evidence (Low, Medium or High)**	**Influence on Likelihood (see Table 28)**
Starting point based on previous assessments (EU-RAR, 2003; EFSA CEF Panel, 2010): EFSA in 2006 noted that BPA is not considered to be genotoxic in bacteria and in mammalian cells, based on previous reviews of BPA genotoxicity (EU-RAR, 2003; Haighton et al., 2002). EFSA (2010) did not consider additional in vitro genotoxicity studies.	Negative	From Medium to High	↓↓
Line of Evidence 1: New evidence of direct damage to DNA (Induction of gene mutation in bacteria e.g.) Gene mutation in the Ames test (Masuda et al., 2005; Tiwari et al., 2012; Fic et al., 2013) *Strengths:* – Ames test well conducted (Tiwari et al., 2012) – Adequate number of concentrations in presence and absence of metabolic activation (S9) (Fic et al., 2013) *Weaknesses:* – Limited number of strains (all studies) – Limitations in the experimental design: single dose (Masuda et al., 2005)	Negative	Low	↓
Line of Evidence 2: New evidence of direct damage to DNA (DNA breakage, DNA adducts, induction of phosphorylated histone γ-H2AX, etc.) Six studies evaluated DNA breakage (Iso et al., 2006; Tayama et al., 2008; Fic et al., 2013), DNA adducts (Izzotti et al., 2009; De Flora et al., 2011) and induction of phosphorylated histone γ-H2AX (Iso et al., 2006; Audebert et al., 2011) in different cell lines *Strengths:* – Sound approach and experimental design (Izzotti et al., 2009: De Flora et al., 2011)	Positive	Low	•

– Three genotoxic endpoints (DNA breakage, SCE and CA) (Tayama et al., 2008) *Weaknesses:* – Experimental procedures questionable: staining procedures (Tayama et al., 2008), single dose level, number of cells examined (De Flora et al., 2011) – Results not clearly reported (Iso et al., 2006) – Inconsistent results in ER-negative and ER-positive cells (different genomic stability) (Iso et al., 2006) – Inconsistent results in the comet assay (e.g. increase in DNA damage not dose-related) (Fic et al., 2013)			
Line of Evidence 3: New evidence of damage at chromosome level (Chromosome aberrations, micronuclei, SCEs) Induction of chromosomal aberrations and SCEs in CHO-K1 cell line (Tayama et al., 2008) *Strengths:* – Adequate range of concentrations – Three genotoxic endpoints (DNA breakage, SCE and CA) – Concentration-related and statistically significant increases of c-metaphases *Weaknesses:* – Experimental procedures questionable: sampling times, cells recovered in the presence of BrdU – Positive effects only at high dose-level in the presence of cytotoxicity which generates false positives	Positive	Low	•
Overall conclusion based on in vitro studies – via non thresholded mechanism: BPA has not been shown to induce gene mutations nor chromosomal aberrations in bacteria and mammalian cells.			**Unlikely**
Q2: Is BPA genotoxic in vitro via a threshold mechanism?	**Answer to the question as reported by the study authors (Positive, Negative or Uncertain)**	**Reliability of evidence (Low, Medium or High)**	**Influence on Likelihood (see Table 28)**
Starting point based on previous assessments (EU RAR, 2003EFSA, 2006 The EU RAR (EU, 2006) reported the potential of BPA to induce aneuploidy by the evidence of microtubule disruption in cell-free system and induction of micronuclei in hamster cell lines. EFSA (2010) did not review *in vitro* genotoxicity studies.	Positive	Medium	↑
Line of Evidence 1: New evidence of genotoxicity in vitro via a threshold mechanism: Aneuploidy (microtubule effects, chromosome loss, non-disjunction, induction of c-metaphases etc.) Evaluation of aneuploidy by analysis of micronuclei in cytochalasin binucleate cells and aberration of mitotic machinery by analysis of multiple spindle poles in human lymphoblastoid cells AHH1 and Chinese hamster V79 cells (Johnson and Parry, 2008)	Positive	High	↑↑↑

Strengths: – Sound experimental design and well documented study – Adequate selection and spacing of concentrations Clear induction of "c-like metaphases", a biomarker of spindle disrupting effects (Tayama et al., 2008) *Strengths:* – Concentration-related and statistically significant increases of c-metaphases *Weaknesses:* – Experimental procedures questionable: sampling times	Positive	High	↑↑↑
Overall conclusion based on in vitro studies – via thresholded mechanism: BPA has been clearly shown to be aneugenic through induction of micronuclei caused by spindle disrupting effects of BPA identified by the use of fluorescently labelled antibodies for α and γ-tubulin to visualize the microtubules and the microtubule organizing centers of the mitotic spindles (Johnson and Parry 2008). Further evidence for spindle disrupting effects of BPA has been also indicated by Tayama et al. (2008) who showed significant increases of colchicine-like metaphases (c-metaphases) in CHO-K1 cells.			**Very Likely**

In vivo studies

Table 47: Assessment of the likelihood that BPA exposure is genotoxic in vivo.

Q1: Is BPA genotoxic in vivo via a non-threshold mechanism?	**Answer to the question as reported by the study authors (Positive, Negative or Uncertain)**	**Reliability of evidence (Low, Medium or High)**	**Influence on Likelihood (see Table 28)**
Starting point based on previous assessments (EFSA, 2006; 2010): The EFSA 2006 opinion noted that BPA is not considered to be genotoxic based on previous reviews of BPA genotoxicity (EC, 2002; EU, 2003; Haighton et al., 2002). In vivo studies had been considered by the EU RAR, mouse micronuclei (negative), DNA adducts (positive) and dominant lethal mutations (abstract only).	Negative	From Medium to High (excl. dominant lethal)	↓↓
Line of Evidence 1: New evidence of genotoxicity in vivo via a non-threshold mechanism involving direct damage to DNA (DNA breakage, DNA adducts etc.) Alkaline comet assay in peripheral blood lymphocytes (De Flora et al., 2011)	Negative	Medium	↓↓

Strength: – Sound approach and experimental design *Weaknesses:* – Experimental procedures questionable: single dose level, number of cell examined Four studies evaluated DNA breakage (Ulutaş et al., 2011; Tiwari et al., 2012; Dobrzyńska and Radzikowska, 2013 2013) and DNA adducts (Izzotti et al., 2009) *Strength:* – Sound approach and experimental design (Izzotti et al., 2009) *Weaknesses:* – Methodological shortcomings: absence of historical control values (Dobrzyńska and Radzikowska, 2013); number of cell examined (Ulutaş et al., 2011); cytotoxicity not evaluated, inadequate sampling times (Ulutaş et al., 2011; Tiwari et al., 2012; Dobrzyńska and Radzikowska, 2013) – Poor study report (Tiwari et al., 2012; Dobrzyńska and Radzikowska, 2013) Alkaline comet assay in the epididymal sperm (Tiwari and Vanage, 2013) *Weaknesses:* – Experimental procedures questionable (e.g. limited number of animals, absence of negative and positive controls, only two dose levels employed and lack of rationale for dose selection) – Results potentially biased by high background/variability for rodent sperm in the alkaline assay – No dose-related increases in dominant lethal mutations – Absence of negative historical controls	Positive Positive	Low Low	• •
Line of Evidence 2: New evidence of genotoxicity in vivo via a non-threshold mechanism involving damage at chromosome level (Chromosome aberrations, micronuclei, SCE's) Analysis of micronuclei in peripheral blood reticulocytes (Masuda et al., 2005) and bone marrow cells of rodents (Masuda et al., 2005; Pacchierotti et al., 2008; Naik and Vijayalaxmi , 2009; De Flora et al., 2011); analysis of chromosomal aberrations in bone marrow cells of rodents (Naik and Vijayalaxmi , 2009) *Strength:* – Sound approach and experimental design (Pacchierotti et al., 2008; Naik et al., 2009; De Flora et al., 2011) *Weaknesses:* – Limitations in the experimental design: single concentration/dose (Masuda et al., 2005); top dose too low (Pacchierotti et al., 2008; Naik et al., 2009), sub-optimal dose and exposure to colchicine (Naik and Vijayalaxmi , 2009)	Negative	Medium	↓

– Experimental procedures questionable: single dose level, number of cells examined (De Flora et al., 2011) Analysis of micronuclei and chromosomal aberrations in bone marrow cells of rodents (Tiwari et al., 2012) *Weaknesses:* – Incidence and type of chromosome aberrations generally not compatible with those seen with other chemical agents; – Methodological shortcomings: inappropriate staining procedures (micronuclei), inappropriate selection of sampling time; mitotic index as a measure of cytotoxicity not determined; sub-optimal exposure to colchicine (chromosomal aberrations) Evaluation of dominant lethal mutations in the different stages of spermatogenesis in the rat (Tiwari and Vanage, 2013) *Weaknesses:* – Experimental procedures questionable; limited number of male animals employed; absence of negative and positive controls, inadequate selection of dose-levels (only two dose levels employed and lack of rationale for dose selection) – Results potentially biased by high background/variability for rodent sperm in the alkaline assay – No dose-related increases in dominant lethal mutations – Absence of negative historical control data	Positive Positive	Low Low	• •
Overall conclusion based on in vivo studies – via non-thresholded mechanism: BPA has not been shown to be clastogenic in vivo (micronuclei and chromosomal aberrations).			**Unlikely**
Q2: Is BPA genotoxic *in vivo* via a threshold mechanism?	**Answer to the question as reported by the study authors (Positive, Negative or Uncertain)**	**Reliability of evidence (Low, Medium or High)**	**Influence on Likelihood (see Table 28)**

Starting point based on previous assessments (EFSA, 2006; 2010): EFSA (2006) could not conclusively evaluate a study by Hunt et al, 2003, showing meiotic disturbances in mouse oocytes, due to deficiencies in the authors' analysis and overall weakness of effects, and did not indicate concern for aneugenicity in vivo. EFSA (2010) considered two additional studies (micronucleus study of Naik and Vijayalaxmi, 2009, Mulhauser et al, 2009 study on mouse oocytes), both showing some effects on spindle structure. EFSA noted the thresholded mechanism for aneuploidy induction and the large margin between the doses tested and the TDI and concluded that the findings of these studies did not alter the previous EFSA conclusion on the lack of aneugenic activity of BPA in mouse germ cells.	Some positive and some negative	Low	•
Line of Evidence 1: New evidence of genotoxicity in vivo via a thresholded mechanism: Aneuploidy (microtubule effects, chromosome loss, non-disjunction, induction of c-metaphases etc.) No effects for induction of aneuploidy; Significant increases in the number of metaphase II oocytes with prematurely separated chromatids of no consequences in terms of fidelity of chromosome segregation during the second meiotic division (Pacchierotti et al., 2008): *Strength:* – Sound approach and experimental design *Weakness:* – Inappropriate dose selection: high dose-levels for single or 7 daily administration apparently low (20 and 0.2, mg/kg bw respectively)	Negative	Medium	↓
Induction of "c-like metaphases" a biomarker of spindle disrupting effects (Naik and Vijayalaxmi , 2009) *Strength:* – Sound approach and experimental design *Weakness:* – Minor limitations in the experimental design: top dose too low, sub-optimal dose and exposure to colchicine	Positive	High	↑
Overall conclusion based on in vivo studies via a thresholded mechanism: The potential of BPA to produce aneuploidy in vitro was not expressed in vivo, based on negative findings obtained for induction of aneuploidy in female and male mouse germ cells (Pacchierotti et al., 2008) and for induction of micronuclei in somatic bone marrow cells (Masuda et al., 2005; Pacchierotti et al., 2008; Naik and Vijayalaxmi , 2009; De Flora et al., 2011). However, BPA induced dose-related increases of c-like metaphases in mouse bone-marrow cells (Naik and Vijayalaxmi , 2009) and significant increases in the number of metaphase II oocytes with prematurely separated chromatids (Pacchierotti et al., 2008) pointing to potential mitotic spindle disrupting effects of BPA in vivo.			**As likely as not**

WoE of carcinogenicity and proliferative changes

Whether BPA has the potential to cause carcinogenicity or proliferqtive changes in tissues was considered using a tabular format for weighing different lines of evidence (WoE evaluation). The WoE evaluation tables for these endpoints are presented in full below.

Animal studies

WoE of the carcinogenicity of BPA in animals and its potential to cause proliferative changes in tissues, that could potentially be linked to development of cancer

Table 48: Assessment of the likelihood that BPA is carcinogenic in animals

Q1: Is BPA genotoxic?	
Overall conclusion on in vivo studies – via non-thresholded mechanism: BPA has not been shown to be clastogenic (micronuclei and chromosomal aberrations).	**Unlikely**
Overall conclusion based on in vivo studies via a thresholded mechanism: The potential of BPA to produce aneuploidy in vitro was not expressed in vivo, based on negative findings obtained for induction of aneuploidy in female and male mouse germ cells (Pacchierotti et al., 2008) and for induction of micronuclei in somatic bone marrow cells (Masuda et al., 2005; Pacchierotti et al., 2008; Naik and Vijayalaxmi , 2009; De Flora et al., 2011). However, BPA induced dose-related increases of c-like metaphases in mouse bone-marrow cells (Naik and Vijayalaxmi , 2009) and significant increases in the number of metaphase II oocytes with prematurely separated chromatids (Pacchierotti et al., 2008) pointing to potential mitotic spindle disrupting effects of BPA in vivo.	**As likely as not**

Q2: Is BPA carcinogenic in animals when exposed during their adult life (post-pubertal) only?	**Answer to the question as reported by the study authors (Positive, Negative or Uncertain)**	**Reliability of evidence (Low, Medium or High)**	**Influence on Likelihood (see Table 28)**
Starting point based on previous assessments (EFSA, 2006; EFSA CEF Panel, 2010): BPA did not show any significant carcinogenic activity in 2 standard oral cancer bioassays in rats and mice (NTP, 1982) *Comment:* Although there were differences between treated groups and controls in some tumour types and NTP had concluded that the data were suggestive of a carcinogenic effect on the haematopoietic system , reviews of the study by the EU RAR and EFSA concluded that these were not toxicologically significant (the main tumour type showing a dose:response relationship, haemopoetic tumours, is of unlikely relevance in humans)	Mainly negative	Medium	↓↓
Line of Evidence 1: **Effects on tumour induction in the mammary glands** (Jenkins et al., 2011)	Positive	Low	●/↑

Strengths: – Number of doses (4) – Large sample size – Phytoestrogen-free diet – Use of non-pc cages and of non plastic bottles *Weaknesses:* – Drinking water consumption not measured: exact doses received not known – Insufficient data reporting (e.g. Data on tumour incidence and histopathology incomplete, type of epithelial cells undergoing proliferation was not specified, time of necropsy not defined)			
Line of evidence 2: effects on tumour induction in the prostate (Prins et al., 2011) *Comments:* unusual early appearance of neoplastic lesions in this model after a very short period of treatment; "prostatic intraductal neoplasia" is a possible response to the prostatic inflammation, not sufficient degree of cellular atypia to be compatible with neoplasia *Strengths:* – Phytoestrogen-free diet – Use of non-PC cages and of non plastic bottles – BPA determination in animal samples *Weaknesses:* – Single dose level study – Possible confounding (BPA exposure was followed by testosterone and oestradiol-17β)	Positive	Low	●
Overall conclusion on carcinogenicity of BPA in animals when exposed during their adult life (post-pubertal) only: Effects on mammary gland growth demonstrated in a transgenic mouse model with post-pubertal exposure (Jenkins et al., 2011) or on prostatic "intraepithelial neoplasia" in rats exposed to BPA in the immediate postnatal period (Prins et al. 2011) do not provide convincing evidence that BPA is carcinogenic in animals when exposed postnatally/during their adult life.			**Unlikely to - as likely as not-**
Q3: Is BPA carcinogenic in animals exposed during pre- and post-natal (during lactation) development?	**Answer to the question as reported by the study authors (Positive, Negative or Uncertain)**	**Reliability of evidence (Low, Medium or High)**	**Influence on Likelihood (see Table 28)**
Carcinogenicity other than mammary carcinogenicity: Starting point on based on previous assessments (EFSA, 2006): Based on studies reviewed by EFSA in the 2006 opinion the AFC Panel concluded that transplacental and lactational exposure to BPA did not affect the incidence of preneoplastic and neoplastic lesions in prostate and seminal vesicle, and had no effects on age-related morphological changes of the reproductive and endocrine organs and uterine carcinogenesis up to 15 months of age (Ichihara et al., 2003; Yoshida et al., 2004), Nor did BPA promote thyroid cancer	Negative	Medium	↑/↓↓

in a thyroid carcinogenesis model (Takagi et al., 2002). In the EFSA CEF PANEL, 2010 opinion studies that addressed proliferative changes in the mammary glands were evaluated, including Jenkins et al., 2009 and Betancourt et al., 2010. The CEF Panel noted that: "*at the highest dose level studied there is a shift of the ratio between cell proliferation and apoptosis in favour of cell multiplication in the mammary gland. In view of the mechanistical data obtained upon in utero exposure in other studies and the implications of an increased cell proliferation/apoptosis ratio in carcinogenesis, the effects reported by Jenkins and Betancourt deserve further consideration.*" In several studies, including those addressing prenatal exposure, no putative pre-neoplastic or neoplastic lesions in the liver were observed (EFSA, 2006; EFSA CEF Panel, 2010; US FDA/NCTR, 2013 and Delclos et al, 2014).			
Line of Evidence 1: effects of BPA on tumour induction in the mammary glands (incidence, multiplicity and/or latency of tumours) (Weber Lozada and Keri, 2011; Acevedo et al., 2013) *Comment:* The tumours identified in the study reported by Weber Lozada and Keri (2011) were all squamous carcinomas whereas DMBA given orally to mice and rats is reported to produce mainly adenocarcinomas. *Strengths:* – Large sample size in most group (Acevedo et al., 2013) – Adequate positive control included (Weber Lozada and Keri, 2011) – Phytoestrogen-free diet (all) – Use of non-PC cages and of non plastic bottles diet (all) – BPA measurements in biological fluids (dams, fetuses and pups) (Acevedo et al., 2013) *Weaknesses:* – Small sample size (Acevedo et al., 2013; Weber Lozada and Keri, 2011) – Insufficient study reporting (e.g. Tumour incidence, timing of necropsy) (Weber Lozada and Keri, 2011)	Positive	Low	●/↑
Line of Evidence 2: effects of BPA on tumour induction in the liver (Weinhouse et al., 2014) Comment: Liver tumours were also seen in male control mice *Strengths:* – Number of BPA doses (3) – Phytoestrogen-free diet – Use of non-PC cages – BPA measurement in biological samples (total liver measurement) *Weaknesses:* – Small sample size (low number of animals/group) – Insufficent reporting (no clear reporting on historical incidence of liver adenomas and carcinomas in female)	**Positive**	Low to Medium	●
Overall conclusion on the carcinogenicity of BPA in animals exposed during pre- and post-natal (during lactation) development: Recent studies			**Unlikely to -as**

(Jenkins et al., 2009, Betancourt et al., 2010; Weber Lozada and Keri, 2011; Acevedo et al., 2013; Weinhouse et al, 2014) investigated the potential carcinogenic effects of fetal or perinatal exposure to BPA. Given the various weaknesses identified in these studies, they do not provide convincing evidence that BPA is carcinogenic in animals when exposed during pre- and post-natal (during lactation) development.	**likely as not-**

Table 49: Assessment of the likelihood that BPA induces proliferative changes in animal tissues

Q1: Does BPA induce proliferative changes in animals when exposed during their adult life?	**Answer to the question as reported by the study authors (Positive, Negative or Uncertain)**	**Reliability of evidence (Low, Medium or High)**	**Influence on Likelihood (see Table 28)**
Starting point based on previous assessments (EFSA, 2006): Findings from the NTP bioassay (NTP, 1982): Histological examination of mammary tissue from all necropsied animals (rats and mice) revealed no evidence of non-neoplastic proliferative changes in either species.	Negative	High	↓↓↓
Line of Evidence 1: BPA induces mammary epithelial cell proliferation and hyperplasia (Jones et al., 2010; Jenkins et al. 2011) *Comment:* The Brca1 mouse model of Jones et al. and the female transgenic MMTV-erbB2 model in mice of Jenkins are not representative of the general population but in the case of the former might reflect increased sensitivity of the subpopulation with defective Brca1 gene. *Comment:* small changes in proliferation and hyperplasia of mammary gland epithelium cells in sensitive mouse models (Jones et al., 2010; Jenkins et al. 2011) *Strengths:* – Number of doses (Jenkins et al., 2011) – Large sample size (Jenkins et al., 2011) – Phytoestrogen-free diet (Jenkins et al., 2011; Jones et al., 2010) – Use of non-pc cages and of non plastic bottles (Jenkins et al., 2011) – Slides were blind-evaluated (Jenkins et al., 2011) *Weaknesses:* – Single dose level study (Jones et al., 2010) – Low number of animal/group for proliferation-drinking water consumption not measured: exact doses received not known (Jenkins et al., 2011) – Insufficient data reporting (e.g. No of animals with tumour and histopathology incomplete) (Jenkins et al., 2011) – Type of cages not evaluated (Jones et al., 2010)	Positive	Medium	●/↑

Line of Evidence 2: **BPA effects on (atypical) hyperplasia (in addition to "intraepithelial neoplasia") in the prostate of rats exposed postnatally** (Prins et al., 2011) Comment: The early appearance of neoplastic lesions in this model after a very short period of treatment is unusual *Strengths:* – Phytoestrogen-free diet – Use of non-PC cages and of non plastic bottles – BPA determination in animal samples *Weaknesses:* – Single dose level study – Possible confounding (BPA exposure was followed by testosterone and oestradiol-17β)	Positive	Low	•
Overall conclusion on proliferative changes of BPA in animals when exposed postnatally/ during their adult life: Both Jones et al (2010) and Jenkins (2011) reported an effect on mammary gland proliferation in sensitive transgenic or gene knock-out models as well as wild-type mice (Jones et al. only) given BPA during adulthood, although proliferative effects in these mouse models are inconsistent with the lack of any proliferative effect on mammary tissue in mice in the two year NTP carcinogenicity study. However, the CEF Panel concluded overall that there is some indication that BPA can cause proliferative changes in the mammary gland of genetically modified animals when exposed postnatally/in adult life. The CEF Panel considered that evidence for proliferative changes induced by BPA in other organs (i.e. prostate) is currently too weak to reach a conclusion.			**As likely as not (for mammary gland proliferation)**

