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Re-evaluation of the risks to public health related to the presence of bisphenol A (BPA) in foodstuffs

EFSA Panel on Food Contact Materials, Enzymes and Processing Aids (CEP),

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Abstract

In 2015, EFSA established a temporary tolerable daily intake (t-TDI) for BPA of 4 µg/kg bw per day. In 2016, the European Commission (EC) mandated EFSA to re-evaluate the risks to public health from the presence of BPA in foodstuffs and to establish a full tolerable daily intake (TDI). For this re-evaluation, a pre-established protocol which had undergone public consultation was used. The CEP Panel concluded that it is Unlikely to Very Unlikely that BPA presents a genotoxic hazard through a direct mechanism. Therefore, it was concluded that the balance of evidence allows a health-based guidance value (HBGV) to be established. The immune system was identified as the most sensitive health outcome category to BPA exposure. Specifically, an increase of Th17 cells was identified as the critical effect; these cells are pivotal in cellular immune mechanisms and involved in the development of allergic lung inflammation. A reference point (RP) of 0.93 ng/kg bw per day, expressed as human equivalent dose, was identified for the critical effect. The uncertainty analysis indicated that it was around 90% probable that no other endpoint was more sensitive than Th17 cells. Therefore, the CEP Panel concluded that no additional uncertainty factor (UF) was needed and that a HBGV based on the identified RP is justified. Applying an UF of 25 to the RP, a TDI of 0.04 ng BPA/kg bw per day was established. Comparison of this TDI with the dietary exposure estimates from the EFSA 2015 opinion showed that both the mean and the 95th percentile dietary exposures in all age groups exceeded the TDI by two to four orders of magnitude. Even considering the uncertainty in the exposure assessment, since the exceedance was so large, the CEP Panel concluded that there is a health concern from dietary BPA exposure for all age groups.

Keywords

Bisphenol A, BPA, hazard, toxicity, health risks, TDI, food contact materials

Summary

In 2015, EFSA established a temporary Tolerable Daily Intake (t-TDI) for BPA of 4 µg/kg bw per day (EFSA CEF Panel, 2015). By comparing this t-TDI with the exposure estimates, it was concluded that there was no health concern for any age group from dietary exposure and low health concern from aggregated exposure. However, uncertainty in the outcome was noted and the European Commission (EC) mandated EFSA to re-evaluate the risks to public health from the presence of BPA in foodstuffs and to establish a full TDI on the basis of the new information available. The current version of the draft opinion has been endorsed by the CEP Panel for public consultation.

The re-evaluation was performed as a systematic approach, including critical appraisal of the studies and weight of evidence (WoE), according to a pre-established protocol which underwent public consultation.

The new evaluation is based on evidence published from January 1, 2013, until October 15, 2018, not previously considered by EFSA. EFSA also launched a call for data in order to obtain from interested parties and/or stakeholders human and animal hazard data relevant to the re-evaluation of BPA and its t-TDI. An important study foreseen in the new evaluation was the BPA NTP CLARITY study and its associated grantees studies, some of which were published after October 15, 2018, but still included in the evaluation. For the health outcome category (HOC) Genotoxicity the time span of the literature search was extended until 21 July 2021 and also the studies assessed in the 2015 EFSA opinion were re-considered.

In the 2015 EFSA opinion on BPA (EFSA CEF Panel, 2015), the extrapolation from the reference point (RP) to the t-TDI was performed using an approach by which the toxicokinetic standard sub-factor for the interspecies extrapolation was substituted by a substance (BPA)-specific Human Equivalent Dose Factor (HEDF). The newly available literature data on toxicokinetics of BPA considered in the present opinion comprised two studies in mice, three studies in rats, three studies in pregnant sheep (ewes) and two studies in humans. The studies in mice and rats did not contribute to a better understanding of the toxicokinetic aspects of BPA. The two human studies showed that BPA is absorbed to nearly 100% and pre-systemically metabolised to a great extent to glucuronide and sulphate conjugates. The area under the curves (AUCs) adjusted for dose were clearly different in the two studies. The CEP Panel decided to use the median value of the AUCs from both studies for the calculation of the HEDF, because both modes of administration used in those studies are realistic for humans. The median AUC was 15.7 nM x h, which is 4-fold higher than the modelled AUC–value used for calculating the HEDF in the 2015 EFSA opinion. In order to calculate the HEDF, the AUC data were used from the 2015 EFSA opinion for mice, rats, monkeys and dogs. For ewes, the data reported in the current opinion were used. The following HEDFs were obtained: 0.0115 for mice, 0.165 for rats, 0.095 for monkeys, 0.1395 for dogs, 0.1197 for ewes (gavage) and 0.4357 for ewes (diet).

In the 2015 EFSA opinion on BPA (EFSA CEF Panel, 2015), BPA effects in liver and kidney were judged as Likely based on results from multigeneration studies in mice and rats. Changes in the mean relative kidney weight in a two-generation toxicity study in mice were considered as the most critical effect at low doses and were used to derive the t-TDI of 4 µg/kg bw per day. The newly available literature data indicated that in the health outcome category (HOC) General