Q2: Does BPA induce proliferative changes in the mammary gland of animals exposed during pre- and/or post-natal (during lactation) development or up to PND 90 (gavage)?	Answer to the question as reported by the study authors (Positive, Negative or Uncertain)	Reliability of evidence (Low, Medium or High)	Influence on Likelihood (see Table 28)
Starting point based on previous assessments (EFSA, 2006, 2010): Based on the reviewed studies (Avecedo et al. 2013, Betancourt et al, 2010; Durando et al, 2007; Jenkins et al, 2009; Moral et al. 2008; Murray et al, 2007; Vandenberg et al, 2007, 2008) the implications of cell proliferation in the mammary gland and the significance of an increased cell proliferation/apoptosis ratio deserve further consideration. Additionally, the Panel noted the findings of a number of earlier s.c. studies (Nikaido et al, 2004, 2005; Markey et al, 2001, 2005; Munoz-de-Toro et al, 2005, Rubin et al, 2006) supporting this conclusion. *Comment*: No dose-effect relationship observed (Jenkins et al, 2009; Betancourt et al, 2010; Durando et al, 2007; Murray et al, 2007; Vandenberg et al, 2007) *Comment:* Differences in architecture/histology was very small (Moral et al, 2008) *Strength:* – Number of doses (Avecedo et al, 2013; Vandenberg et al., 2008; Murray et al, 2007) – Large sample size (Betancourt et al, 2010, not for proliferation assessment; Moral et al. 2008; Vandenberg et al., 2007) – Oral administration by gavage (Jenkins et al, 2009; Betancourt et al, 2010; Moral et al. 2008) – Phytoestrogen-free diet (Jenkins et al, 2009; Vandenberg et al., 2008; Betancourt et al, 2010; Moral et al. 2008; Murray et al, 2007, Markey et al, 2001) – Use of non-PC cages and of non plastic bottles (Jenkins et al, 2009; Durando et al, 2007; Vandenberg et al, 2008; Betancourt et al, 2010; Murray et al, 2007) – Study design not appropriate to the scope (Comprehensive histology of TEB, AB and Lobules type 1 (Moral et al., 2008) – Food tested negative for estrogenicity (Munoz de Toro et al., 2005) – Cages and bedding tested negative for estrogenicity (Markey et al, 2001; Munoz de Toro et al., 2005; Vandenberg et al, 2007) *Weaknesses:* – Only one tumour/animal selected for histopathology (Betancourt et al, 2010) – Only one dose-level tested for cell proliferation (Betancourt et al., 2013; Jenkins et al., 2009; Vandenberg et al., 2007)	Mainly Positive	Low to medium	↑

– Tubular epithelium not further specified (not TED (Betancourt et al., 2010) – Cell proliferation and apoptosis was measured at 12 months of age in TEB only (Jenkins et al., 2009) – No quantitative proliferation measurement (only hystopathological examination) (Avecedo et al., 2013) – Low number of animals/group for proliferation (Markey et al, 2001; Avecedo et al, 2013; Betancourt et al, 2010; Vandenberg et al, 2007) – Low number of animals tested for histological examination) (Jenkins et al., 2009) – Low/unclear number of animals for the dose-groups (Munoz de Toro et al., 2005) – Time-points for BrdU is not indicated (Moral et al., 2008) – Short exposure period (Vandenberg et al, 2007)			
Line of Evidence 1: Changes in number of mammary (terminal end) buds volume fraction of (alveolar) buds, and/or (atypical) intraductal epithelial hyperplasia/proliferation (Ayyanan et al., 2011, Tharp et al., 2012, Vandenberg, 2013c; US FDA/NCTR 90-day study, 2013, Acevedo et al., 2013) *Comment*: Increase in TEBs at one low dose only; small changes (Ayyanan et al., 2011) *Comment*: No dose-response relationship (Acevedo et al., 2013, Vandenberg et al., 2013c) *Strengths:* – Number of doses in some of the dose groups (U.S FDA/NCTR, 2013; Vandenberg et al, 2013; Acevedo et al., 2013) – Large sample size (US FDA/NCTR, 2013 and Delclos et al., 2014; Ayyanan et al., 2011) – Large sample size in most group (Acevedo et al., 2013) – Both naive and vehicle controls available (US FDA/NCTR, 2013 and Delclos et al., 2014) – Oral administration by gavage (US FDA/NCTR, 2013 and Delclos et al., 2014; Tharp et al, 2012) – Measurement of BPA concentration in the serum (US FDA/NCTR, 2013 and Delclos et al., 2014) – Primate study judged to have particular relevance for humans (Tharp et al., 2012) – Diet with low content or free of phytoestrogens (US FDA/NCTR, 2013 and Delclos et al., 2014; Ayyanan et al., 2011; Vandenberg et al, 2013c; Acevedo et al., 2013) – Use of non-pc cages and of non plastic bottles (US FDA/NCTR, 2013 and Delclos et al., 2014; Ayyanan et al., 2011; Vandenberg et al, 2013c; Acevedo et al., 2013) – Study performed according to GLP regulations (US FDA/NCTR, 2013 and Delclos et al., 2014) – BPA measurements in biological fluids (Tharp et al, 2012; Acevedo et al., 2013) – Mammary glands were analyzed in a treatment-blind manner (Tharp et al., 2012, US FDA/NCTR, 2013 and Delclos et al., 2014) – Study performed according to OECD guidelines, under GLP (US FDA/NCTR, 2013 and Delclos et al., 2014) – Three statistical methods applied (US FDA/NCTR, 2013 and Delclos et al., 2014)	Positive	Low to High	↑↑

– Re-evaluation of the lesions by a pathology working group (US FDA/NCTR, 2013 and Delclos et al., 2014) *Weaknesses:* – Small sample size (Tharp et al, 2012; Vandenberg et al, 2013c; Acevedo et al., 2013; Ayyanan et al., 2011) – Single dose level study (Tharp et al, 2012) – Statistics (because of limited sample size) (Tharp et al, 2012) – Inconsitent results within groups (females not sensitive to EE2 effects) (US FDA/NCTR, 2013 and Delclos et al., 2014) – Drinking water consumption not measured (Ayyanan et al., 2011) – Animal diet and phytoestrogen content not measured (Tharp et al, 2012) – Low No of animals tested for histological examination (Ayyanan et al., 2011) – Unclear/insufficient study reporting (Vandenbergh et al., 2013c; Ayyanan et al., 2011) – Reproductive cycling not controlled (Ayyanan et al., 2011, Tharp et al., 2012) – Unintended exposure of the control groups (US FDA/NCTR, 2013 and Delclos et al., 2014, see Churchwell et al, 2014)			
Line of Evidence 2: BPA has been reported to cause Leydig cell division in the prepubertal period and increased Leydig cell numbers were shown in the testes of adult male rats at 90 days (Nanjappa et al., 2012). *Comment* Rat strain highly disposed to Leydig cell proliferation *Strengths:* – Phytoestrogen free-diet – Use of non-pc cages and of non plastic bottles – Multiple tests to address the same endpoint – Correlation between morphological and functional changes assessed *Weaknesses:* – Results interpretation (biological relevance debatable)	Positive	Low	•
Overall conclusion on induction of proliferative changes by BPA in animals exposed during pre- and/or post-natal (during lactation) development or up to PND 90 (gavage): The EFSA opinion of 2010 noted potential proliferative effects of fetal or perinatal exposure to BPA. Since 2010 additional studies including a study in non-human primates **(**Ayyanan et al., 2011, Tharp, 2012, Vandenberg, 2013c, Acevedo, 2013, US FDA/NCTR, 2013 and Delclos et al., 2014) have also suggested that BPA can have proliferative effects on mammary tissues and strengthen the evidence for an effect of BPA on mammary gland proliferation in animals exposed during pre- and post-natal development. The CEF Panel considered that evidence for proliferative changes induced by BPA in other organs (e.g. testis: Nanjappa et al., 2012) is currently too weak to reach a definite conclusion.			**Likely (for mammary gland proliferation)**

Table 50: Summary of the WoE assessment of the likelihood that BPA is carcinogenic in animals

Overall conclusion on carcinogenicity of BPA in animals when exposed during their adult life (post-pubertal) only: Effects on mammary gland growth demonstrated in a transgenic mouse model with post-pubertal exposure (Jenkins et al., 2011) or on reported prostatic "intraepithelial neoplasia" in rats exposed to BPA in the immediate postnatal period (Prins et al., 2011) do not provide convincing evidence that BPA is carcinogenic in animals when exposed postnatally/during their adult life.	**Unlikely to -as likely as not-**

Overall conclusion on the carcinogenicity of BPA in animals exposed during pre- and post-natal (during lactation) development: Recent studies **(**Jenkins et al., 2009, Betancourt et al., 2010; Weber Lozada and Keri, 2011; Acevedo et al, 2013) investigated the potential carcinogenic effects of fetal or perinatal exposure to BPA. Given the various weaknesses identified in these studies, they do not provide convincing evidence that BPA is carcinogenic in animals when exposed during pre- and post-natal (during lactation) development.	**Unlikely to as like as not**

Table 51: Summary of the WoE assessment of the likelihood that BPA causes cell proliferation in tissues of animals exposed post- or pre-natally

Overall conclusion on proliferative changes of BPA in animals when exposed postnatally/ during their adult life: Both Jones et al (2010) and Jenkins (2011) reported an effect on mammary gland proliferation in sensitive transgenic or gene knock-out models as well as wild-type mice (Jones et al. only) given BPA during adulthood, although proliferative effects in these mouse models are inconsistent with the lack of any proliferative effect on mammary tissue in mice in the two year NTP carcinogenicity study. However, the CEF Panel concluded overall that there is some indication that BPA can cause proliferative changes in the mammary gland of genetically modified animals when exposed postnatally/in adult life. The Panel considered that evidence for proliferative changes induced by BPA in other organs (i.e. prostate) is currently too weak to reach a conclusion.	**As likely as not (for mammary gland proliferation)**
Overall conclusion on BPA- induced proliferative changes/ developmental advancement in the mammary gland of animals exposed during pre- and/or post-natal (during lactation) development or up to PND 90 (gavage): The EFSA opinion of 2010 noted potential proliferative effects of fetal or perinatal exposure to BPA. Since 2010 additional studies including a study in non-human primates **(**Ayyanan et al., 2011, Tharp, 2012, Vandenberg, 2013c, Acevedo, 2013, US FDA/NCTR, 2013 and Delclos et al., 2014) have also suggested that BPA can have proliferative effects on mammary tissues and strengthen the evidence for an effect of BPA on mammary gland proliferation in animals exposed during pre- and post-natal development. The CEF Panel considered that evidence for proliferative changes induced by BPA in other organs (e.g. testis: Nanjappa et al., 2012) is currently too weak to reach a definite conclusion.	**Likely (for mammary gland proliferation)**

Appendix D. Uncertainty analysis: (I) Dermal absorption (II) HEDF (III) Hazard characterisation

(i) Dermal penetration and absorption studies and uncertainties affecting the assessment of BPA absorption from dermal contact with thermal paper

In vitro studies on dermal penetration

Five in vitro studies on dermal penetration are available (Table 52 of this Appendix). The most recent study by Demierre et al. (2012) used non-viable (defrosted) human skin in a flow-through Franz cell system and performed the experiments according to the OECD test guideline 428 for in vitro skin absorption. Skin samples were obtained from the dorsal part of the upper leg from two human deceased donors, and seven skin sections dermatomed to a thickness of 200 µm were analysed. Skin integrity was checked with ^{3}H-water, yielding permeability coefficients within the acceptance range. Water was used as vehicle for the donor solution, and radiolabelled ^{14}C-BPA was applied in concentration of 193.6 mg/l slightly below the, aqueous solubility limit of ~250 mg/l. The applied surface density was 1.82 µg/cm^2. The donor chamber was covered with permeable tape (non-occluded conditions) to mimic real exposure conditions. Physiological saline was used as receptor fluid. The experiments were conducted at 30–32 °C for 24 h, and the receptor fluid was collected initially in 1 h and 2 h intervals. After 24 h incubation, the percutaneous penetration (i.e. the relative amount present in the receptor fluid) was 8.6%, the skin deposition 35.5%, and the recovery 101.5 %. Consecutive stripping of the stratum corneum (SC) with adhesive tape (15 tape strips) recovered 34.9% of the external dose in the SC with the main portion located in the most external SC layers. The kinetics of cumulative percutaneous penetration revealed a lag time of 1 h and a maximum penetration flux of 0.022 µg/cm^2/h, reflecting the penetration rate under steady-state conditions in the initial linear phase from 1–4 h. In the later time period, the penetration rate decreased to a ~6-fold lower level in the terminal linear phase from 11–24 h, indicating a change in the diffusion process, which is possibly (but not necessarily exclusively) resulting from the evaporation of the aqueous vehicle (applied volume 6 µl) on the skin surface. Dividing the maximum percutaneous penetration flux by vehicle concentration yielded a permeability coefficient (a dose-independent measure) K_p of 11×10^{-5} cm/h. Given the good quality of the study and the detail of reporting, the CEF Panel used the study of Demierre et al. (2012) as a reference for comparison with the other studies.

Marquet et al. (2011) used a static Franz diffusion cell and analyzed viable and non-viable (defrosted) human skin from 6 patients undergoing plastic surgery. The skin was dermatomed to a thickness of 500 µm, and the skin integrity was checked by measuring the transepidermal water loss. Acetone was used as vehicle, and ^{14}C-BPA was applied in a high concentration of 4000 mg/l, resulting in a surface density of 200 µg/cm^2. The receptor fluid consisted of cell culture medium with 2% BSA (BPA solubility ≥300 mg/l). The experiments were conducted at 32 ± 1 °C for 24 h, and receptor-fluid samples were taken on regular intervals. Permeation experiments with 15 non-viable human skin sections revealed a recovery of 95.6% and a maximum percutaneous flux of 0.12 µg/cm^2/h occurring at the end of the incubation period at 23.5 h. The quotient of maximum percutaneous flux and vehicle concentration yielded a permeability coefficient of 3.0×10^{-5} cm/h, which was 3.7-fold lower than in Demierre et al. (2012) but still comparable given the differences in vehicle type, surface density, and diffusion-cell design. Additional permeation experiments with non-viable rat skin under identical conditions revealed a ~12-fold higher permeability for rat skin compared to human skin. Finally, the authors used viable human and rat skin to estimate the extent of skin metabolism by measuring the BPA metabolites in the receptor fluid after 24 h of exposure. For both human and rat skin, metabolised BPA accounted for ~3% of the permeant.

Mørck et al. (2010) used a a static Franz diffusion cell and analyzed non-viable human skin from breast-surgery patients according to the OECD TG 428. For experimental details, the authors referred to the paper of Nielsen et al. (2009) from which some of information described below was taken. Full thickness skin (800–1000 µm) was used, and the skin integrity was checked by capacitance measurements. A diluted ethanol solution was used as vehicle, and ^{14}C-BPA was applied in a high concentration of 4000 mg/l, resulting in a surface density of 259 µg/cm^2. The receptor fluid consisted

of physiological saline solution containing 5% BSA. The experiments were carried out at ~32 °C for 48 h, and receptor-fluid samples were taken at regular time intervals. Experiments with 11 skin sections after 48 h incubation showed a percutaneous penetration of 13.0%, a skin deposition of 24.6%, and a recovery of 82.1%. A more detailed analysis of skin deposition showed 7.4% and 17.2% of the applied dose to be in the epidermis and dermis, respectively, which is in contrast to Demierre et al. (2012) who found the main portion of the skin deposition to be located in the stratum corneum of the epidermis. The CEF Panel noted that the percutaneous penetration of 13.0% is in good agreement with the 8.6% determined by Demierre et al. (2012) if the different incubation times (48 h vs. 24 h) are accounted for.

Kaddar et al. (2008) analyzed shaved pig skin from the flanks in a static Franz diffusion cell. Physiological serum was used as vehicle, and ^{14}C-BPA was applied in a concentration of 10 mg/l. The applied surface density was not reported, but the applied dose of 0.7 µg was comparable to the dose of 1.16 µg applied by Demierre et al. (2012). The experiments were carried out at ~32 °C, either for 24 h with repeated sampling in regular intervals (transfer kinetics experiment) or for 2, 5, and 10 h with single sampling (skin distribution experiment). For the skin distribution experiment, six replicates were used per exposure duration. Additional methodical details (e.g., skin thickness, applied surface concentration) were not reported. Analysis of skin distribution after the longest exposure time of 10 h showed that 5.4% and 8.8% of the applied dose to be in the epidermis and dermis, respectively, which is in contrast to human-skin study of Demierre et al. (2012) where the main portion of skin deposition was in the stratum corneum of the epidermis. The transfer kinetics experiment revealed a lag time of ~3 h and a percutaneous penetration of 4.1% after 24 h, which the CEF Panel noted was in good agreement with the 8.6% determined by Demierre et al. (2012) when taking the different skin types into account.

Zalko et al. (2011) examined the diffusion and metabolism of BPA using viable human skin explants from the abdominal region of female donors. The skin was dermatomed to a thickness of 500 µm and then seeded in cell culture inserts, where the explants were maintained at the air/liquid interface with dermal/epidermal feeding by diffusion of nutrients from the culture medium (1.5 ml) across the insert. Ethanol/phosphate buffer 0.1 M pH 7.4 (1:2, v/v) was used as vehicle, and ^{14}C-BPA was applied in a surface density of 2.75 µg/cm^2. The experiments were carried out at 37 °C, and culture media were collected at 24, 48, and 72 h. Experiments with 3 skin sections after 72 h incubation showed a percutaneous penetration 45.6%, a skin deposition of 41.5%, a residual amount of 2.5 % on the skin surface, and a recovery 92.6%. The CEF Panel noted that the reported skin penetration and deposition are not reliable estimates for in vitro skin absorption since several methodical features (e.g. use of cell culture inserts as diffusion cells, missing skin integrity check, exposure times largely exceeding 24 h, 33% ethanol solution as vehicle) did not conform with the OECD TG 428. Additional experiments with viable human skin and pig ear skin were carried out to analyze the extent of skin metabolism. Major skin metabolites were BPA mono-glucuronide and BPA mono-sulfate, which were reported to account for 73% and 27% of the dose in porcine and human skin after 72 h of incubation. The CEF Panel considered that the transferability of these results to the in vivo situation in humans as questionable. First, there was almost a complete depletion of the permeant on the skin surface. Second, the concentrations of BPA equivalents in the culture medium (i.e. the receptor compartment) reached values well above 1 µM, which is not really the "sink" condition prevailing in vivo with serum concentrations for BPA equivalents being generally far below 10 nM. As a consequence, there was no longer a one-directional transport of the permeant from the donor compartment to the receptor compartment, and a re-uptake of BPA from the culture medium with subsequent metabolism in the skin cannot be excluded. The CEF Panel considered that ignoring these methodical flaws would lead to an overestimation of the extent of in vivo skin metabolism.

In conclusion, a consistent picture of in vitro skin absorption of BPA emerged from the available studies with the exception of Zalko et al. (2011), which was not further considered for the methodological reasons given above. The CEF Panel regarded the study of Demierre et al. (2012) as a key study and considered other acceptable studies as supporting evidence. Demierre et al. (2012) used water as the vehicle, which is more comparable to a consumer exposure scenario to thermal paper than

e.g. acetone or diluted ethanol solutions, and the applied surface density of 1.82 µg/cm^2 is comparable to exposure estimates as derived for thermal paper (1.375–5.5 µg BPA per ~2 cm^2 finger tip). The experiment was conducted for 24 h, which again is comparable to a scenario with a daily exposure to thermal paper. Demierre et al. (2012) reported a percutaneous penetration of 8.6% and a skin deposition of 35.5% after 24 h. It is important to note that a somewhat depleted but still large portion of the applied dose (57%) remained on the skin after 24 h. The relatively high fraction of 35.5% in the skin is physiologically plausible as a steep concentration gradient is needed in the stratum corneum (SC), along which BPA can diffuse from the skin surface to the deeper skin layers. That this high fraction is present in the SC was corroborated by tape stripping of successive corneocyte layers (Demierre et al., 2012) and by the findings of Biedermann et al. (2010). Seemingly contradictory findings by Mørck et al. (2010) and Kaddar et al. (2008) concerning the distribution between the epidermis and dermis would need to be considered in terms of dermis thickness and the extent of deviation from the sink condition in the receptor compartment. In Demierre et al. (2012), the specific permeation kinetics with an initial high penetration rate and a subsequent low penetration rate are suggestive of effects arising from finite dosing (i.e. partial depletion of the dose on the skin surface) and/or evaporation of the aqueous vehicle (→ reduced hydration of the SC), which both are realistic conditions applicable to consumer exposure. In spite of differences in the diffusion-cell design, skin type, vehicle type and applied dose, the in vitro studies of Marquet et al. (2011), Mørck et al. (2010), and Kaddar et al. (2008) support the percutaneous penetration estimate of 8.6 % of Demierre et al. (2012), although tending to somewhat lower values: a rough calculation based on the comparison of permeability coefficients or the normalization of percutaneous penetration to 24 h incubation yielded estimates of 2.3 % (Marquet et al., 2011) and 6.5 % (Mørck et al., 2010) for human skin, and of 4.1 % (Kaddar et al., 2008) for pig skin.

Given a percutaneous penetration of <10%, a skin deposition in the stratum corneum of ~35%, and a residual amount on the skin surface of >50% after 24 h incubation, the question arises as to whether or not the amount that is deposited in the skin may reach the systemic circulation. The CEF Panel noted that if a simple consumer scenario with a repeated single daily exposure to thermal paper is considered, and if it is further assumed that any BPA remaining on the skin surface after 24 h is removed (e.g. by hand washing, sloughing or by touching things) before the next dose is applied, then a stationary state will prevail, with a more or less stable concentration gradient in the stratum corneum (SC), along which BPA diffuses across the skin to reach the circulation. As a consequence, it is the <10% fraction of the external dermal dose that reaches the systemic circulation within the time period of 24 h.

Concerning the metabolic capacity of the skin, there were two in vitro dermal penetration studies providing information on BPA metabolism. Marquet et al. (2011) analysed human and rat skin and reported that metabolized BPA accounted for ~3% of the permeant, which is a negligible fraction. Zalko et al. (2011) reported that skin metabolites accounted for 73% (pig skin) and 27% (human skin) of the dose. This study was not further considered because of methodological shortcomings. The CEF Panel noted that the available information does not permit to arrive at a reliable estimate of the extent of skin metabolism. For the estimation of internal exposure from dermal exposure to thermal paper, it was conservatively assumed that no considerable skin metabolisation occurs.

In vivo studies on percutaneous absorption

The study of Marquet et al. (2011) in rats is the only in vivo study of BPA dermal absorption. The absorbed dose of total ^{14}C-BPA after application of a concentrated acetone solution (4000 mg/l, 500 µl total volume) in a surface density of 200 µg/cm^2 and a 72 hr sample collection interval was 23% (i.e. fraction of total radioactivity found in urine + feces + carcass). The total recovery was in the range of 90–100% of the administered dose. There was a linear relationship between the cumulative absorption and exposure time over the experimental period of 0–30 h, and the slope of this line corresponded to an absorption flux of 2.5 µg/cm^2/h. The quotient of absorption flux and vehicle concentration yielded a permeability coefficient of 62.5×10^{-5} cm/h.

Marquet et al. (2011) also compared the maximum percutaneous fluxes ex vivo from rat and human frozen dermatomed skin explants and found the human flux to be 8% of the rat value under identical conditions using the acetone vehicle. Using this figure as an "interspecies factor" permits extrapolation of the in vivo rat permeability coefficient of 62.5×10^{-5} cm/h to humans. This extrapolation yielded an in vivo human permeability coefficient of 5.0×10^{-5} cm/h, which is somewhat lower than but still comparable to the value of 11×10^{-5} cm/h determined by Demierre et al. (2012) for human skin ex vivo. This additional plausibility check not only corroborates the findings of Demierre et al. (2012) but also suggests a 24-h percutaneous penetration that is approximately half the value of 8.6% reported by Demierre et al. (2012).

Table 52: Overview of in vitro studies on percutaneous penetration of BPA. Data are given as means ± SD.

Parameter	Kaddar et al. (2008)	Mørck et al. (2010)	Marquet et al. (2011)	Zalko et al. (2011)	Demierre et al. (2012)
Skin type	pig skin from the flanks	human skin samples from breast surgery	human skin from 6 patients undergoing plastic surgery	human skin explants from abdominal region	dorsal part of the upper leg from 2 human cadavers.
Number of skin sections	6 (?)	11	15	3	7
Skin viability		non-viable	non-viable (and viable)	viable skin	non-viable
Skin Section thickness		800–1000 µm	500 µm	500 µm	200 µm
Exposed area		2.12 cm^2	1.76 cm^2	6.2 cm^2 (Ø28 mm)	0.64 cm^2
Applied volume	70 µl	32.6 µl		60 µl	6 µl
Applied volume per area		15.4 µl/cm^2	50 µl/cm^2	9.7 µl/cm^2	9.4 µl/cm^2
Applied concentration	10 mg/l	3995 mg/l (= 17.5 mM)	4000 mg/l	284 mg/l	194 mg/l
Applied surface density		259 µg/cm^2	200 µg/cm^2	2.75 µg/cm^2	1.82 µg/cm^2
Applied dose	0.7 µg	452 µg	352 µg	17 µg	1.16 µg
Temperature	32.0 ± 0.1 °C	≈32 °C	32 ± 1 °C	37 °C	30–32 °C
Method	static Franz diffusion cell	static Franz diffusion cell OECD TG 428	static Franz diffusion cell	organ culture in Transwell cell culture inserts	flow-through Franz cell OECD TG 428
Skin integrity check		capacitance measurement	measurement of trans-epidermal water loss		permeability coefficient within acceptance range
Occlusion conditions		Parafilm cover	no cover	no cover	permeable-tape cover
donor solution (vehicle)	physiological serum	0.9% NaCl + 2% EtOH	acetone	EtOH/P-buffer (1:2, v/v)	water
receptor fluid		physiol. saline + BSA	culture medium	culture medium	physiological saline
Duration of incubation	24 h	48 h	24 h	72 h	24 h
Recovery	84.3 ± 9.0 % at 10 h	82.1 %	96.5 ± 1.9 %	92.6 ± 5.8 %	101.5 ± 1.6 %
Skin deposition		24.6 ± 5.8 %		41.5 ± 10.8 %	35.5 ± 6.6 %
Percutaneous penetration	4.1 % at 24 h	13.0 ± 5.4 %		45.6 ± 6.2 % at 72 h	8.6 ± 2.1 %

Parameter	Kaddar et al. (2008)	Mørck et al. (2010)	Marquet et al. (2011)	Zalko et al. (2011)	Demierre et al. (2012)
				(15.2% when down-scaled to 24 h)	
Maximum penetration flux			0.12 µg/cm^2/h		0.022 µg/cm^2/h
Permeability coefficient K_p			3.0×10^{-5} cm/h		11×10^{-5} cm/h
Remark	also data for 2, 5 and 10 h, lag time ≈ 3 h	some experimental details were taken from Nielsen et al. (2009)	additional data for rat skin, information on metabolites	similar data for pig skin, information on metabolites	lag time ≈ 1 h, biphasic time course

Evaluation of uncertainties affecting the assessment of dermal absorption of BPA after dermal exposure to BPA from thermal paper

This evaluation of uncertainties surrounding the estimate for dermal absorption of BPA starts with a definition and clarification of the processes involved. Dermal / percutaneous absorption is the movement of a chemical from the outer surface of the skin into the circulatory system leading to systemic exposure (EFSA, 2011). Dermal penetration is the movement of a chemical from the outer surface of the skin into the epidermis, but not necessarily into the circulatory system (EFSA, 2011).

The study of Biedermann et al. (2010) on the transfer of BPA from thermal paper to the skin and the dermal penetration study of Demierre et al. (2011) suggest that ~30% of the external dermal exposure may penetrate into the skin and become available for subsequent systemic uptake. Demierre et al. (2011) also showed that 8.6% of the applied dose passed through the human skin within 24 h. Given the uncertainties around this value, and taking the evidence from the other dermal absorption studies into account, a dermal absorption fraction of 10% was assumed in the present opinion for the exposure scenarios with dermal contact to thermal paper. This is considered as fairly realistic. Further specifications in the PBPK modelling of these exposure scenarios comprised (i) the assumption of a BPA depot (receiving 100% of the external dermal dose) in the moisture film on the skin and (ii) the assumption of a first order process for dermal absorption. To keep the PBPK model as simple as possible (in terms of the number of assumptions and parameters), it was further assumed (conservatively) that the BPA depot remains on the skin surface during the whole day and that 10% of the initial depot content is absorbed within 24 h. BPA remaining on the skin surface after 24 h (i.e. 90% of the initial depot content) is assumed to be completely removed (e.g. by hand washing, abrasion etc.), and the skin surface depot is then reloaded with 100% of the new dermal dose. The impact of this assumption, which could potentially result in severe underestimation of the contribution of dermal exposure, is however limited, as discussed in the uncertainty table (Table 53) below. In other words, the BPA depot is assumed to be periodically replenished to 100% after 24 h by a new dermal contact to thermal paper. An important consequence of assuming the BPA depot to be depleted to only a small extent within 24 h is that the dermal absorption process is in a steady state with a virtually stable and permanent concentration gradient in the stratum corneum (SC), along which BPA is diffusing through the skin to reach the systemic circulation. In the PBPK modelling of internal exposure (i.e. serum concentration of unconjugated BPA) resulting from dermal contact to dermal paper, it was made sure that the modelled system was in a steady steady state by running the simulation for 10 days. For the HEDF calculations the AUC value from day 10 was used.

Given the above specifications, the amount of BPA absorbed per unit time and area J (µg cm^{-2} h^{-1}) is described according to a first-order process as

$$J = k \cdot X / A,$$

where k is the first-order rate constant (h^{-1}), X is the amount of BPA (µg) in the skin surface depot, and A is the skin surface area (cm^2).

The assumption of a first-order process for dermal absorption and of 10% absorption during 24 h leads to a rate constant (k) of –ln(0.9)/24 h = 0.00439 h^{-1}. For the external dermal dose that is loaded into the skin surface depot (X/A), an average estimate of 0.69 µg/cm^2 was derived e.g. for adult males based on the transfer of 1.375 µg BPA from thermal paper to the finger tips (surface area per finger tip: 2 cm^2) following a single handling event per day. The high estimate of 3.17 µg/cm^2 for adult males was based on 4.6 handling events per day and by further assuming that each new handling event adds 0.69 µg/cm^2 to the already existing BPA depot on the skin surface.

Table 53 contains the evaluation of uncertainties surrounding the rate constant estimate (k) and the built-up and maintenance of the BPA depot (X/A). Concerning the BPA depot, the uncertainty assessment is focussed on the uncertainty in the surface dose X/A and not on the uncertainty in the individual parameters X (amount) and A (exposed surface area) as these are treated in the uncertainty evaluation of the external dermal exposure (see Appendix H in Part I-Exposure assessment). The scale used to evaluate the impact of the source of uncertainty on the estimates of dermal absorption is shown in Figure 18. Plus symbols indicate the real value could be higher than the estimate, while minus symbols indicate the real value could be lower than the estimate. The evaluations are approximate expert judgements and should not be interpreted as precise estimates.

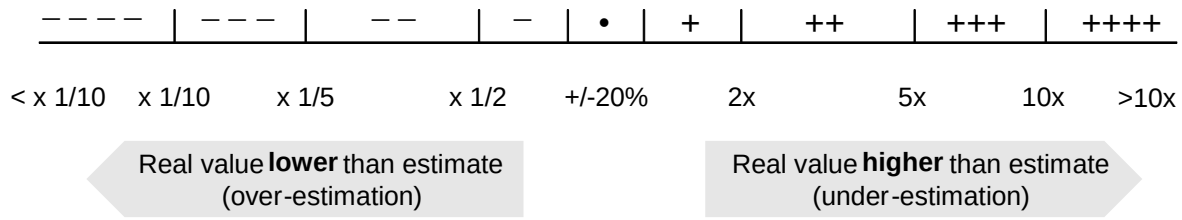

Figure 18: Scale used for evaluating the impact of uncertainties on estimates of total exposure to BPA.

Table 53: Assessment of the uncertainty in the dermal absorption fraction, as reflected by the first order rate constant k, and in the BPA depot in the moisture film on the skin surface, as reflected by the variable X/A, as related to the dermal exposure to BPA in thermal paper (See Figure 18).

Source of uncertainty	Variable affected	Uncertainty of the estimate
Extent of dermal absorption Available evidence from in vitro dermal absorption studies with human skin explants from breast, abdomen, and upper leg suggests a 24-h dermal absorption for human skin of 2.3–8.6%. The upper limit was reported by Demierre et al. (2012) as the fraction of the applied dose that passed through human skin explants within 24 h. By taking the amount of BPA in the viable part of the epidermis into account, Demierre et al. (2012) reported a bioavailable fraction of 9.6%, which was rounded up in the present opinion to 10% to reflect the uncertainties around this number. The impact of uncertainty is judged to be within the range of 0.5–1.2 times the estimate.	k (first-order rate constant for 10% dermal absorption during 24 h)	– / ●
Skin viability and skin metabolism The non-viability of the human skin explants in Demierre et al.	k	– / no impact

Source of uncertainty	Variable affected	Uncertainty of the estimate
(2011) may influence the extent of the dermal absorption. However, it is generally accepted that the non-viable SC is the main diffusion barrier for absorption (EFSA, 2011), so that the effect on percutaneous diffusion can be assumed to be small. The tape-stripping results of Demierre et al. (2011) support the assumption that the SC is the main diffusion barrier. Skin metabolism, however, may reduce the extent of dermal absorption of unconjugated BPA. There are two *in vitro* dermal absorption studies on skin metabolism. Marquet et al. (2011) analysed human and rat skin and reported ~3% of the permeant being metabolized BPA. Zalko et al. (2011) reported that skin metabolites accounted for 73% (pig skin) and 27% (human skin) of the applied dose. The available information did not permit to arrive at a reliable estimate of extent of skin metabolism. Not considering skin metabolism may overestimate the amount of unconjugated BPA that reaches the systemic circulation. This uncertainty is within the range of 1/2–1 times the estimate.		
Thickness of the Stratum corneum (SC) Dermal absorption studies normally use the back (*in vivo* studies) or breast/abdomen or upper leg (*in vitro* studies), which are considered to provide realistic dermal absorption values (EFSA, 2011). The thickness of the SC is several times greater in the palms than in other parts of the body (Egawa et al., 2007; US EPA, 2011; EFSA, 2011). The extent of dermal absorption across the skin of the finger tips may therefore be smaller than across other body part such as those normally used for dermal absorption studies. The impact of uncertainty is judged to be within the range of 1/5–1/2 times the estimate.	*k*	– –
Age-related differences in dermal absorption Possible minor differences in skin absorption due to age appear to be limited to certain skin areas only and do not call for any correction factor or any specific default figures to be applied (EFSA, 2011).	*k*	No impact
Sweating and skin hydration Sweating and skin hydration were reported to increase dermal absorption < 2 fold (EFSA, 2011). Two variables are affected. Compared to dry fingers, sweaty fingers have a thicker moisture film on the skin surface, which would enable a larger BPA depot on the skin surface (*X/A*). This potential effect is already covered under "Wet and oily/greasy fingers". An increased hydration of the Stratum corneum (SC) could increase the diffusion of BPA through the SC. However, this potential effect is already covered by the dermal absorption fraction of 10% (from which the first-order rate constant *k* was derived), because the aqueous vehicle that was used in the in-vitro dermal penetration study of Demierre et al. (2012), likely resulted in an increased skin hydration. The impact of uncertainty is judged to be within the range of 1–1.5 times the estimate.	*k* *X/A* (BPA depot in the moisture film on the skin surface)	● to ●/+
Saturation of BPA in the skin moisture film Biedermann et al. (2010) reported a BPA transfer from thermal paper to dry finger tips of 1.13 µg BPA per finger. They also showed that the BPA load to the skin did not increase when the paper was touched for longer times or repeatedly. This could suggest a saturation of BPA in the skin moisture film. Further evidence for a saturation effect can be derived from analyses of the film thickness of liquids on the skin. E.g., the contact of dry hands with water and subsequent full wipe of the hands resulted in an aqueous water film on the skin of ~20 µm thickness (US EPA, 2011).	*X/A* (BPA depot in the moisture film on the skin surface)	scenario for average expo.: ● scenario for high exposure: –

Source of uncertainty	Variable affected	Uncertainty of the estimate
Taking the aqueous solubility of BPA of 250 mg/l into account, the maximum BPA load into this aqueous surface film would be 0.5 µg/cm^2 or about 1 µg per finger tip when assuming a surface area of 2 cm^2 per finger tip. This agrees well with the observed transfer of 1.13 µg BPA per finger in Biedermann et al. (2010) and of 11.3 µg to 8 fingers (= 1.41 µg per finger) in Lassen et al. (2011) under dry-hand conditions. So, if the skin moisture film is already saturated, the BPA depot would not increase with further touching of thermal paper. This limitation would specifically affect the dermal absorption of high dermal doses. The impact is judged to be within the range of 1/2–1 times the estimate.		
Wet and oily/greasy fingers Biedermann et al. (2010) reported a limited transfer of BPA from thermal paper to dry fingers but a comparatively increased transfer to wet or oily fingers. Similarily, Lassen et al. (2011) reported a ~9-fold higher BPA transfer to humid fingers compared to dry fingers. The comparatively higher BPA transfer from thermal paper into an oily or greasy surface film can be explained by the higher solubility of the lipophilic BPA and also by a possibly thicker surface film. A higher BPA concentration in the skin surface film increases the dermal absorption. For wet or sweaty fingers, however, the BPA concentration in the skin surface film remains limited by the aqueous solubility. Compared to the dry-finger scenario, the main difference in the humid-finger scenario is the larger depot volume on the skin surface and, consequently, the reduced depletion of the BPA depot. For a chronic daily exposure to BPA in thermal paper, the assumption of having always wet or greasy/oily fingers when touching thermal paper is unlikely for the general population. The impact of uncertainty is judged to be within the range of 1–2 times the estimate.	*X/A*	● / +
Hand washing and desquamation Processes such as hand washing and desquamation (i.e. shedding of the outermost skin layers) that could additionally deplete the BPA depot on the skin surface have not been taken into account in the scenarios for dermal absorption. Not considering these effects leads to an overestimation of dermal absorption. The impact of uncertainty is judged to be within the range of 1/2 times to –20% of the estimate.	*X/A*	–
Replenishment of the skin surface depot Another simplification concerns neglecting a possible built-up of the BPA amount on and in the skin (i.e. scenarios with contact to thermal paper repeated on consecutive days). Biedermann et al. (2010) determined that 2 h after finger contact to thermal paper only 70% of applied substance could be removed from the finger tips with ethanol, which is in accordance with the in vitro dermal penetration study of Demierre et al. (2012), where ~15% of applied surface dose were found in most external SC layer (tape strip 1), which can be regarded as potentially extractable and therefore to belong to the BPA depot, and where ~20% of BPA were found in the deeper parts of the SC (tape strip 2-10). Therefore, if it is assumed that a maximum of 30% BPA penetrates the skin, of which 10% are internally absorbed, 20% may still be present in deeper layers of the SC. The second dosing would therefore initially raise the amount on and in the skin to 120%. On the second day 20% of these 120% (i.e. 24%) would remain in the skin, which raises the dose on the third day to 124%. After around 5 days a plateau of around 126% will be reached. The impact of uncertainty of daily dosing on consecutive days therefore is judged to	*X/A*	+

Source of uncertainty	Variable affected	Uncertainty of the estimate
be slightly larger than 20% in the direction of underestimation.		
Overall assessment:		
The main sources of uncertainty in the first-order rate constant (*k*) for 10% dermal absorption during 24 h resulting from the dermal exposure to BPA in thermal paper are (i) the extent of dermal absorption and (ii) the increased thickness of the Stratum corneum (SC) of the finger tips. The combined impact of both factors is judged to be within the range of 1/5–1/2 times the estimate (– –). Based on this proposed uncertainty range, the experts were asked to make their personal judgements. Five experts gave their personal view on that; some shifted the upper limit of the proposed uncertainty range to a higher value, others specified a somewhat different uncertainty range (Table 54). The differences among the experts' individual judgements are covered by the uncertainty range of 1/5–2 times the estimate (– – / +).		Uncertianty for the first-order rate constant *k*: – – / +
The main sources of uncertainty in the BPA depot in the moisture film on the skin surface (*X/A*) are (i) the potential saturation of BPA in the skin moisture film, (ii) the possibility of having wet or oily/greasy fingers, (iii) hand washing and desquamation, and (v) the replenishment of the skin surface depot. The combined impact of all factors is judged to be within the range of 1/2–2 times the estimate (– / +). This uncertainty is already covered by the uncertainty evaluation of external dermal exposure given in Appendix H in Part I-Exposure assessment.		Uncertainty in the skin depot *X/A*: – / +

Table 54: Individual expert judgements of the uncertainty around the dermal absorption fraction of 0.1 (= 10%) relating to the dermal exposure to BPA in thermal paper. Each expert was asked to consider the proposed uncertainty range of 1/5–1/2 times the estimate (– –) and to make a personal judgement about the uncertainty range (See Figure 18 for key to symbols).

Expert	Reasoning	Uncertainty of the estimate
1	The description of dermal absorption as a first-order process is an abstraction of the real process and includes simplifying assumptions. The upper uncertainty limit is therefore increased to equal the estimate of 10% dermal absorption. A further increase of the upper uncertainty limit to 2–5 times the estimate is regarded as overly conservative as it would generate a substantial disagreement in the internal exposure estimates as obtained by biomonitoring and forward exposure modelling.	– – / ●
2	The study of Biedermann et al. (2010) on the transfer of BPA from thermal paper to the skin and the dermal penetration study of Demierre et al. (2011) suggest that ~30% of the external dermal exposure may penetrate into the skin and may become available for subsequent systemic uptake. This should be covered to some extent by the uncertainty range. In addition, skin contact to ethanolic solutions may increase dermal uptake. Overall, the uncertainty around the dermal absorption fraction of 0.1 is assessed as –/+ (×1/2 – ×2).	– / +
3	The 10% dermal absorption is conservative and sufficient as an upper uncertainty limit.	– – / ●
4	From the controlled exposure study to BPA in thermal paper (Ehrlich et al., 2014), it can be estimated that 0.112 µg/kg bw per day is excreted in urine above the background excretion level after handling thermal paper without gloves for 2 hours (see Section 3.1.2.4). The forward exposure modelling (see Section 4.5.3.3 in Part I-Exposure assessment) estimated the average external exposure to be 0.06 µg/kg bw per day (= 3 fingers × 1.4 µg per finger × 1 occasion/day/70 kg) and the high external exposure to be 0.55 µg/kg bw per day (= 2×3 fingers [both hands] × 1.4 µg per finger × 4.6 occasions/day/70 kg). Assuming 10% dermal absorption, these external exposure estimates translate into internal exposures of 0.006 µg/kg bw per day (average) and 0.055 µg/kg bw per day (high). The amount of 0.112 µg/kg/day (according to Ehrlich et al., 2014) is still 2-fold higher than the calculated internal high exposure. Thus, 10% dermal absorption is a	– / +

Expert	Reasoning	Uncertainty of the estimate
	rather plausible estimate without an extreme uncertainty. Overall, the uncertainty around the dermal absorption fraction of 0.1 is assessed as –/+ (×1/2 – ×2).	
5	Given the information and the stengths/weaknesses of the various studies, the estimate of 10% as central estimate for the dermal absorpion is realistic to conservative, but not over-conservative. However, one shows that 30% of the dermal load may be present in the skin. For this 30% the reloading (replenishment) principle is applicable, and has been addressed in the reloading / replenishing uncertainty discussion. Consequently, the impact of this depot and the reloading of it was quantified to be around 20–30% (under-estimation). Assuming that the dermal uptake can be 5 times higher than what we currently assume (i.e. a ++ score) is very conservative, taking into account that several other studies, among which the reliable study by Demiere et al. (2011), have measured less intensive absorption than the 10% that we already assume as central estimate. It seems that an upper limit for the dermal absorption factor of 15–20% (i.e. 1.5 to 2 times as high as we have used) would be sufficient to cover the uncertainty in this parameter. For the lower bound, 5% absorption (i.e. 1/2 times the estimate) seems a reasonable assumption.	– / +

(ii) Evaluation of uncertainties affecting the determination of Human-Equivalent Dose Factor (HEDF) for BPA

Table 55 contains the evaluations of uncertainties affecting the determination of the *average* HEDF. The scale used to evaluate the impact of the source of uncertainty is shown in Figure 19. Plus symbols indicate the real value could be higher than the estimate, while minus symbols indicate the real value could be lower than the estimate. These evaluations are approximate expert judgements and should not be interpreted as precise estimates.

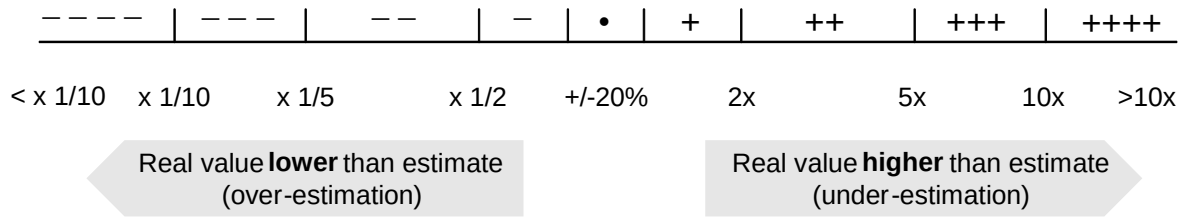

Figure 19: Scale used for evaluating the impact of uncertainties on estimates of total exposure to BPA.

Table 55: Evaluation of uncertainties affecting the determination of the average Human-Equivalent Dose Factors (HEDF = AUC_{Animal}/AUC_{Human}) for BPA. See Figure 19 here above for key to symbols.

Source of uncertainty	Variable affected	Impact on the HEDF
Analytical uncertainty for serum concentrations of unconjugated BPA (C_{BPA}) above the LOD. Recovery: Not a problem since all studies used isotope-dilution mass spectrometry with recovery correction. Repeatability (CV): <20%. Accuracy: < ±20%. These percentages refer to the uncertainty in the measurement of C_{BPA} at a single time point. A serum concentration-time profile consists of 3–8 data points, so that the imprecision in the measurement of a single concentration value will average out when calculating the AUC. The overall impact of the analytical uncertainty on the AUC estimate is regarded to be within ±20%.	AUC_{Animal} (nM×h)	●
Contamination of serum samples. Not a problem since all studies used isotope-labelled (deuterated) BPA for administration.	AUC_{Animal} (nM×h)	none
Inter-individual variability and uncertainties in experimental procedures and toxicokinetic analysis Variability in the experimental animals and in the dosing and sampling procedures results in a variability in the individual AUC_{Animal} values. An additional source of variability is the toxicokinetic analysis, which is based on the application of the trapezoidal area method to estimate the AUC for the observed serum concentration-time profile but which additionally includes the extrapolation to the AUC from zero time to infinity. For this extrapolation, the elimination rate constant and the last observed quantifiable C_{BPA} are required, both of which are associated with uncertainties. All these sources of variability are covered by the reported standard deviation (SD) of AUC_{Animal}. Based on the estimates for the mean and the SD, and on the number of animals, a relative standard error (RSE) can be calculated as a measure of uncertainty around the mean AUC_{Animal}. RSE values of 34–37% can be derived for adult rats and monkeys with oral administration and for PND 77 monkeys with oral administration. In addition, RSE values of 4–21% can be derived for adult rats and monkeys with IV injection. Translating these RSE values into naive 95% confidence intervals (i.e. 1.96×RSE), and taking into account a log-normal distribution for the serum concentration values as a reasonable assumption, the true mean AUC_{Animal} value for given tested species is judged to be within the range of 0.5–2 times the estimated mean AUC_{Animal}. Experimental designs can incorporate sequential blood sampling from individual animals of sufficient body size and blood volumes (e.g., rats and monkeys) to calculate individual serum concentration-time profiles that are used to produce group mean PK parameters and reliable estimates of inter-animal variability. On the other hand, studies in small animals that have insufficient blood volumes for repeated withdrawals (e.g., mice), samples can only be collected from multiple animals at each time point to calculate an average serum concentration-time profile that is used to produce a single set of PK parameters. In this experimental design, no statistical analysis is possible except for C_{max} and estimation of inter-animal variability in other PK parameters is not possible. An example for the latter case is the AUC_{Animal} for adult mice with oral administration. A detailed uncertainty discussion for this case is given in Section 3.1.6.1.	AUC_{Animal} (nM×h)	–/+
Uncertainty due the laboratory-specific bias	AUC_{Animal} (nM×h)	●

Source of uncertainty	Variable affected	Impact on the HEDF
The administration procedure can have a significant effect on the AUC_{Aminal} estimate. For HEDF determination, it is noted that the administration procedures in animal and human toxicokinetic studies should be comparable. The animal toxicokinetic studies of Doerge et al. (2010a/b, 2011a/b, 2012) used gastrointestinal bolus gavage with aqueous vehicle solutions, which permitted fast gastrointestinal absorption and excluded potential sources of variability associated with digestive processes. The obtained values for AUC_{Animal} (and AUC_{Human}, see below) are therefore internally consistent. There are no known or suspected sources of bias apart from those already mentioned (i.e. analytical uncertainty and contamination). If present, it would affect all the data in the same way.		
Uncertainty about the serum concentration-time course of unconjugated BPA as predicted by PBPK modelling The AUC_{Human} values for human adults and infants were obtained from PBPK modelling (Yang et al., 2013) by using a monkey-based PBPK model with bolus-gavage dosing (Fisher et al., 2011). The human PBPK model was evaluated against the results of a toxicokinetic study in humans with gelatine-capsule administration (Völkel, et al. 2002), which is consistent with the administration procedure used in the animal studies. A sensitivity analysis revealed the volume of the liver, the hepatic and small-intestine metabolism, and the oral uptake rate constant to be sensitive in predicting the unconjugated BPA concentration in serum. The impact of this uncertainty is judged within the range of 0.5–2 times the estimated mean AUC_{Human}.	AUC_{Human} (nM×h)	–/+
Overall assessment: The main sources of uncertainty in the determination of HEDF are (i) the variabilities in the experimental animals and in the dosing and sampling procedures, and (ii) the uncertainty about the serum concentration-time course of unconjugated BPA in humans as predicted by PBPK modelling (Fisher et al., 2011; Yang et al., 2013). These sources of uncertainty influence the AUC_{Aminal} and AUC_{Human}, which are ratioed to yield the HEDF. The overall uncertainty is judged to be within the range of 0.5–2 times the estimate. A specific case is the toxicokinetic study in adult mice with oral administration, for which a dedicated uncertainty assessment was performed (see Section 3.1.6.1). Multiplying the HEDF with the reference point of a toxicity study with oral administration re-raises the issue of uncertainty in the extrapolation to the human situation. The question of uncertainty is whether the type of oral administration in the toxicity study is comparable to the typical exposure situation in humans. The exposure of animals *via* dosed feed has been shown to lead to a serum concentration-time profile for unconjugated BPA which was different from that observed under oral-bolus dosing (Sieli et al., 2011); the AUC, however, was not affected. Since the typical exposure to BPA in humans is *via* dietary exposure, there is no reason to assume a large uncertainty when extrapolating from a toxicity study with dietary exposure to the human situation.		–/+

(iii) Additional results from the uncertainty analysis for aggregate exposure

The main results of the uncertainty analysis for aggregate exposure is reported in Section 5.1.2. This section reports additional results from specific parts of that analysis.

Parameters for PBPK modelling

Table 56: Parameter values for the male adult were taken from Mielke et al. (2011). Parameter values for the children were taken from Mielke and Gundert-Remy (2009). In the latter publication, the muscle and skin tissues were combined to a single muscle/skin compartment. Additional information from Edginton and Ritter (2009) was used for assigning organ weights and blood flow rates to the muscle and skin compartments of the children. The values

for the extent of absorption for the oral and dermal routes were those set in the present opinion.

Parameter / Age group	Children 1.5–4.5 years	Male adult
Body weight (kg)	19	73
Organ weights (g)		
Adipose tissue	5500	18200
Liver	570	1800
Brain	1310	1450
Kidney	110	310
Muscle	0.86×6170	29200
Skin	0.14×6170	2708
Other vessel-rich organs	1141	3768
Skeleton	2090	9330
Blood flow rates (l/h)		
Fat	9.7	19.5
Brain	55.8	46.8
Kidney	27.1	74.1
Muscle	0.55×26.7	65.8
Skin	0.45×26.7	20
Other vessel-rich organs	29.8	56.5
Skeleton	2.9	7.8
Liver	52	99.5
Tissue:blood partition coefficients		
Brain	1.06	
Kidney	1.35	
Liver	1.46	
Fat	3.31	
Muscle	1.35	
Skin	5.7	
Other vessel-rich organs	1.43	
Skeleton	0.5	
Metabolic parameters		
Glucuronidation K_m (µM)	8.5	
Glucuronidation V_{max} (nmol min^{-1} g liver^{-1})	54.9	
Sulfation = 0.08 × V_{max} / K_m	0.08 × 54.9 / 8.5	
Absorption half-life (h)		
Oral route	0.25	
Dermal route (Thermal paper)	158	
Extent of absorption (fraction of the dose)		
Oral route	1.0	
Dermal route (Thermal paper)	0.1	
Dermal route (Cosmetics)	0.5	

Uncertainty in PBPK modelling

The PBPK model of Mielke et al. (2011), hereinafter called the Mielke/Gundert-Remy model, was used to convert an external dermal exposure into an equivalent oral exposure (see Section 3.1.7.3). The use of the Mielke/Gundert-Remy model introduced an uncertainty, because the PBPK model of Fisher et al. (2011) was selected as the model of first choice for deriving the HED (see Section 3.1.3.4). The structure of both models is described in the Sections 3.1.3.1 and 3.1.7.3. In the following, the functional differences of both models are described and the overall impact of these differences on the dermal-to-oral route equivalence factor (EF_{D_O}) is quantified.

Whereas intrinsic liver clearance in both models is similar, a difference exists in the implementation of the first-pass metabolism. This leads to different systemic availabilities of BPA following oral exposure. In the Fisher model the first pass takes place in the gut wall and in the liver, which reduces the systemic availability of unconjugated parent compound to about 1.3 % of the oral dose. The Mielke/Gundert-Remy-Modell contains only the hepatic first pass, because the intestinal metabolism was considered to be so small to be negligible, being less than 10% of the liver clearance according to the in vitro data of Trdan Lušin et al. (2012). Compared to the Fisher model, the Mielke/Gundert-Remy model predicts a higher systemic availability of about 10 %. More precisely, for a defined oral exposure, the Mielke/Gundert-Remy model predicts an area under the curve (AUC) for the serum concentration of unconjugated BPA which is 8.1-fold higher than the AUC predicted by the Fisher model (Yang et al., 2013, and calculation by the experts using the Mielke/Gundert-Remy model). Accordingly, $AUC_{oral.MGR} / AUC_{oral.F} = 8.1$.

For the dermal exposure, the magnitude of the AUC depends on how much of the dermal dose is assumed to penetrate the skin and to enter the blood. The present opinion assumed 10 % dermal absorption for the exposure to BPA in thermal paper and 50 % dermal absorption for the exposure to BPA in cosmetics. For a dermal absorption fraction being equal in both PBPK models, the AUC predicted by the Fisher model is slightly lower (by a factor of 0.94) than AUC predicted by the Mielke/Gundert-Remy model (the value of 0.94 is the ratio of the intrinsic liver clearances of the two models). Accordingly, $AUC_{dermal.F} / AUC_{dermal.MGR} = 0.94$.

Having quantified the model differences in the internal dose metric (AUC) for defined oral and dermal exposures, it becomes possible to evaluate the model uncertainty in the dermal-to-oral route equivalence factor ($EF_{D\rightarrow O}$) which was used to convert an external dermal exposure into an equivalent oral exposure (see Section 3.1.7.3). $EF_{D\rightarrow O}$ values were calculated by using the Mielke/Gundert-Remy model (see Section 3.1.7.3). Using the Fisher model instead of the Mielke/Gundert-Remy model would have resulted in 7.6-fold higher $EF_{D\rightarrow O}$ values. This difference arises from the fact that (i) the AUC for oral exposure is considerably higher in the Mielke/Gundert-Remy model than in the Fisher model and that (ii) the AUC for dermal exposure is only slightly higher in the Mielke/Gundert-Remy model than in the Fisher model:

$$(AUC_{dermal.F} / AUC_{dermal.MGR}) \times (AUC_{oral.MGR} / AUC_{oral.F}) = 0.94 \times 8.1 \approx 7.6$$

Uncertainty regarding the relative contribution of different sources to aggregate exposure

Estimates of aggregated exposure were presented graphically in Figure 16 (Section 5.1.2). The numerical results are shown in Table 57.

Table 57: Evaluation of uncertainties affecting assessment of aggregate equivalent oral exposure for females aged 18-45. Based on estimates for individual sources of exposure from Table 24 in the Section 5.1.1).

AGGREGATE EXPOSURES:		
Average Dietary + Average Non-Dietary		
Estimate with Mielke PBPK model (µg/kg bw per day)	0.22	
- range based on uncertainties for each route of exposure (µg/kg bw per day)		0.068 - 0.99
Estimate with Mielke model + model uncertainty factor (µg/kg bw per day)	0.76	
- range based on uncertainties for each route of exposure (µg/kg bw per day)		0.08 - 6.4
High Dietary + Average Non-Dietary		
Estimate with Mielke PBPK model (µg/kg bw per day)	0.47	
- range based on uncertainties for each route of exposure (µg/kg bw per day)		0.20 - 1.3
Estimate with Mielke model + model uncertainty factor (µg/kg bw per day)	1.0	
- range based on uncertainties for each route of exposure (µg/kg bw per day)		0.21 - 6.7
Average Dietary + High Non-Dietary		
Estimate with Mielke PBPK model (µg/kg bw per day)	0.81	
- range based on uncertainties for each route of exposure (µg/kg bw per day)		0.093 - 6.9
Estimate with Mielke model + model uncertainty factor (µg/kg bw per day)	5.3	
- range based on uncertainties for each route of exposure (µg/kg bw per day)		0.27 - 51
High Dietary + High Non-Dietary		
Estimate with Mielke PBPK model (µg/kg bw per day)	1.1	
- range based on uncertainties for each route of exposure (µg/kg bw per day)		0.22 - 7.2
Estimate with Mielke model + model uncertainty factor (µg/kg bw per day)	5.5	
- range based on uncertainties for each route of exposure (µg/kg bw per day)		0.40 - 52

The importance of the uncertainty regarding the exposure from thermal paper and the choice of the PBPK model is apparent from the results and discussion in Section 5.1.2. This is further emphasised when the contributions of the main sources of exposure are expressed as a percentage of the aggregate, shown in Figure 20. Average exposures were expressed as a percentage of the aggregate of averages (Aggregate 1), and high exposures as a percentage of the aggregate of high exposures (Aggregate 4). Upper bound estimates were calculated by substituting the upper bound for the source of interest into both the numerator and denominator when calculating its percentage contribution. Figure 20 shows that the contribution from cosmetics tends to be small either with or without the factor of about 7.6 for model uncertainty, whereas this factor is critical in determining the relative contributions of the dietary exposure and thermal paper. The dietary route is important if the Mielke/Gundert-Remy model is more realistic, but less important if the Fisher model is more realistic. If the high estimates for thermal paper prove realistic, however, this source will then dominate under either model.

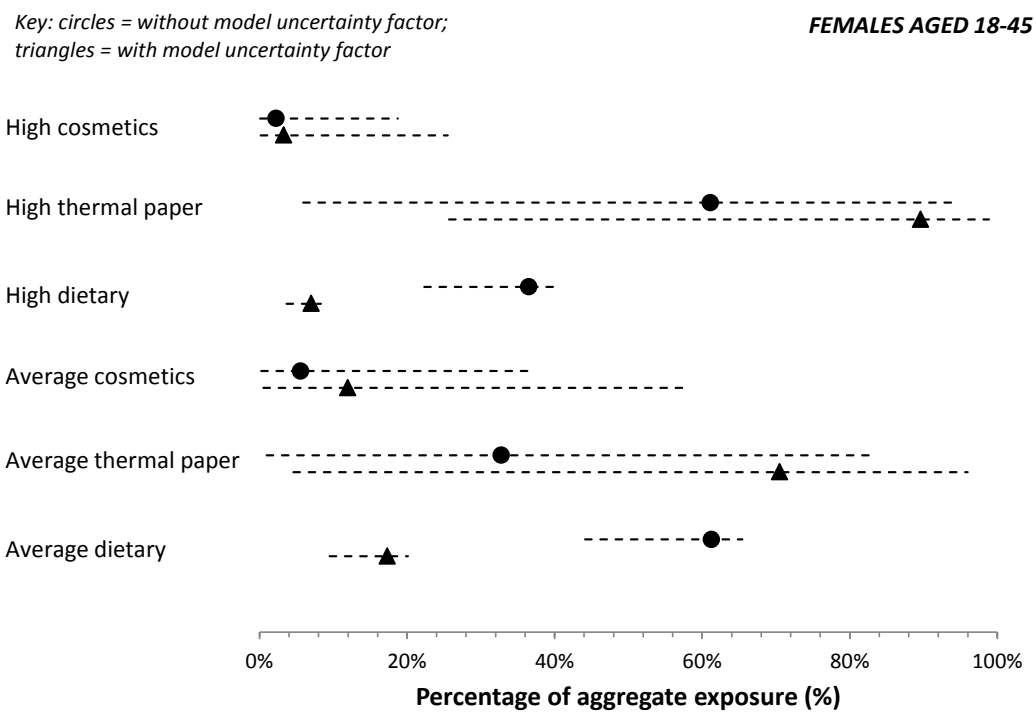

Figure 20: Relative contribution of the main sources of exposure to BPA for females aged 18-45, expressed as a percentage of aggregate equivalent oral exposure. Average exposures were expressed as a percentage of the aggregate of averages (Aggregate 1), with and without inclusion of the PBPK model uncertainty factor. High exposures were expressed as a percentage of the aggregate of high exposures (Aggregate 4). Dashed lines show approximate uncertainty ranges for each estimate.

Additional uncertainties affecting the assessment

Section 5.1.2 brings together qualitative and quantitative evaluations of uncertainty in all parts of the aggregate exposure assessment, and quantitatively examines their impact on risk characterisation (a form of sensitivity analysis). Uncertainties in the hazard assessment were taken into account quantitatively in deriving the t-TDI. However, it is also important to consider, at least qualitatively, the potential impact of other uncertainties that have not been included in the quantitative evaluations (EFSA, 2006, 2009). Additional uncertainties identified by the CEF Panel in this case are summarised and evaluated in Table 58.

Overall, the CEF Panel concludes that these additional uncertainties are smaller than those examined in the quantitative uncertainty analysis and are not sufficiently large to alter the main conclusion, which is that most uncertainty is associated with assessing exposure from thermal paper.

Table 58: Qualitative evaluation of additional uncertainties affecting the assessment.

Source of uncertainty	Evaluation of impact
Uncertainty within the Fisher PBPK model (separate from the difference between it and the Mielke/Gundert-Remy model)	May cause over or underestimation of the AUC by a factor of 2. Limited overall impact because it would affect the exposure and hazard assessments in opposite ways and hence tend to cancel out.
Uncertainty in extrapolating the Mielke/Gundert-Remy model, calibrated for adult males, to other age groups	May cause over or underestimation of the AUC by a factor of 2. This will have a minor influence on the results, compared to the difference between the Fisher and Mielke/Gundert-Remy models (a factor

	of 7.6).
Human equivalent oral dose factor (HEDF) used in setting the t-TDI.	Assessed in Section 3.1.6.1 as –/++ for oral studies with adult mice and in Appendix D II as +/- for all other studies. This is not expected to cause large uncertainty in the human toxicity assessment (Appendix D).
Dependencies between the uncertainties analysed quantitatively	Difficult to assess qualitatively, but unlikely to change the conclusion that uncertainty in exposure and risk characterisation is dominated by the dermal route.
The inhalation and dust absorption fractions were both assumed to be 1.	The true fractions will be lower, by an uncertain amount. However this will have little impact because the contributions of these sources are very minor.
Absorption fraction of 1 assumed for dietary exposure.	1 is an upper bound. The true value is expected to be between 0.8 and 1
Using full range of uncertainty scale intervals when converting to numerical ranges	Will overstate assessors' judgement of uncertainty to varying degrees, but within the same interval on the uncertainty scale.
Assessment of aggregate exposure by examining different combinations of average and high exposures.	Combining average and high exposures is unlikely to cause underestimation of the 95th percentile overall, since using only high estimates causes little increase in the total.
Evaluation of uncertainty of aggregate exposure by combining upper bounds of all sources of exposure to give aggregate upper bound, and lower bounds of all sources to give aggregate lower bound.	Will over-estimate the uncertainty of the aggregate exposure estimates, possibly by a substantial amount. This will exaggerate the degree by which the upper bounds exceed the t-TDI. However, this will not change the CEF Panel's main conclusions regarding the uncertainty of the risk characterisation, since the central estimates with and without model uncertainty (excluding other uncertainties) already span the t-TDI for Aggregate 3 (Figure 16), and most of the overall uncertainty derives from only two sources (estimation of thermal paper exposure and PBPK model uncertainty).
The evaluations used for some uncertainties were considered in less detail than others.	The effect of this is likely to be small compared to the large uncertainties in estimating thermal paper exposure. The uncertainty ranges for average exposures may be somewhat over-estimated, since some were assumed to be similar to those for high exposure, which would generally be more uncertain.
Exposure from inhalation is excluded from the estimates of aggregate exposure.	Average and high exposure from inhalation are both negligible compared to other sources, even when uncertainties affecting that route (- -/+) are taken into account.

(iv) Uncertainty analysis of hazard characterisation for the mammary gland, and the reproductive, neurobehavioural, immune and metabolic systems

For the reasons outlined in section 3.2.6, the CEF Panel used the HED of the BMDL for increased relative kidney weight in mice as the reference point for establishing a TDI. In setting the TDI, the CEF Panel assessed whether an extra factor (besides the uncertainty factors to account for inter- and intra-species differences) should be included to account for the uncertainty in the database concerning the mammary gland, and the reproductive, neurobehavioural, immune and metabolic systems. According to the outcome of the WoE evaluation, an association between BPA and these hazards was considered "as likely as not"– with the exception of proliferative changes in mammary gland and immunotoxicity, which were assessed respectively as "likely" or "-as likely as not- to likely". The

CEF Panel considered that the uncertainty about these effects contributes to uncertainty around in the hazard characterisation for BPA. For each effect a specific set of endpoints and criteria were used for inclusion of animal studies in the uncertainty analysis which were as follows:

- Proliferative changes in the mammary gland: all studies – both before and after 2010, addressing effects on ductal hyperplasia (including terminal end buds and ductal area), intraductal hyperplasia and epithelial cell proliferation.
- Reproductive and developmental toxicity: all publications (new and pre-2010 studies) addressing effects on endometrial hyperplasia, ovarian cysts and anogenital distance (AGD) at levels below the HED of 3.6 mg/kg bw per day. These endpoints were selected because other evaluations regarded these as critical endpoints.
- Neurobehavioural effects: all studies published after 2010 which contribute more than negligibly to the WoE.
- Immunotoxicity: all animal studies published after 2010.
- Metabolic effects: all publications addressing effects on glucose and insulin published after 2010, for which parameters there were clearly indications of effects in in vitro studies.

The WoE evaluation (Appendix C) allowed an estimation of the overall likelihood that BPA is hazardous with respect to certain toxicological endpoints in animal studies, considering the findings for all of the dose levels tested (with the exception of reproductive effects for which a cut-off dose level was taken as a study inclusion criterion). However, to assess whether an extra UF (and its magnitude) in the health-based guidance value would be appropriate to account for uncertainties in the database, the following were considered: (i) the likelihood that BPA exposure is associated with a certain outcome in animals at particular dose intervals (and especially at doses below the HED for the increase in relative mean kidney weight), (ii) the relevance of this effect to humans and (iii) its adversity in humans, if it occurs.

The CEF Panel approached the evaluation of uncertainty in three stages. In the first stage, it carried out the following steps for each of the selected types of effect in turn, to assess the likelihood that BPA might affect a certain endpoint in animals at each dose interval.

1. The experts reviewed the studies previously considered in the WoE assessment (see bulleted list above).
2. The experts extracted key information from each study and collated this in a graphical format (Section 4.3.2). The graphs summarise, for each study, the life stage of the animals at treatment onset, duration of treatment and sampling time for measurements, the doses tested, whether there was a statistically significant effect at any dose. They also show the number of strengths and weaknesses of the study, as assessed in the WoE assessment but excluding any strengths or weaknesses that did not apply to the effect of interest. In addition, they show the CEF Panel's evaluation of the reliability of the study and its relevance to the effect of interest, taking into account all the preceding information. When assessing reliability and relevance, the strengths and weaknesses were not weighted equally but according to the CEF Panel's evaluation of their relative importance.
3. In order to allow studies for different species using different routes of exposure to be plotted on the same dose scale scale, as well as on the same scale as the reference dose for the increase in relative kidney weight – all doses were converted to HED.
4. An expert elicitation procedure[21] was conducted to ensure that all the experts were familiar with making probabilistic judgements before they were engaged in assessing likelihoods for the endpoint of interest. Experts used a defined scale of likelihood categories (Table 59), based on a similar table used by the Intergovernmental Panel on Climate Change (Mastrandrea et al, 2010). Experts were offered the option to use more than one category if they wished to

[21] A simplified expert elicitation process was performed, based on the procedure described in the EFSA (2014) guidance document.

express a broader range of likelihoods, or to respond with a question mark ('?') if they felt unable to give any estimate.

Table 59: Terms and abbreviations used to express likelihood in the uncertainty evaluation for hazard characterisation (from Mastrandrea et al. 2010).

Virtually certain (VC)	99 - 100% probability
Very likely (VL)	90 - 100% probability
Likely (L)	66 - 100% probability
As likely as not (ALAN)	33 to 66% probability
Unlikely (U)	0 - 33% probability
Very unlikely (VU)	0 - 10% probability
Exceptionally unlikely (EU)	0 - 1% probability

The experts were then asked to review the graphical summary of studies (e.g. Figure 10) and assess the likelihood that BPA has the inherent ability to cause effects in each logarithmic interval of the dose scale (expressed as HED), which is indicated at the bottom of the graph, for one or more combinations of the animal species tested, exposure period and measurement time. For neurobehavioural endpoints, the experts considered it necessary to assess likelihoods separately for males and females, otherwise both sexes were considered together. The participants did not assume that likelihoods necessarily increase with increasing dose, but rather assessed the likelihood in each dose interval based on the evidence shown for that interval, taking account also of evidence in the adjacent dose intervals if they considered that appropriate. After discussion of the methodology with the CEF Panel, it was decided to only assess likelihoods for dose intervals within the range of the studies considered.

The assessment was conducted in three rounds. In the first round, each WG member made his assessment of the likelihoods. These were collectively displayed in a table, and discussed, focussing mainly on dose intervals where the differences between estimates were largest, and the reasoning for the estimates. In a second round, members were asked to review their individual assessments of the likelihoods in the light of the discussion. The second round estimates were again collected and the highest and lowest estimates in each dose interval were combined to form a provisional range, expressing the variation of estimates within the group. In a third round, these ranges were discussed, and adjusted if appropriate to represent the range of opinion in the group. The final ranges were added to the graph for each endpoint, where they are shown below the dose scale on the horizontal axis (e.g. Figure 10).

The steps described above were carried our separately for all of the 5 effects being considered.

In the next stage of the uncertainty evaluation, the experts met to assess the relevance of each effect to humans, and its adversity in humans, if it occurs. This stage was carried out for all 5 effects together. For each effect, a list of evidence and reasoning relevant to assessing its human relevance, and a second list for adversity: evidence for and against, and sources of uncertainty, had been prepared in advance and discussed and amended, if needed, at the meeting. Participants took note of the EFSA Scientific Committee's definition[22] of biological relevance (EFSA, 2011). The experts then made judgements about the likelihood of relevance and adversity for each effect using the same scale of likelihood terms as before (Table 59). The likelihoods were conditional, i.e. the likelihood of each effect being relevant in humans if it occurred in animals, and the likelihood of each effect being adverse in humans, if it occurred in humans. The experts expressed their judgements. The judgements were elicited in two rounds. In the first round, individuals judgements were collected and displayed on

[22]The Scientific Committee definition is: 'A biologically relevant effect can be defined as an effect considered by expert judgement as important and meaningful for human, animal, plant or environmental health. It therefore implies a change that may alter how decisions for a specific problem are taken.' It was noted that 'important and meaningful' could considered in the judgements made in this Opinion about adversity, while the judgements about human relevance refer to occurrence in humans, rather than importance.

screen for discussion by the group, focussing especially on the judgements where the likelihoods differed most between experts. In the second round, the experts again made individual judgements, which were collected and a summary was produced, in which the range of individual likelihoods was assigned to each judgement. The results of all these assessments are presented in Tables 60-75.
The final stage of the uncertainty evaluation was conducted in the same meeting as the preceding stage. The experts were asked to make judgements about the overall likelihood, in each HED dose interval, that BPA has the inherent ability to cause one or more type of effect in animals and that it is relevant and adverse in humans, based on the group summaries of the judgements that had been made in the earlier stages of the assessment (summarised in Table 18). The overall assessment was considered by, first, considering how the likelihoods of causation, human relevance and adversity combine for each effect separately and, second, combining likelihood across effects in each dose interval. The judgements were then summarised into a range of likelihoods for each dose interval, spanning the range of those expressed by individual experts. The experts then discussed which dose levels would need to be covered by an uncertainty factor, taking account of the group's conclusions regarding the likelihood of BPA having the inherent ability to cause human-relevant adverse effects in each dose interval. This led to further discussion, in which account was taken of the range of individual likelihood judgements in each dose interval and their associated probabilities (based on table 59). A final vote was then taken, which showed unanimous agreement on the final conclusion (see Section 4.3.2).

INDIVIDUAL and GROUP ASSESSMENT of the LIKELIHOODS FOR DIFFERENT EFFECTS IN ANIMALS

Reproductive effects

The precise question to consider for each dose interval is as follows:
"What is the likelihood that BPA has the capability to cause reproductive effects (of one or more of the types listed in the summary graph) in this dose interval, for one or more combinations of the animal species tested, exposure period and measurement time. In other words, if large, well-conducted experiments were done for the same species with a range of combinations of exposure period and time, what is the likelihood that one or more of the types of reproductive effect listed in the summary graph would be found in this dose interval?"

Table 60: First round estimates of the likelihood that BPA has the capability to cause reproductive effects (of one or more of the types listed in the summary graphic) in each dose interval, for one or more combinations of the animal species tested, exposure period and measurement time.

Dose interval (µg BPA/kg bw per day)	**10^{-2}-10^{-1}**	**10^{-1}-10^{0}**	**10^{0}-10^{1}**	**10^{1}-10^{2}**	**10^{2}-10^{3}**	**10^{3}-10^{4}**	**10^{4}-10^{5}**	**10^{5}-10^{6}**
Expert 1	VU	VU	VU	ALAN	ALAN	L	L	L
Expert 2	VU	VU	VU	U	U	U	U	L
Expert 3	U	U	U	ALAN	ALAN	ALAN	ALAN	L
Expert 4	U	VU	VU	ALAN	ALAN	ALAN	ALAN	L
Expert 5	U	VU	VU	U	U to ALAN	ALAN	ALAN	ALAN
Expert 6	U	U	U	U	ALAN	ALAN	ALAN	L
Expert 7	VU	VU	VU	VU	ALAN	ALAN	L	VL
Expert 8	U	U	U	ALAN	ALAN	ALAN	ALAN	L
Expert 9	VU	VU	VU	VU	VU	VU	VU	VL

Table 61: Second round estimates and group assessment (bottom two rows) of the likelihood that BPA has the capability to cause reproductive effects (of one or more of the types listed in

the summary graphic) in each dose interval, for one or more combinations of the animal species tested, exposure period and measurement time.

Dose interval (µg BPA/kg bw per day)	**10^{-2}-10^{-1}**	**10^{-1}-10^{0}**	**10^{0}-10^{1}**	**10^{1}-10^{2}**	**10^{2}-10^{3}**	**10^{3}-10^{4}**	**10^{4}-10^{5}**	**10^{5}-10^{6}**
Expert 1	VU	VU	VU	ALAN	ALAN	ALAN	L	L
Expert 2	VU	VU	VU	U	U	U	U	L
Expert 3	U	U	U	ALAN	ALAN	ALAN	ALAN	L
Expert 4	VU	VU	VU	U	ALAN	ALAN	ALAN	L
Expert 5	U	VU	VU	U	U to ALAN	ALAN	ALAN to U	L
Expert 6	U	U	U	U	ALAN	ALAN	ALAN	L
Expert 7	VU	VU	VU	VU	U to ALAN	ALAN	L	VL
Expert 8	U	U	U	ALAN	ALAN	ALAN	ALAN	L
Expert 9	VU	VU	VU	VU	VU	VU	VU	VL
Group evaluation	**VU/ U**	**VU/U**	**VU/U**	**VU/ALAN**	**VU/ALAN**	**VU/ALAN**	**VU/L**	**L to VL**

Neurobehavioural effects

The precise question to consider for each dose interval is as follows:
"What is the likelihood that BPA causes neurobehavioural effects (of one or more of the types listed in the summary graph) in this dose interval, for one or more combinations of animal species that were tested, exposure period and measurement time?. In other words, if large, well-conducted experiments were done with a wide range of combinations of species, exposure periods and time, what is the likelihood that one or more of the types of neurobehavioural effect listed in the summary graph would be found in this dose interval?"

Table 62: First round estimates of the likelihood that BPA has the capability to cause neurobehavioural effects (of one or more of the types listed in the summary graphic) in each dose interval, for one or more combinations of animal species, exposure period and measurement time in males.

Dose interval (µg BPA/kg bw per day)	**10^{-3}-10^{-2}**	**10^{-2}-10^{-1}**	**10^{-1}-10^{0}**	**10^{0}-10^{1}**	**10^{1}-10^{2}**	**10^{2}-10^{3}**	**10^{3}-10^{4}**	**10^{4}-10^{5}**
Expert 1	U?	U?	U	ALAN	ALAN	ALAN	L?	VL
Expert 2	U?	U?	ALAN	ALAN	L	L	L?	VL?
Expert 3	VU	VU	VU	U	U	U	U-ALAN	
Expert 4	EU	EU	U	ALAN	ALAN	ALAN	L	
Expert 5	VU	U	U	ALAN	ALAN-L	ALAN-L	ALAN-L	
Expert 6	VU	U	ALAN	ALAN	L	L	L	VL?
Expert 7	U	ALAN	ALAN	ALAN	ALAN	ALAN	ALAN-L	U?

Table 63: Second round estimates and group assessment (bottom two rows) of the likelihood that BPA has the capability to cause neurobehavioural effects (of one or more of the types listed in the summary graphic) in each dose interval, for one or more combinations of animal species, exposure period and measurement time in males.

Dose interval (µg BPA/kg bw	**10^{-3}-10^{-2}**	**10^{-2}-10^{-1}**	**10^{-1}-10^{0}**	**10^{0}-10^{1}**	**10^{1}-10^{2}**	**10^{2}-10^{3}**	**10^{3}-10^{4}**	**10^{4}-10^{5}**

per day)								
Expert 1	VU	VU	U	ALAN	ALAN	ALAN	L	VL
Expert 2	VU	VU	ALAN	ALAN	L	L	L	VL
Expert 3	VU	VU	VU	U	U/ALAN	U/ALAN	ALAN	
Expert 4	VU	VU	U	ALAN	ALAN	ALAN	L	
Expert 5	VU	VU-	U	ALAN	ALAN-L	ALAN-L	ALAN-L	
Expert 6	VU	VU	U	ALAN	ALAN	ALAN	L	
Expert 7	U	U-ALAN	ALAN	ALAN	ALAN	ALAN	ALAN-L	U?
Group evaluation	**VU-U**	**VU-U**	**U-ALAN**	**ALAN**	**ALAN**	**ALAN**	**ALAN-L**	

Table 64: First round estimates of the likelihood that BPA has the capability to cause neurobehavioural effects (of one or more of the types listed in the summary graphic) in each dose interval, for one or more combinations of animal species, exposure period and measurement time in females.

Dose interval (µg BPA/kg bw per day)	**10^{-3}-10^{-2}**	**10^{-2}-10^{-1}**	**10^{-1}-10^{0}**	**10^{0}-10^{1}**	**10^{1}-10^{2}**	**10^{2}-10^{3}**	**10^{3}-10^{4}**	**10^{4}-10^{5}**
Expert 1	U?	U?	U	ALAN	ALAN	ALAN	ALAN	VL
Expert 2	U?	U?	U	U	U	ALAN	L	VL?
Expert 3	VU	VU	VU	VU	U	U	U	
Expert 4	EU	EU	U	U	ALAN	ALAN	U	
Expert 5	VU	U	U	U	ALAN	ALAN	ALAN	
Expert 6	VU	U	ALAN	ALAN	L	L	L?	
Expert 7	U	U	ALAN	ALAN	ALAN	ALAN	U?	

Table 65: Second round estimates and group assessment (bottom two rows) of the likelihood that BPA has the capability to cause neurobehavioural effects (of one or more of the types listed in the summary graphic) in each dose interval, for one or more combinations of animal species, exposure period and measurement time in females.

Dose interval (µg BPA/kg bw per day)	**10^{-3}-10^{-2}**	**10^{-2}-10^{-1}**	**10^{-1}-10^{0}**	**10^{0}-10^{1}**	**10^{1}-10^{2}**	**10^{2}-10^{3}**	**10^{3}-10^{4}**	**10^{4}-10^{5}**
Expert 1	VU	VU	U	ALAN	ALAN	ALAN	ALAN	VL
Expert 2	VU	VU	U	U	U	ALAN	L	VL
Expert 3	VU	VU	VU	VU	U	U	U	
Expert 4	VU	VU	U	U	ALAN	ALAN	U	
Expert 5	VU	VU	U	U	ALAN	ALAN	ALAN	
Expert 6	VU	VU	U	U	ALAN	ALAN	L?	
Expert 7	U	U	U-ALAN	ALAN	ALAN	ALAN	U?	
Group evaluation	**VU-U**	**VU-U**	**U**	**U**	**U-ALAN**	**ALAN**	**U-L**	

Immune effects

The precise question to consider for each dose interval is as follows:
"What is the likelihood that BPA has the capability to cause immune effects (of one or more of the types listed in the summary graph) in this dose interval, for one or more combinations of the animal species tested, exposure period and measurement time. In other words, if large, well-conducted experiments were done for the same species with a range of combinations of exposure period and time, what is the likelihood that one or more of the types of immune effect listed in the summary graph would be found in this dose interval?"

Table 66: First round estimates of the likelihood that BPA has the capability to cause immune effects (of one or more of the types listed in the summary graphic) in each dose interval, for one or more combinations of the animal species tested, exposure period and measurement time.

Dose interval (µg BPA/kg bw per day)	**10^{-2}-10^{-1}**	**10^{-1}-10^{0}**	**10^{0}-10^{1}**	**10^{1}-10^{2}**	**10^{2}-10^{3}**	**10^{3}-10^{4}**	**10^{4}-10^{5}**
Expert 1	VU	VU	ALAN	U	L	?	ALAN
Expert 2	VU	VU	ALAN	ALAN	L	?	ALAN
Expert 3	VU-to U	VU-to U	ALAN	ALAN to L	ALAN	ALAN	ALAN
Expert 4	U	U	ALAN to L	L	L	ALAN to L	ALAN to L
Expert 5	VU	VU	ALAN	ALAN	L	L	L
Expert 6	U	U	ALAN	ALAN	ALAN	?	ALAN to L
Expert 7	VU	VU	U to ALAN	U to ALAN	ALAN to L	?	ALAN
Expert 8	VU	VU	ALAN	ALAN to L	ALAN to L	?	ALAN to L
Expert 9	EU	EU	VU	VU	VU	?	U to ALAN
Expert 10	U	U	ALAN	ALAN to L	ALAN to L	?	?
Expert 11	U	U	ALAN	ALAN	ALAN	?	ALAN
Expert 12	ALAN	ALAN	ALAN	ALAN	ALAN	?	ALAN
Expert 13	VU	VU	ALAN	ALAN	L	?/L	L

Table 67: Second round estimates and group assessment (bottom two rows) of the likelihood that BPA has the capability to cause immune effects in each dose interval, for one or more combinations of the animal species tested, exposure period and measurement time.

Dose interval (µg BPA/kg bw per day)	**10^{-2}-10^{-1}**	**10^{-1}-10^{0}**	**10^{0}-10^{1}**	**10^{1}-10^{2}**	**10^{2}-10^{3}**	**10^{3}-10^{4}**	**10^{4}-10^{5}**
Expert 1	VU	VU	ALAN	U	L	?	ALAN
Expert 2	VU	VU	ALAN	ALAN	L	?	ALAN
Expert 3	VU to U	VU to U	ALAN	ALAN	ALAN to L	?	ALAN
Expert 4	VU to U	U	ALAN	L	L	ALAN to L	ALAN to L
Expert 5	VU	VU	ALAN	ALAN	L	L	L
Expert 6	VU	VU	ALAN	ALAN	ALAN	?	ALAN to L
Expert 7	VU	VU	U to ALAN	ALAN	ALAN to L	?	ALAN
Expert 8	VU	VU	ALAN	ALAN to L	ALAN to L	?	ALAN to L
Expert 9	VU	VU	U	U	U	?	U to ALAN
Expert 10	U	U	ALAN	ALAN to L	ALAN to L	ALAN to L	ALAN to L
Expert 11	U	U	ALAN	ALAN	ALAN	ALAN	ALAN
Expert 12	U	U	ALAN	ALAN	ALAN	?	ALAN
Expert 13	VU	VU	ALAN	ALAN	L	ALAN	ALAN
Group evaluation	**VU to U**	**VU to U**	**U to ALAN**	**U to L**	**U to L**	**U to L**	**U to L**

Metabolic effects

The precise question to consider for each dose interval is as follows:

"What is the likelihood that BPA has the capability to cause metabolic effects (of one or more of the types listed in the summary graph) in this dose interval, for one or more combinations of animal species, exposure period and measurement time. In other words, if large, well-conducted experiments were done for the same species with a range of combinations of exposure period and time, what is the likelihood that one or more of the types of metabolic effect listed in the summary graph would be found in this dose interval?"

Table 68: First round estimates of the likelihood that BPA has the capability to cause metabolic effects (of one or more of the types listed in the summary graphic) in each dose interval, for one or more combinations of the animal species tested, exposure period and measurement time.

Dose interval (µg BPA/kg bw per day)	**10^{-4}-10^{-3}**	**10^{-3}-10^{-2}**	**10^{-2}-10^{-1}**	**10^{-1}-10^{0}**	**10^{0}-10^{1}**	**10^{1}-10^{2}**	**10^{2}-10^{3}**	**10^{3}-10^{4}**	**10^{4}-10^{5}**	**10^{5}-10^{6}**
Expert 1	U	U	U	U	U	ALAN	ALAN	L	ALAN	U
Expert 2	VU	U	U	U	U	U	U	U	U	U
Expert 3	VU	VU	VU	VU	VU	VU	VU	VU	VU	VU
Expert 4	VU	U	U	U	U	U	U to ALAN	ALAN	ALAN	ALAN
Expert 5	U	U	U	ALAN	U	ALAN	ALAN	U to ALAN	L	ALAN
Expert 6	VU	VU	U	U	U	U	U	U	ALAN	ALAN
Expert 7	U	U	U	U	U	ALAN	ALAN	ALAN	L	L
Expert 8	U	U	U	U	U	U	U	U	U to ALAN	U to ALAN
Expert 9	VU to ALAN	VU to ALAN	VU to ALAN	VU to ALAN	U	VU to ALAN	VU to ALAN	VU to ALAN	VU to ALAN	VU to ALAN

Table 69: Second round estimates and group assessment (bottom two rows) of the likelihood that BPA has the capability to cause metabolic effects (of one or more of the types listed in the summary graphic) in each dose interval, for one or more combinations of the animal species tested, exposure period and measurement time.

Dose interval (µg BPA/kg bw per day)	**10^{-4}-10^{-3}**	**10^{-3}-10^{-2}**	**10^{-2}-10^{-1}**	**10^{-1}-10^{0}**	**10^{0}-10^{1}**	**10^{1}-10^{2}**	**10^{2}-10^{3}**	**10^{3}-10^{4}**	**10^{4}-10^{5}**	**10^{5}-10^{6}**
Expert 1	U	U	U	U	U	U TO ALAN	U TO ALAN	U TO ALAN	U TO ALAN	U
Expert 2	VU	U	U	U	U	U	U	U	U	U
Expert 3	VU	VU	VU	VU	VU	VU	VU	VU	U	U
Expert 4	VU	U	U	U	U	U	U to ALAN	ALAN	ALAN	ALAN
Expert 5	U	U	U	ALAN	U	ALAN	ALAN	U to ALAN	ALAN TO L	ALAN
Expert 6	VU	U	U	U	U	U	U	U	ALAN	ALAN
Expert 7	U	U	U	U	U	U TO ALAN	U TO ALAN	U TO ALAN	L	L
Expert 8	U	U	U	U	U	U	U	U	U to ALAN	U to ALAN
Expert 9	VU to ALAN	VU to ALAN	VU to ALAN	VU to ALAN	U	VU to ALAN	VU to ALAN	VU to ALAN	VU to ALAN	VU to ALAN
Group evaluation	**VU to ALAN**	**VU to ALAN**	**VU to ALAN**	**VU to ALAN**	**VU to U**	**VU to ALAN**	**VU to ALAN**	**VU to ALAN**	**VU to L**	**VU to L**

Proliferative changes in mammary gland

The precise question to consider for each dose interval is as follows:

"What is the likelihood that BPA has the capability to cause proliferative changes in mammary gland (of one or more of the types listed in the summary graph) in this dose interval, for one or more combinations of animal species, exposure period and measurement time. In other words, if large, well-conducted experiments were done for the same species with a range of combinations of exposure period and time, what is the likelihood that one or more of the types of proliferative changes in mammary gland listed in the summary graph would be found in this dose interval?"

Table 70: First round estimates of the likelihood that BPA has the capability to cause proliferative changes in mammary gland (of one or more of the types listed in the summary graphic) in each dose interval, for one or more combinations of the animal species tested, exposure period and measurement time.

Dose interval (µg BPA/kg bw per day)	**$<10^{-1}$**	**10^{-1}-10^{0}**	**10^{0}-10^{1}**	**10^{1}-10^{2}**	**10^{2}-10^{3}**	**10^{3}-10^{4}**	**10^{4}-10^{5}**	**10^{5}-10^{6}**
Expert 1	U	U	U	U	ALAN	ALAN	L	L
Expert 2	VU	U	U	U	ALAN	ALAN	L	L
Expert 3	VU	U	ALAN	ALAN	L	L	L	L
Expert 4	VU	U	ALAN	ALAN	L	L	L	L
Expert 5	VU	U	ALAN	ALAN	L	L	U	U
Expert 6	VU	U	U	U	ALAN	ALAN	ALAN	ALAN

Table 71: Second round estimates and group assessment (bottom two rows) of the likelihood that BPA has the capability to cause proliferative changes in mammary gland (of one or more of the types listed in the summary graphic) in each dose interval, for one or more combinations of the animal species tested, exposure period and measurement time.

Dose interval (µg	**$<10^{-1}$**	**10^{-1}-**	**10^{0}-10^{1}**	**10^{1}-10^{2}**	**10^{2}-10^{3}**	**10^{3}-10^{4}**	**10^{4}-10^{5}**	**10^{5}-10^{6}**

BPA/kg bw per day)		**10^0**						
Expert 1	VU	VU	U	U	ALAN	ALAN	ALAN	ALAN
Expert 2	VU	VU	U	U	ALAN	ALAN	ALAN	ALAN
Expert 3	VU	U	ALAN	ALAN	L	L	L	L
Expert 4	VU	U	ALAN	ALAN	L	L	ALAN	ALAN
Expert 5	VU	U	ALAN	ALAN	L	L	ALAN	ALAN
Expert 6	VU	U	U	U	ALAN	ALAN	ALAN	ALAN
Overall group assessment	**VU**	**VU**	**U/ALAN**	**U/ALAN**	**ALAN/L**	**ALAN/L**	**ALAN/L**	**ALAN/L**

Table 72: Summary of second round group assessments of the likelihood that BPA has the capability to cause each of the effects considered in the uncertainty analysis, in each dose interval, for one or more combinations of the animal species tested, exposure period and measurement (compiled from the bottom rows of Tables 60, 62, 64, 66, 68 and 70

	BPA DOSE INTERVALS Human equivalent dose (HED) (µg BPA/kg bw per day)									
ENDPOINT	**10^{-4}-10^{-3}**	**10^{-3}-10^{-2}**	**10^{-2}-10^{-1}**	**10^{-1}-10^{0}**	**10^{0}-10^{1}**	**10^{1}-10^{2}**	**10^{2}-10^{3}**	**10^{3}-10^{4}**	**10^{4}-10^{5}**	**10^{5}-10^{6}**
Mammary proliferation	VU	VU	VU	VU	U - ALAN	U - ALAN	ALAN - L	ALAN - L	ALAN - L	ALAN - L
Effects on reproductive system	-	-	VU - U	VU - U	VU - U	VU - ALAN	VU - ALAN	VU - ALAN	VU - L	L - VL
Metabolic effects	VU - ALAN	VU - ALAN	VU - ALAN	VU - ALAN	VU - U	VU - ALAN	VU - ALAN	VU - ALAN	VU - L	VU - L
Effects on immune system	-	-	VU - U	VU - U	U - ALAN	U - L	U - L	U - L	U - L	-
Neurobehavioural effects in males	-	VU - U	VU - U	U - ALAN	ALAN	ALAN	ALAN	ALAN - L	-	-
Neurobehavioural effects in females	-	VU-U	VU-U	U	U	U-ALAN	ALAN	U-L	-	-

Relevance of effects to humans

The precise question considered for each effect is as follows:

"What is the likelihood of this effect being relevant in humans, if it occurred in animals?"

Table 73: First round estimates of the likelihood of each effect being relevant in humans, if it occurred in animals.

Expert	**Mammary proliferation**	**Effects on reproductive system**	**Metabolic effects**	**Effects on immune system**	**Neurobehavioural effects**
Expert 1	ALAN	ALAN	ALAN	U TO ALAN	U TO ALAN
Expert 2	ALAN TO L	ALAN	ALAN	ALAN	U
Expert 3	L	ALAN TO L	ALAN TO L	ALAN	ALAN
Expert 4	L	L	ALAN	ALAN TO L	ALAN
Expert 5	L	L	ALAN TO L	L	U TO ALAN
Expert 6	ALAN	ALAN TO L	ALAN TO L	ALAN TO L	ALAN TO L
Expert 7	VL	VL	VL	VL	ALAN

Expert 8	L	L	L	L	ALAN
Expert 9	L	L	ALAN	L	ALAN
Expert 10	L	ALAN	ALAN	U TO ALAN	U TO ALAN

Table 74: Second round estimates of the likelihood of each effect being relevant in humans, if it occurred in animals. The overall range of the individual estimates is shown in the bottom row.

Expert	Mammary proliferation	Effects on reproductive system	Metabolic effects	Effects on immune system	Neurobehavioural effects
Expert 1	ALAN TO L	ALAN TO L	ALAN TO L	U TO ALAN	U TO ALAN
Expert 2	ALAN TO L	ALAN	ALAN	ALAN	U
Expert 3	L	ALAN TO L	ALAN TO L	ALAN TO L	ALAN TO L
Expert 4	L	L	ALAN	ALAN TO L	ALAN
Expert 5	L	L	ALAN TO L	L	U TO ALAN
Expert 6	ALAN TO L	ALAN TO L	ALAN TO L	ALAN TO L	ALAN TO L
Expert 7	L	L	L	ALAN TO L	ALAN
Expert 8	L	L	L	L	ALAN
Expert 9	L	L	ALAN	L	ALAN
Expert 10	L	ALAN	ALAN	U TO ALAN	U TO ALAN
Range	**ALAN - L**	**ALAN - L**	**ALAN - L**	**U - L**	**U - L**

Adversity of effects in humans

The precise question considered for each effect is as follows:

Table 75: "What is the likelihood of this effect being adverse in humans, if it occurred in humans?" First round estimates of the likelihood of each effect being adverse in humans, if it occurred in humans.

Expert	Mammary proliferation	Effects on reproductive system	Metabolic effects	Effects on immune system	Neurobehavioural effects
Expert 1	U TO ALAN	ALAN	ALAN	ALAN	U TO ALAN
Expert 2	L	ALAN	ALAN	ALAN	U TO ALAN
Expert 3	L	L	L	L	ALAN TO L
Expert 4	L	L	ALAN	ALAN TO L	ALAN
Expert 5	L	L	ALAN TO L	ALAN	ALAN
Expert 6	ALAN	L	ALAN	ALAN	ALAN
Expert 7	ALAN	L	U TO L	ALAN	U TO ALAN
Expert 8	L	L	L	L	ALAN
Expert 9	L	L	ALAN	ALAN TO L	?
Expert 10	VL	VL	ALAN TO L	ALAN TO L	ALAN

Table 76: Second round estimates of the likelihood of each effect being adverse in humans, if it occurred in humans. The overall range of the individual estimates is shown in the bottom row.

Expert	Mammary proliferation	Effects on reproductive system	Metabolic effects	Effects on immune system	Neurobehavioural effects

Expert 1	ALAN TO L	ALAN TO L	ALAN TO L	ALAN TO L	U TO ALAN
Expert 2	L	ALAN TO L	ALAN TO L	ALAN TO L	U TO ALAN
Expert 3	L	L	L	L	ALAN TO L
Expert 4	L	L	ALAN	ALAN TO L	ALAN
Expert 5	L	L	ALAN TO L	ALAN	ALAN
Expert 6	ALAN	L	ALAN	ALAN	ALAN
Expert 7	ALAN	L	U TO L	ALAN	U TO ALAN
Expert 8	L	L	L	L	ALAN
Expert 9	L	L	L	L	L
Expert 10	L	L	ALAN TO L	ALAN TO L	ALAN
Range	**ALAN TO L**	**ALAN TO L**	**U TO L**	**ALAN TO L**	**U TO L**

Appendix E. Report on BMD calculations on general toxicity and proliferative changes in the mammary gland

General introduction

In this Annex the results of the BMD modelling for "likely"effects (kidney and liver effects; proliferative changes in mammary gland) are presented. For the liver and kidney effects data as reported in two rodent multigeneration studies (Tyl et al., 2002 and Tyl et al., 2008) have been considered (Part A). For the evaluation of mammary duct hyperplasia, the data from the recent US FDA/NCTR study (2013) have been used (Part B).

For all the new dose-response modelling of the individual organ weight data (Tyl et al, 2008), the statistical package PROAST (version 40.7) has been used, obtained directly from RIVM, through personal contact. For the previous dose-response modelling of the histopathological data (liver hypertrophy and mammary duct hyperplasia), PROAST version 38.6 has been used. This version is no longer available at the website (www.proast.nl), but a more recent version (38.9) can be downloaded. Using the PROAST package, the 95 % lower confidence limit (one-sided) of the Benchmark dose (BMDL) was calculated. For each evaluation, depending on the type of data evaluated, the statistical models for continuous data (organ weights) or for quantal data (histopathology) were used.

All evaluations were carried out with the following setting:

- Benchmark dose-response (BMR, or CES = critical effect size) 10 % extra risk for all effects
- No restrictions for model parameters to limit e.g. steepness of the fitted dose-response curves.

For all evaluations a P-value for goodness of fit of 0.05 was used as criterion to decide on acceptability of modelling output.

PART A

Report on BMD calculation on general toxicity from Tyl et al. (2008)

In compliance with the Opinion of the EFSA Scientific Committee on the use of the Benchmark dose (BMD) approach in Risk Assessment (EFSA, 2009), the results obtained on general toxicity in the reproductive toxicity studies with BPA in rats (Tyl et al., 2002) and mice (Tyl et al., 2008) were submitted to statistical dose-response modelling. From these studies kidney weight accompanied by nephropathy (mice), liver weight (mice and rats) and histological changes in liver have been identified as critical effects (see Section 3.2.4). Given that a NOAEL of 5 mg/kg bw per day has been established from both the rat (Tyl et al., 2002) and the mouse study (Tyl et al., 2008), but that the HEDFs for the mouse are much lower than for rats, the focus of the BMD analysis was on the Tyl et al. (2008) study in mice.

The necessary data (see Table 77) were retrieved from the paper (Tyl et al., 2008), the supplementary file available through the respective journal's website and the full study report with individual data, which were kindly provided to EFSA shortly after the end of the public consultation (March 2014).

1.1 Experimental data

The BMD and BMDL were estimated using data collected by Tyl et al. (2008). For the body and organ weights individual data from the full study report (Tyl et al., 2008) were used. The data on the histological changes (centrilobular hepatocellular hypertrophy) were taken from the publication and the supplementary data (Tyl et. al., 2008). The information used is presented in Table 77 in a summarised form. Since left and right kidney weights are not independent variables, the kidney weights have been evaluated as mean kidney weight per animal (i.e. left + right kidney weight divided by 2). Relative kidney weights are the mean kidney weights divided by the body weight. Relative liver

weight is liver weight divided by body weight. A lack of dose-response relationship was observed for nephropathy in both sexes. Therefore no model was obtained with acceptable fit and no BMDL could be calculated for this effects.

Table 77: Critical general toxicological effects in mice in the adult F0 and F1 generations from Tyl et al. (2008).

Generation / Sex	Endpoint	BPA mg/kg bw per day						
		0	**0.003**	**0.03**	**0.3**	**5**	**50**	**600**
ORGAN WEIGHTS[&]								
F0-males	Number of animals	55	28	28	28	28	28	27
	Absolute liver weight (g)	2.123±0.204	2.168±0.271	2.180±0.297	2.215±0.218	2.246±0.223	2.226±0.243	2.498±0.324[@]
	Relative liver weight (% bw)	5.399±0.414	5.419±0.601	5.545±0.369	5.490±0.413	5.573±0.367	5.628±0.478	6.317±0.883
	Abs mean kidney weight (g)	0.386±0.041	0.385±0.051	0.383±0.045	0.397±0.041	0.408±0.064	0.417±0.043	0.464±0.110[@]
	Rel mean kidney weight (% bw)	0.983±0.097	0.961±0.112	0.979±0.119	0.985±0.100	1.008±0.113	1.057±0.113	1.171±0.110
F1-parental males#	Number of animals	54	27	27	28	28	27	27
	Absolute liver weight (g)	2.074±0.289*	2.121±0.204[@]	2.088±0.226	2.158±0.256	2.105±0.247	2.139±0.271[@]	2.428±0.449
	Relative liver weight (% bw)	5.311±0.432	5.289±0.277	5.249±0.435	5.467±0.509	5.450±0.375	5.551±0.387	6.480±1.037
	Abs mean kidney weight (g)	0.368±0.049	0.396±0.069	0.382±0.049	0.393±0.034	0.408±0.053	0.402±0.035	0.432±0.059
	Rel mean kidney weight (% bw)	0.947±0.105	0.988±0.0139	0.963±0.112	1.000±0.108	1.058±0.117	1.042±0.099	1.153±0.131
F0-females	Number of animals	56	28	27	27	27	28	28
	Absolute liver weight (g)	2.733±0.481	2.871±0.451	2.752±0.510	2.785±0.421	2.603±0.270	2.710±0.465	3.293±0.802
	Relative liver weight (% bw)	7.808±1.061	8.088±0.821	7.750±1.034	7.857±0.820	7.491±0.508	7.837±1.051	9.162±1.705
	Abs mean kidney weight (g)	0.307±0.045	0.311±0.039	0.320±0.038	0.319±0.035	0.323±0.028	0.321±0.043	0.350±0.044
	Rel mean kidney weight (% bw)	0.881±0.111	0.878±0.078	0.908±0.095	0.904±0.075	0.933±0.084	0.936±0.141	0.985±0.118
F1-parental females#	Number of animals	55	28	27	27	26	27	27
	Absolute liver weight (g)	2.939±0.0507	2.889±0.512	2.845±0.492	3.089±0.476	2.825±0.470	2.776±0.585	3.1065±0.711
	Relative liver weight (% bw)	7.951±0.823	7.690±0.922	7.815±0.917	8.078±0.871	7.756±0.832	7.549±0.985	8.727±1.543
	Abs mean kidney weight (g)	0.324±0.039	0.311±0.031	0.318±0.037	0.348±0.035	0.321±0.044	0.323±0.041	0.334±0.051
	Rel mean kidney weight (% bw)	0.883±0.096	0.834±0.077	0.882±0.114	0.915±0.076	0.885±0.098	0.886±0.075	0.949±0.111

Generation / Sex	Endpoint	BPA mg/kg bw per day						
		0	**0.003**	**0.03**	**0.3**	**5**	**50**	**600**
HISTOPATHOLOGICAL OBSERVATIONS[$]								
	Centrilobular hepatocyte hypertrophy							
F0-males		6/56 (10.7)	1/10 (10)	2/10 (20)	2/10 (20)	0/10 (0)	4/10 (40)	10/10 (100)
F1-males		7/55 (12.7)	0/10 (0)	0/10 (0)	4/10 (40)	2/10 (20)	1/10 (10)	4/10 (40)
F0-females		1/56 (1.8)	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)	1/10 (10)	6/10 (60)
F1-females		2/55 (3.6)	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)	3/11 (27.3)	7/10 (70)
	Nephropathy							
F0-males		12/56 (21.4)	0/10 (0.0)	3/10 (30.0)	2/10 (20.0)	2/10 (20.0)	1/10 (10.0)	4/10 (40.0)
F1-parental males		6/55 (10.9)	2/10 (20.0)	0/10 (0.0)	1/10 (10.0)	2/10 (20.0)	0/10 (0.0)	4/10 (40.0)
F1-retained males		8/50 (16.0)	1/10 (10)	0/10 (0.0)	0/10 (0.0)	2/10 (20.0)	0/10 (0.0)	3/10 (10.0)

& mean ± SD; # animals were randomly selected from the dose groups, but it is not clear if littermates could be present in each selected group. Brother-sister combinations for mating were not allowed. $ number observed/ number examined (percentage); @ N=28; * N=55.

1.2 Results

1.2.1 Kidney Data

BMD assessment for absolute mean kidney weight

Table 78 shows the BMD confidence intervals for the absolute mean kidney weights for the four subgroups male/female F0/F1, using generation and sex as a covariate. Figure 21 shows the associated data and the dose-response curves for the two models that described the data most adequately (i.e. Exponential and Hill model 3). The data for the F1 females contained insufficient information, which resulted in a very wide confidence interval around the BMD. Also for the other groups (F0 males/females and F1 males) rather wide confidence intervals around the BMD were obtained. Nevertheless, from this analysis the F0 males were found to be the most sensitive group, with a BMD of 22 (Exponential 3) or 22.4 (Hill 3) mg/kg bw per day, a BMDL of 3.42 mg/kg bw per day and a BMDU of 91.69 mg/kg bw per day (encompassing the results of both models).

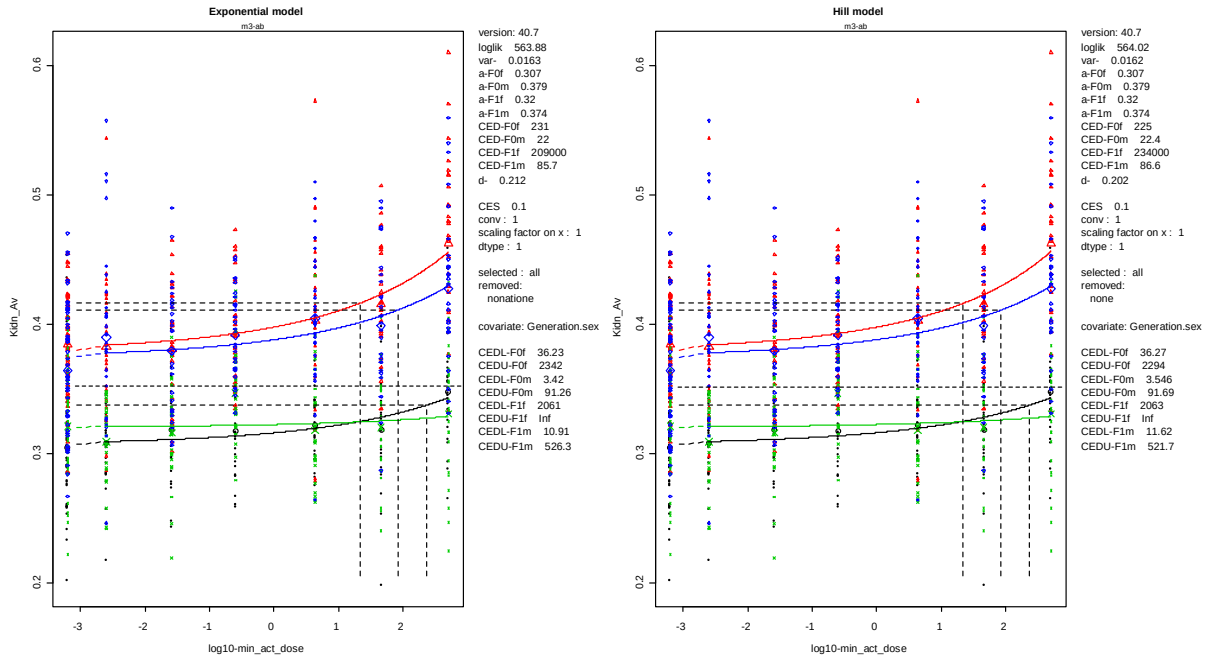

Figure 21: Dose-response modelling for absolute mean kidney weight. CED (critical effect dose) is the BMD for a 10% increase in absolute mean kidney weight. Green crosses: females F1; blue diamonds: males F1; black circles: females F0; red triangles: males F0. Horizontal axis: log dose; Vertical axis: absolute mean kidney weight (g).

Table 78: Summary of the results obtained for the models fitted considering generations F0 and F1 and sex for absolute mean kidney weight changes.

	Model used	Full	Exp 3	Hill 3
	N° of parameter	29	10	10
	LL*	576.65	563.88	564.02
F0-Female	BMD_{10}	NA	231	225
	$BMDL_{10}$	NA	36.23	36.27
	$BMDU_{10}$	NA	2342	2294
F0-Male	BMD_{10}	NA	22	22.4
	$BMDL_{10}$	NA	3.42	3.55
	$BMDU_{10}$	NA	91.26	91.69

F1-Female	BMD_{10}	NA	209000	234000
	$BMDL_{10}$	NA	2061	2063
	$BMDU_{10}$	NA	∞	∞
F1-Male	BMD_{10}	NA	85.7	86.6
	$BMDL_{10}$	NA	10.91	11.62
	$BMDU_{10}$	NA	526.3	521.7

* LL: Log-likelihood

BMD assessment for relative mean kidney weight

Table 79 shows the BMD confidence intervals for the relative mean kidney weights for the four subgroups male/female F0/F1, using generation and sex as a covariate. Figure 22 shows the associated data and the dose-response curves for the two models that described the data most adequately (i.e. Exponential and Hill model 3). In comparison with the analysis for absolute mean kidney weight, the data for the F1 females contained now sufficient information to estimate a BMD with a fairly narrow confidence interval. Also for the other groups (F0 males/females and F1 males) similar narrow confidence intervals around the BMD were obtained. By correcting the individual kidney weights for the individual body weights an additional source of uncertainty has been removed. From this analysis the F1 males were found to be the most sensitive group, with a BMD of 34.8 (Exponential 3) or 36.7 (Hill3) mg/kg bw per day, a BMDL of 8.96 mg/kg bw per day and a BMDU of 108.9 mg/kg bw per day (encompassing the results of both models).

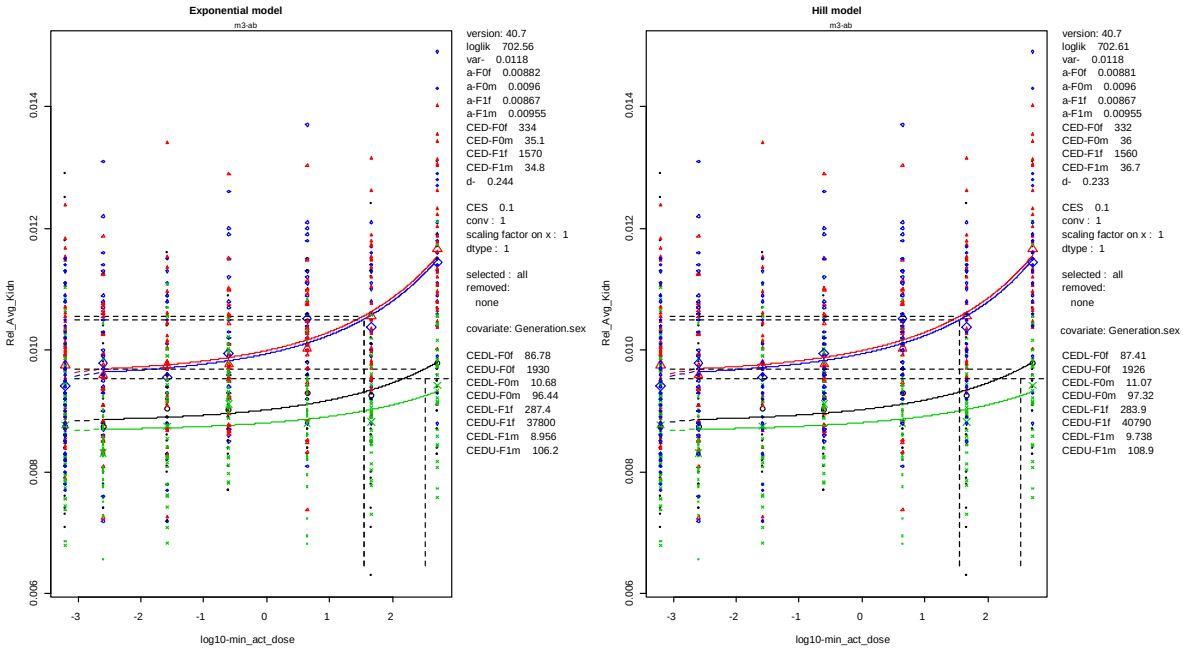

Figure 22: Dose-response modelling for relative mean kidney weight. CED (critical effect dose) is the BMD for a 10% increase in relative mean kidney weight. Green crosses: females F1; blue diamonds: males F1; black circles: females F0; red triangles: males F0. Horizontal axis: log dose; Vertical axis: Relative mean kidney weight.

Table 79: Summary of the results obtained for the models fitted considering generations F0 and F1 and gender for relative mean kidney weights changes.

	Model used	Full	Exp 3	Hill 3
	N° of parameter	29	10	10
	LL*	714.07	702.44	702.61
F0-Female	BMD_{10}	NA	334	332
	$BMDL_{10}$	NA	86.78	87.41
	$BMDU_{10}$	NA	1930	1926
	$BMD_{10}/BMDL_{10}$	NA	3.85	3.8
F0-Male	BMD_{10}	NA	35.1	36
	$BMDL_{10}$	NA	10.68	11.1
	$BMDU_{10}$	NA	96.44	97.3
	$BMD_{10}/BMDL_{10}$	NA	3.29	3.24
F1-Female	BMD_{10}	NA	1570	1560
	$BMDL_{10}$	NA	287.4	283.9
	$BMDU_{10}$	NA	37800	40790
	$BMD_{10}/BMDL_{10}$	NA	5.46	5.49
F1-Male	BMD_{10}	NA	34.8	36.7
	$BMDL_{10}$	NA	8.96	9.7
	$BMDU_{10}$	NA	106.2	108.9
	$BMD_{10}/BMDL_{10}$	NA	3.88	3.78

* LL: Log-likelihood

1.2.2 Liver Data

BMD assessment for absolute liver weight

Table 80 shows the BMD confidence intervals for the absolute liver weights for the four subgroups male/female F0/F1, using generation and sex as a covariate. Figure 23 shows the associated data and the dose-response curves for the two models that described the data most adequately (i.e. Exponential and Hill model 2). The data for the F1 females contained insufficient information, which resulted in a very wide confidence interval around the BMD. For the other groups (F0 males/females and F1 males) rather narrow confidence intervals around the BMD were obtained. From this analysis the F0 males were found to be the most sensitive group, with a BMD of 290.3 (Exponential 2) or 300.4 (Hill 2) mg/kg bw per day, a BMDL of 213.1 mg/kg bw per day and a BMDU of 456.8 mg/kg bw per day (encompassing the results of both models).

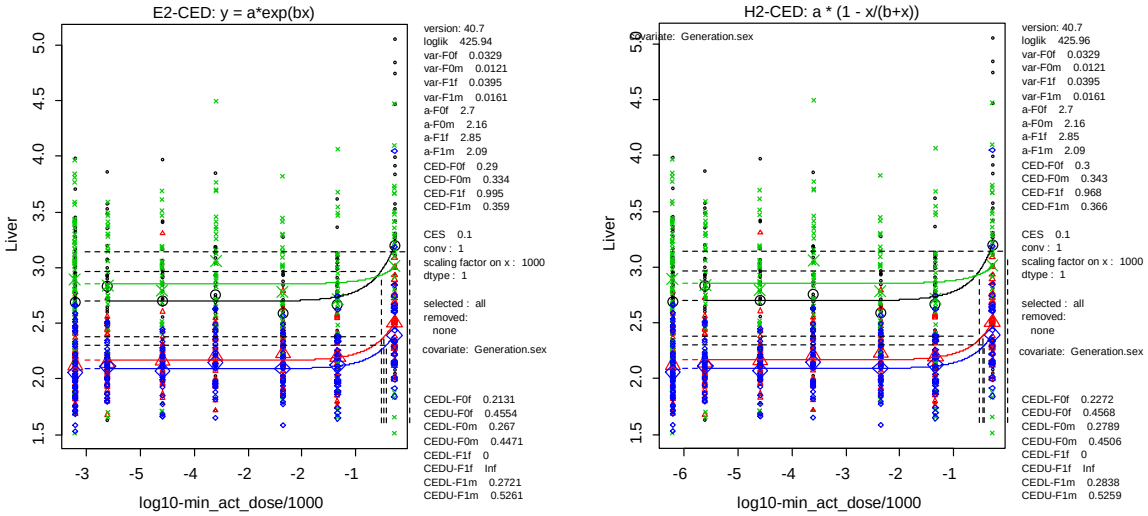

Figure 23: Dose-response modelling for absolute liver weight. CED (critical effect dose) is the BMD for a 10% increase in absolute liver weight. Green crosses: females F1; blue diamonds: males F1; black circles: females F0; red triangles: males F0. Horizontal axis: log dose/1000; Vertical axis: absolute liver weight (g). Panels E2 and H2 show the dose-response curves calculated with Exponential model 2 and Hill model 2, respectively.

Table 80: Summary of the results obtained for the models fitted considering generations F0 and F1 and gender for absolute liver weight changes.

	Model used	Full	Exp 2	Hill 2
	N° of parameter	32	12	12
	LL*	434.85	425.9	425.96
F0-Female	BMD_{10}	NA	290.3	300.4
	$BMDL_{10}$	NA	213.1	227.2
	$BMDU_{10}$	NA	455.4	456.8
	$BMD_{10}/BMDL_{10}$	NA	1.36	1.32
F0-Male	BMD_{10}	NA	334.3	343.1
	$BMDL_{10}$	NA	266.9	278.9
	$BMDU_{10}$	NA	447.1	450.6
	$BMD_{10}/BMDL_{10}$	NA	1.25	1.23
F1-Female	BMD_{10}	NA	995	967.9
	$BMDL_{10}$	NA	1.00E-15	1.00E-15
	$BMDU_{10}$	NA	∞	∞
	$BMD_{10}/BMDL_{10}$	NA	9.95E+17	9.679E+17
F1-Male	BMD_{10}	NA	358.7	366.3
	$BMDL_{10}$	NA	272.1	283.8
	$BMDU_{10}$	NA	526.1	525.9
	$BMD_{10}/BMDL_{10}$	NA	1.32	1.29

* LL: Log-likelihood

BMD assessment for relative liver weight

Table 81 shows the BMD confidence intervals for the relative liver weights for the four subgroups male/female F0/F1, using generation and sex as a covariate. Figures 24 shows the associated data and the dose-response curves for the two models that described the data most adquately (i.e. Exponential and Hill model 2). In comparison with the analysis for absolute liver weight, the data for the F1 females contained now sufficient information to estimate a BMD with a fairly narrow confidence interval. Also for the other groups (F0 males/ females and F1males) similar narrow confidence intervals around the BMD were obtained. Obviously, by correcting the individual liver weights for the individual body weights an additional source of uncertainty has been removed. From this analysis, the F1 males were found to be the most sensitive group, with a BMD of 266 (Exponential 2) or 278.9 (Hill 2) mg/kg bw per day, a BMDL of 229 mg/kg bw per day and a BMDU of 328.7 mg/kg bw per day (encompassing the results of both models).

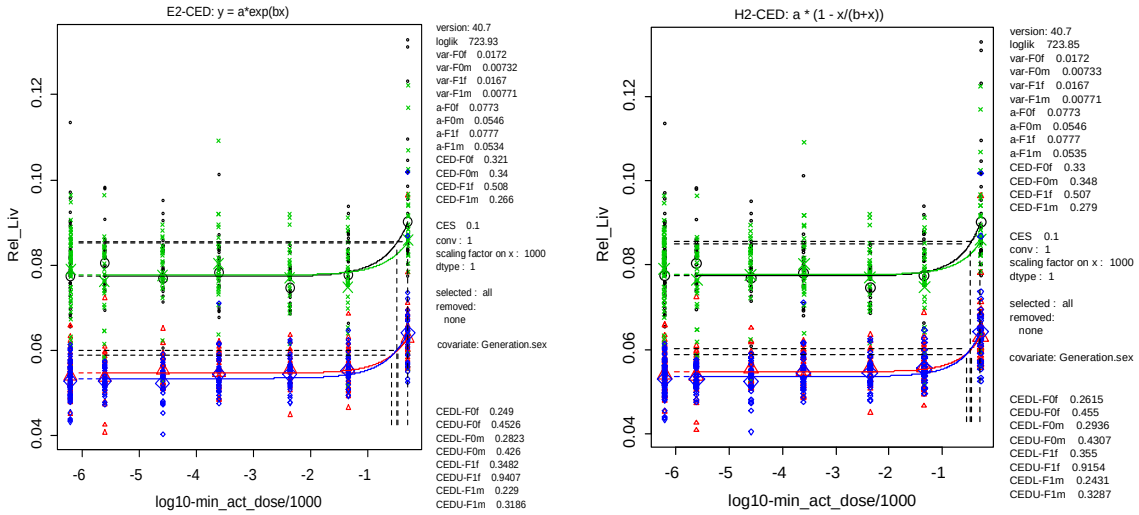

Figure 24: Dose-response modelling for relative liver weight changes. CED (critical effect dose) is the BMD for a 10% increase in relative liver weight. Green crosses: females F1; blue diamonds: males F1; black circles: females F0; red triangles: males F0. Horizontal axis: log dose; Vertical axis: relative liver weight. Panels E2 and H2 show the dose-response curves calculated with Exponential model 2 and Hill model 2, respectively.

Table 81: Summary of the results obtained for the models fitted considering generations F0 and F1 and gender for relative liver weight changes.

	Model used	Full	Exp 2	Hill 2
	N° of parameter	32	12	12
	LL*	735.14	723.93	723.85
F0-Female	BMD_{10}	NA	321.2	330.1
	$BMDL_{10}$	NA	249	261.5
	$BMDU_{10}$	NA	452.6	454.9
	$BMD_{10}/BMDL_{10}$	NA	1.29	1.26
F0-Male	BMD_{10}	NA	339	348.2
	$BMDL_{10}$	NA	282.3	293.7
	$BMDU_{10}$	NA	426	430.7
	$BMD_{10}/BMDL_{10}$	NA	1.2	1.19
F1-Female	BMD_{10}	NA	508	506.6
	$BMDL_{10}$	NA	348.2	355
	$BMDU_{10}$	NA	940.7	915.4
	$BMD_{10}/BMDL_{10}$	NA	1.46	1.43
F1-Male	BMD_{10}	NA	266	278.9
	$BMDL_{10}$	NA	229	243.1
	$BMDU_{10}$	NA	318.6	328.7
	$BMD_{10}/BMDL_{10}$	NA	1.16	1.15

* LL: Log-likelihood

BMD assessment for centrilobular hepatocellular hypertrophy

Table 82 shows the BMD confidence intervals for centrilobular hypertrophy of liver cells for the F0 males. As can be seen in Figure 25, all four goups were analysed, but the F0 males gave the lowest BMD values. Since histopathological data are quantal rather than continous, for these data additional ("classical"models were also included in the assessment (the "classical models" cannot be used to evaluate continuous data). From this analysis, the lowest BMDL of 3.46 mg/kg bw per day was obtained with the Gamma model. The BMD ranged from 12.8 to 43.5 mg/kg bw per day and the highest BMDU of 67.2 was obtained with Exponential model 5.

Table 82: Dose-response modelling results for centrilobular hepatocellular hypertrophy.

model.list	npar	loglik	accepted	bmd	bmdl	bmdu	ab.txt	sens.lev
null	1	-184	NA	NA	NA			
full	28	-118	NA	NA	NA			
two.stage	9	-128	yes	13.6	6.19	39.2	ab	F0m
log.logist	9	-129	yes	13.3	4.26	34.9	ab	F0m
Weibull	9	-128	yes	12.5	3.60	36.2	ab	F0m
log.prob	9	-128	yes	13.1	4.43	33.3	ab	F0m
gamma	9	-128	yes	12.6	3.46	35.5	ab	F0m
logistic	8	-131	yes	21.4	12.70	66.4	ab	F0m
LVM:E5-	10	-127	yes	43.5	7.27	67.2	ab	F0m
LVM:H2-	8	-129	yes	12.8	6.02	29.7	ab	F0m

data: mouse; response: Centr_hyperthrophy; covariate: gnrtn*sex; CED: 0.1 extra risk; constraint: no; P-value GoF: 0.05

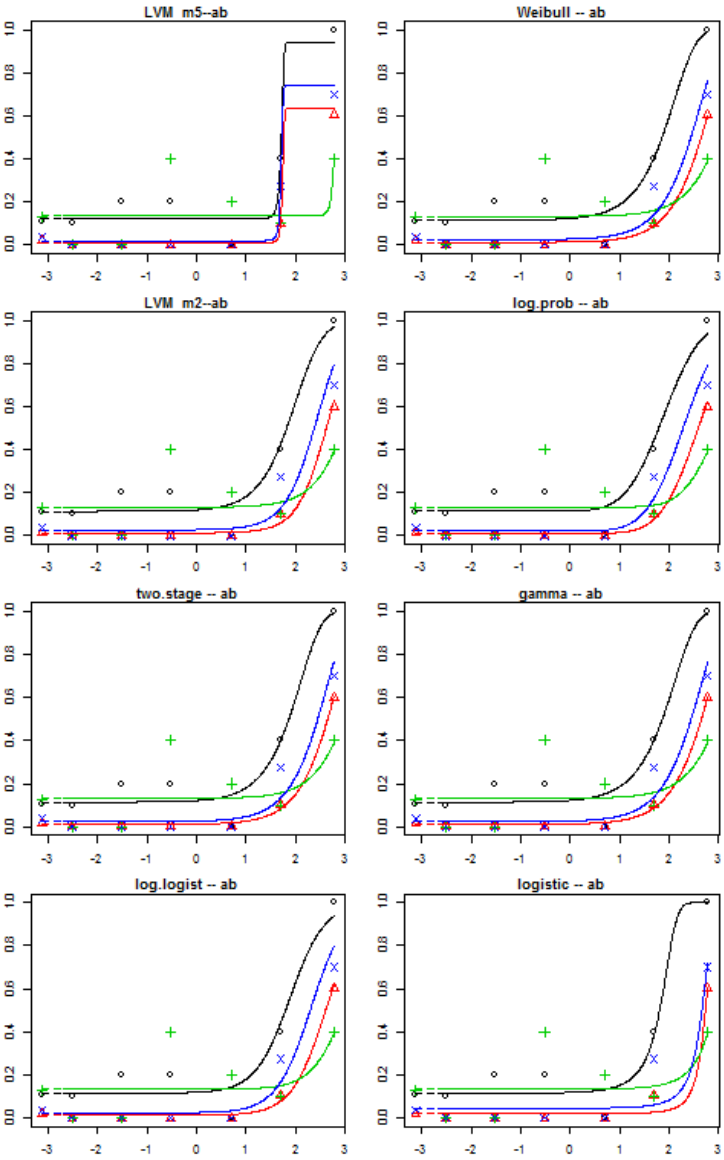

Figure 25: Dose-response curves for centrilobular hepatocellular hypertrophy. CED (critical effect dose) is the BMD for a 10% increase in incidence of hepatocellular hypertrophy. Green crosses: males F1; blue x-es: females F1; black circles: males F0; red triangles: females F0. Horizontal axis: log dose; Vertical axis: fraction responding.

In general, models from both exponential and Hill families were fitting the dose-response data for the absolute and relative liver and kidney weight changes well, except for the absolute liver and kidney weight changes in F0 females, where very wide confidence intervals around the BMDs were obtained, because of lack of dose-response information in the data. However, considering that organ weights are dependent on body weight, after correction for body weight (i.e. by calculation of relative organ weights) also for the F0 females adequate dose-response information could be identified in these studies, and for the relative organ weight changes the information in the F0 female groups has been included.

PART B

Dose-response modelling on mammary duct hyperplasia after exposure to BPA from the US FDA/NCTR (2013) / Delclos et al. (2014)

In compliance with the Opinion of the EFSA Scientific Committee on the use of the Benchmark dose (BMD) approach in Risk Assessment (EFSA, 2011), the results obtained on mammary duct hyperplasia in a subchronic toxicity study with BPA in rats have been submitted to statistical dose-response modelling. A full study report with the individual data (US FDA/NCTR, 2013 and Delclos et al., 2014) was available for dose-response modelling. In the study, animals were administered BPA by oral gavage from GD 6 through the start of labour and then directly to pups from PND 1 until termination at PND 90 at the doses of 0, 2.5, 8, 25, 80, 260, 840, 2 700, 100 000 and 300 000 µg/kg bw per day.
Microscopic evaluation of the mammary gland was performed at PND 21 and PND 90. An increase in the incidence of mammary duct hyperplasia in females was observed at both times, with statistically significant effects at the BPA doses of 2 700 and 100 000 µg/kg bw per day at PND 21 and at the doses of 2 700, 100 000 and 300 000 µg/kg bw per day at PND 90. The severity of the duct hyperplasia was minimal in all the observed findings and in all dose groups.

BMD calculations were performed for mammary duct hyperplasia at both PND 21 and PND 90, and with PND 21 and PND 90 as a covariate.

Table 83: Dose-response information for mammary duct hyperplasia in BPA exposed rats (data from FDA/NCTR, 2013; Delclos et al, 2014)

	Summary data on incidence of mammary duct hyperplasia in female rats at PND 21 (US FDA/NCTR, 2013 and Delclos et al., 2014)		**SUMMARY DATA ON INCIDENCE OF MAMMARY DUCT HYPERPLASIA IN FEMALE RATS AT PND 90 (US FDA/NCTR, 2013 AND DELCLOS ET AL., 2014)**	
Dose (µg/kg bw per day)	**Incidence**	**Group size**	**Incidence**	**Group size**
0.0	0	16	7	20
2.5	2	19	11	23
8	1	13	6	18
25	4	19	11	21
80	1	20	8	20
260	1	13	8	20
840	2	18	9	20
2 700	5	17	11	20
100 000	6	17	13	20
300 000	3	12	14	19

The severity of the mammary duct hyperplasia was also reported and was minimal hyperplasia for all the observations in all doses. This will therefore not influence the BMD calculations.

BMD calculation on mammary duct hyperplasia females with PND 21 and PND 90 as covariate

Table 84: Summary of BMD analysis on mammary duct hyperplasia. The column "covar" indicates which parameters were found to differ significantly between the subgroups. The column "level" indicates which subgroup was found to be most sensitive.

Results from benchmark dose calculations for mammary duct hyperplasia in female rats with PND 21 and PND 90 as covariate (US FDA/NCTR, 2013 and Delclos et al., 2014)								
Model	**covar**	**npar**	**loglike**	**accept**	**BMD**	**$BMDL_{10}$**	**BMDU**	**level**
null	NA	1	-233.24	--	NA	NA	NA	--
full	NA	20	-194.76	--	NA	NA	NA	--
Two-stage	a	4	-204.14	yes	62 200	36 100	147 000	--
Log-logistic	a	4	-199.97	yes	17.3	0.15	197	--
Weibull	b	4	-210.14	no	1.00E-06	NA	NA	PND 90
Log-prob	a	4	-200.08	yes	16.8	0.184	162	--
Gamma	a	4	-199.89	yes	14.9	0.085	220	--
Logistic	ab	4	-205.33	yes	46 100	28800	113000	PND 90
LMV:E5-	a	5	-199.84	yes	943	2.09E-06	3 020	PND 90
LVM:H5-	a	5	-200.26	yes	1.3	4.70E-06	2 390	PND 90

BMR: 0.1 extra risk; P-value GoF: 0.05; constraint: no

From the data provided in Table 84 the CEF Panel concluded that given the wide differences in the results obtained with various models (see also Figure 26) and wide confidence intervals, obtained with some of the models, the dose-response information in the FDA/NCTR study is too limited to derive with any confidence BMDL for this endpoint.

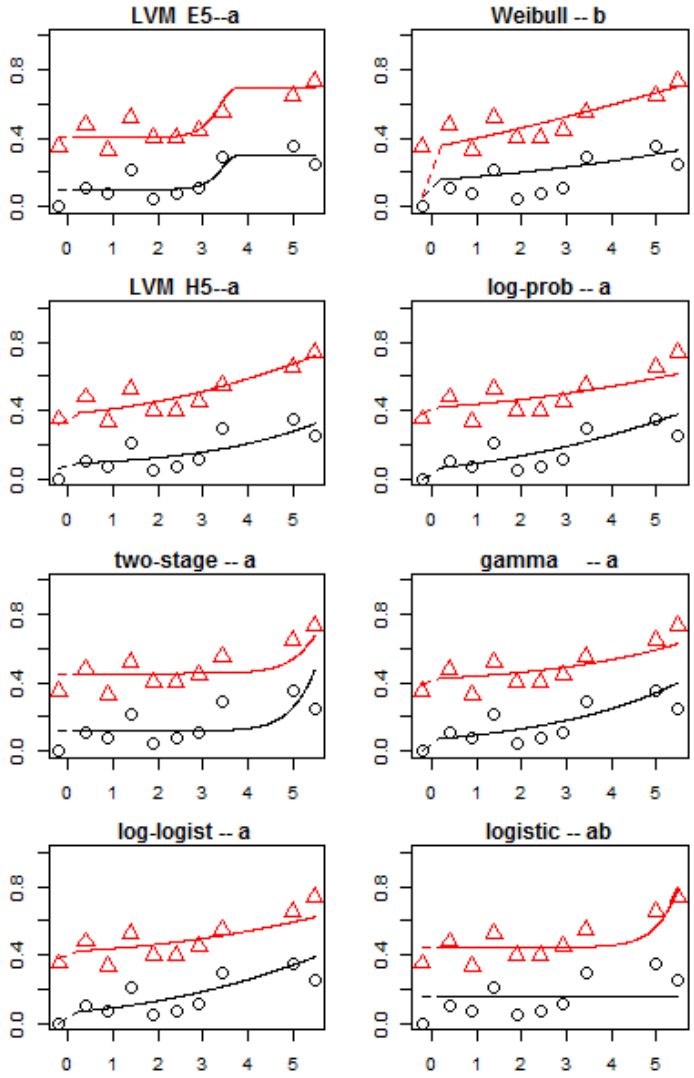

Figure 26: The various models fitted to mammary duct hyperplasia, observed in the two subgroups (PND21 (black circles) and PND 90 (red triangles) females). Some models did not distinguish between the two subgroups with respect to sensitivity; for other models the PND 90 females turned out to be the most sensitive subgroup. Horizontal axis: log dose; Vertical axis: fraction responding.

Conclusions of the BMD calculation on general toxicity and on mammary duct hyperplasia

The summary Table 85 below shows the $BMDL_{10}$ values obtained for liver and kidney effects in the F0 and F1 generations of mice. A lack of dose-response relationship was observed in the effect on nephropathy in both sexes. Therefore no model was obtained with acceptable fit and no $BMDL_{10}$ could be calculated for these toxic effects.

A benchmark response (BMR) of 10% was chosen both for the kidney and liver effects, based on the view of the CEF Panel that changes in the kidney and liver weight, and hepatocyte hypertrophy of less than 10% should not be regarded as adverse. The CEF Panel also took into account that the adaptive nature of the liver changes and that pathological changes in the kidney were marginal and were only observed at the highest dose level.

Table 85: Dose-response relationships for general toxicity of BPA in mice (Tyl et al., 2008)

Group	Toxic end point	$BMDL_{10}$[$]	BMD_{10}[$]	$BMDU_{10}$[$]
F0 males	Absolute mean kidney weight	3.42	22 – 22.4	91.69
F1 males	Relative mean kidney weight	8.96	35.1 - 36	108.9
F0 males	Absolute liver weight	213.1	290.3 -300.4	456.8
F1 males	Relative liver weight	229	266 – 278.9	328.7
F0 males	Centrilobular hepatocellular hypertrophy	3.46[@1]	12.8 – 43.5[@2]	67.2[@3]

$: values expressed as mg/kg bw per day; for the BMDL and BMDU the lower and upper bound values are given, irrespective of the model with which they were obtained; for the BMD the two values for the two separate models are given. @1: lowest of 7 models, @2: range of 7 models, @3: highest of 7 models

Although the lowest $BMDL_{10}$ from the modelling was observed for hepatocyte hypertrophy, the effect of BPA on hepatocyte hypertrophy was regarded by the CEF Panel as adaptive and as a less critical effect than the effect in the kidney. In addition, the analysis of the liver weight changes indicated a much higher level for the BMD for this endpoint, while a relationship between liver weight changes and liver hypertrophy is known to exist. That supports that the liver hypertrophy observed here has only marginal relevance if any at all. The CEF Panel has therefore selected the endpoint of relative mean kidney weight in the mouse, for which a $BMDL_{10}$ of 8 960 µg/kg bw per day was derived. This BMDL can be used as a PoD in the risk characterisation.

The CEF Panel noted that there is uncertainty regarding the robustness of the BMD modeling for on mammary duct hyperplasia. This is further discussed in section 3.9.6. of the main text. The CEF Panel concluded that given the wide differences in the results obtained with the various models and the wide confidence intervals, obtained with some of the models, the dose-response information in the FDA/NCTR study is too limited to derive with any confidence BMDL for this endpoint.

ABBREVIATIONS

ABC	Atp-Binding Cassette
Abs	Alveolar Buds
ACHN	Human Kidney Adenocarcinoma Cells
ADHD	Attention Deficit Hyperactivity Disorder
ADME	Absorption, Distribution, Metabolism And Excretion
AFC Panel	Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food
AFSSA	Agence Française de Sécurité Sanitaire des Aliments
AGD	Ano Genital Distance
AGRP	Agouti-Regulated Protein
AhR	Aryl Hydrocarbon Receptor
AIST	Japanese Institute of Advanced Industrial Science and Technology
ALARA	As Low As Reasonably Achievable
ALP	Alkaline Phosphatase
AMPA	A-Amino-3-Hydroxy-5-Methyl-4-Isoxazolepropionic Acid
AMY	Amygdala
ANOVA	Analysis of Variance
ANSES	French Agency For Food, Environmental And Occupational Health And Safety
ARC	Arcuate Nucleus
ASD	Autistic Spectrum Disorders
ASIP	Agouti Signaling Protein
ATP III	Adult Treatment Panel Iii
AUC	Area Under The Curve
AVPV	Anteroventral Periventricular Nucleus
BASC-2	Behaviour Assessment System For Children 2
BAT	Brown Adipose Tissue
BBB	Blood-Brain Barrier
BCRP	Breast Cancer–Resistant Protein
BDNF	Brain-Derived Neurotrophic Factor
BfR	Federal Institute For Risk Assessment
BMD	Benchmark Dose
BMDL	Benchmark Dose (Lower Confidence Limit)
BMI	Body Mass Index
BMR	Benchmark Response
BPA	Bisphenol A
BPADS	BPA Disulfate
BPAG	BPA-Glucuronide
BrdU	Bromodeoxyuridine
BRCA	Breast Cancer
BRIEF-P	Behaviour Rating Inventory of Executive Function-Preschool
BUS	Blood, Urine, and Sweat
bw	Body Weight
CA	Chromosome Aberration
CAD	Coronary Artery Disease
CADS	Conners' Adhd/Dsm-Iv Scales
CASA	Computer-Assisted Sperm Analysis
CBCL	Child Behavioural Checklist
CBMA	Cytokinesis Blocked Micronucleus Assay
CBX	Hemisuccinateester Carbenoxolone
CDC	Center For Disease Control And Prevention
CDI	Children Depression Inventory
CED	Critical Effect Dose
CEF Panel	Panel on Food Contact Materials, Enzymes, Flavourings And Processing Aids
CERHR	Center for the Evaluation of Risks to Human Reproduction
CES	Critical Effect Size
CFSAN	Center for Food Safety and Applied Nutrition
CHD	Coronaryheart Disease
CI	Confidence Interval
CKD-EPI	Chronic Kidney Disease Epidemiology Collaboration
C_{max}	Maximum concentration

CMV	Cytomegalo Virus
COX	Cyclooxygenase
CPT	Continuous Performance Test
CREB	Camp-Response Element Binding Protein
CRH	Corticotrophin-Releasing Hormone
Css	Calculate Steady State Plasma Concentrations
CTB	Cudrania Tricuspidata Bureau
CV	Cardiovascular
CVD	Cardiovascular Disease
CYP	Cytochrome P450
DA	Dopamine
DAF	Dosimetric Adjustment Factor
DAP	Dialkyl Phosphate
DBP	Diastolic Blood Pressure
2,5-DCP	2,5-Dichlorophenol
DD	Dermal Doses
DEHP	Di(2-Ethylhexyl)Phthalate
DES	Diethylstilboestrol
D/LT	Dark–Light Transition
DMBA	Dimethylbenzanthracene
DMEM	Dulbecco's Modified Eagle's Medium
DMNT	DNA Methyltransferases
DMSO	Dimethylsulfoxide
DNA	Desoxyribonucleic Acid
DNEL	Derived No Effect Level
DNMT	DNA Methyltransferase
DO	Oral Doses
DOAJ	Directory of Open Access Journals
DOPAC	3,4-Dihydroxyphenylacetic Acid
DOV	Day Of Vaginal Opening
DXA	Dual-Energy X-Ray Absorptiometry
E2	Oestradiol
EC	European Commission
ECG	Electrocardiogram
ECHA	European Chemical Agency
ECN	Embryo Cell Number
EDCs	Endocrine-Disrupting Compounds
EE, EE2	Ethinyl Oestradiol
EEC	European Economic Commission
EFS	Embryo Fragmentation Score
EFSA	European Food Safety Authority
EGFR	Epidermal Growth Factor Receptor
EHR	Enterohepatic Recirculation
ELISA	Enzyme-Linked Immunosorbent Assay
EPA	Exposure Factors Handbook
EPM	Elevated Plus Maze
ER	Estrogen Receptor
ERα	Estrogen Receptor Alpha
ERRγ	Estrogen-Related Receptor Gamma
ERβ	Estrogen Receptor Beta
ERK	Extracellular Signal-Regulated Kinases
ESR	Estrogen Receptor Beta
EU	European Union
EU-RAR	European Union -Risk Assessment Report
EZH2	Enhancer of Zeste Homolog 2
FAO/WHO	Food and Agriculture Organization /World Health Organization
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FENO	Fraction of Exhaled Nitric Oxide
FEV	Forced Expired Volume

FLD	Fluorescence Detection
FS	Forced Swim
FSANZ	Food Standard Australia New Zealand
FSH	Folliculum Stimulant Hormon
FST	Forced Swimming Test
GC	Gas Chromatography
GC-ECNI/MS	Gas Chromatography Coupled With Mass Spectrometry Operated In Electron Negative Ionization Mode
GC/EI-MS/MS	Gas Chromatography/Tandem Mass Spectrometry
GC-MSD	Gas Chromatography Coupled to a Mass Selective Detector
GD	Gestational Day
GEN	Genistein
GFR	Glomerular Filtration Rate
GI	Gastrointestinal
GLP	Good Laboratories Practice
GluR1	Glutamate Receptor 1
GnRH	Gonadotropin-Receptor Hormone
GPR30	G-Protein Coupled Receptor
GSD	Geometric Standard Deviation
GSM	Grey Scale Median
HbA1c	Glycated Hemoglobin
HDL	High Density Lipoproteins
HED	Human Equivalent Dose
HEDF	Human Equivalent Dose Factor
HepG2	Human Hepatocellular Carcinoma Cells
HFD	High-Fat Diet
HFEA	Human Fertilisation and Embryology Authority
HIP	Hippocampus
HLP	High Leptin
Hpcal1	Hippocalcin-Like 1
HPLC	High-Performance Liquid Chromatography
HPLC-ESI-MS/MS	High Performance Liquid Chromatography – Electrospray Tandem Mass Spectrometry
HPLC-MS/MS	High-Performance Liquid Chromatography Tandem Mass Spectrometry
HRBEC	High Risk Donor Breast Epithelial Cells
HRT	Hormone Replacement Therapy
HSD	Hydroxysteroid Dehydrogenase
IAP	Intracisternal A Particle
ICSI	Intracytoplasmic Sperm Injection
ID LC-MS-MS	Isotope Dilution High-Performance Liquid Chromatography-Tandem Mass-Spectrometry
IgE	Immunoglobuline E
IM-GSM	Intima–Media Complex-Grey Scale Median
IMT	Intima-Media Thickness
INSL3	Insulin-Like3
ISHH	In Situ Hybridization Histochemistry
ISI	Institute For Scientific Information
IV	Intravenous
IVF	In Vitro Fertilization
JAK/Stat	Janus Kinase/Signal Transducer And Activator Of Transcription
JAM-A	Junction Adherence Molecular A
JRC	Joint Research Center
Kiss1	Kisspeptin
LAD	Low Adiponectin
LAMP3	Lysosomal-Associated Membrane Protein 3
LBW	Low Birth Weight
LC	Liquid Chromatography
LC-ED	Liquid Chromatography With Electrochemical Detection
LC-MS	Liquid Chromatography Coupled With Mass Spectrometry
LC/MS/MS	Liquid Chromatography Coupled To Tandem Mass Spectrometer
LD	Lactation Day

LDES	Learning Disability Evaluation Scale
LDL	Low-Density Lipoprotein
LH	Luteinizing Hormone
LLE	Liquid-Liquid Extraction
LLOD	Lower Level Of Detection
LOAEL	Lowest Observed Adverse Effect Level
LOD	Limit Of Detection
LOQ	Limit Of Quantification
LPL	Lipoprotein Lipase
MaGiCAD	Metabonomics And Genomics In Coronary Artery Disease
MAO	Monoamine Oxidase
MAPK	Mitogen-Activated Protein Kinase
Mc3r, Mc4r	Melanocortin Receptors
MDR	Multidrug Resistance
MDRD	Modification of Diet In Renal Disease
MED	Medulla oblongata
MEHP	Mono-(2-Ethylhexyl) Phthalate
MEP	Monoethyl Phthalate
MiBP	Monoisobutyl Phthalate
MLH	Mutl Homolog
MM	Mirrored Maze
MMP	Mono- Methyl Phthalate
MN-PCE	Micronucleated Polychromatic Erythrocytes
MOA	Mode of Action
MOCEH	Mothers and Children's Environmental Health
MOEs	Margins of Exposure
mPFC	Medial Prefrontal Cortex
mPSc	Mid-Pachytene Spermatocytes
mRNA	Messenger RNA
miRNA	Microrna
MRI	Magnetic Resonance Imaging
MRP	Multidrug Resistance-Associated Proteins
MSTFA	N-Methyl-N-(Trimethylsilyl)Trifluoro-Acetamide
MTOCs	Modification Of The Microtubule Organizing Centers
MTOCs	Microtubule Organizing Centers
MWM	Morris Water Maze
NCTR	National Center For Toxicological Research
ND	Non-Detectable
NFG	Nerve Growth Factor
NHANES	National Health and Nutrition Examination Survey
NIEHS	National Institute of Environmental Health Sciences
NMDA	N-Methyl-D-Aspartate
NMDAR	N-Methyl-D-Aspartate Receptor
NMDR	Non-Monotonic Dose-Response
NMDR	Non-Monotonic Dose-Response
NNS	Network Neurobehavioral Scale
NO	Nitric Oxide
NOAEL	No Observed Adverse Effect Level
NOD	Non-Obese Pre-Diabetic
NP	Nonylphenol
NTP	National Toxicology Program
ODF	Outer Dense Fiber Protein
OECD	Organisation for Economic Co-Operation and Development
OF	Open Field
OFT	Open-Field Test
OJ	Official Journal
OP	Object Placement
OP	Octylphenol
OR	Object Recognition
OR	Odds Ratio

OVAR	Epithelial Ovarian Cancer
OVCAR	Ovarian Epithelial Carcinoma Cells
OVX	Ovariectomized
OW	Overweight
PAD	Peripheral Arterial Disease
PBDE	Polybrominated Diphenyl Ether
PBG	Phenylbiguanide
PBPK	Physiologically Based Pharmacokinetic Modelling
PBTK	Physiologically Based Toxicokinetic
PC	Polycarbonate
PCOS	Polycystic Ovarian Syndrome
pCREB	p-Camp Response Element-Binding Protein
PDE4D4	Phosphodiesterase Type 4 Variant 4
PDI	Protein Disulphide Isomerase
PGR	Progesterone Receptor
PI3K/Akt	Phosphatidylinositide 3-Kinases/Protein Kinase B
PIN	Prostate Intraepithelial Neoplasia
PIVUS	Prospective Investigation of the Vasculature in Uppsala Seniors
PK	Protein Kinase
PKC	Protein Kinase C
PKG	Protein Kinase G
PLSD	Protected Least Significant Difference
PND	Postnatal Days
POL	Postimplantation Loss
PP	Polypropylene
PR	Progesterone Receptor
PRL	Prolactin
PSD	Post Synaptic Density
PVC	Poly Vinyl Chloride
pWAT	Perigonadic White Adipose Tissue
QPCR	Quantitative Real Time Polymerase Chain Reaction
RES	Resveratrol
RGCs	Radial Glial Cells
RIA	Radioimmunoassay
RSE	Relative Standard Error
RT	Real-Time
SBP	Systolic Blood Pressure
SC's	Sister Chromatid Exchanges
SCENIHR	Scientific Committee On Emerging And Newly Identified Health Risks
SC	Subcutaneous
SCF	Scientific Committee On Food
s.d.	Standard Deviation
SD	Standard Deviation
SDN-POA	Sexually Dimorphic Nucleus Of The Preoptic Area
SEM	Standard Error Of The Mean
SERM	Estrogen Receptor Modulator
SGA	Small For Gestational Age
SHBG	Sex Hormone-Binding Globulin
SMART	Study Of Metals And Assisted Reproductive Technologies
SML	Specific Migration Limit
SPE	Solid Phase Extraction
SRC	Steroid Receptor Activator
SRD5A1	Steroid 5-Alpha- Reductase Type I
SRS	Social Responsiveness Scale
StAR	Steroidogenic Acute Regulatory
SULT	Sulfotransferases
SVZ	Sub-Ventricular Zone
TEBs	Terminal End Buds
TED	Tubular Epithelium
TDI	Tolerable Dailay Intake

t-TDI	Temporary- Tolerable Daily Intake
TDs	Terminal Ducts
TNP	Transition Protein
TP	Testosterone Propionate
TR	Thyroid Receptor
TSH	Thyroid-Stimulating Hormone
TUNEL	Terminal Deoxynucleotidyl Transferase Dutp Nick End Labeling
TWA	Time Weighted Average
UF	Uncertainty Factor
UGT	UDP-Glucuronyl-Transferase
UNEP	United Nations Environment Programme
UTD	Undescended Testes
UWW	Uterine Wet Weight
VD	Volume of Distribution
VDR	Vitamin D Receptor
VEGF	Vascular Endothelial Growth Factor
WoE	Weight of Evidence
WT	Wild-Type
ZEA	Zearalenone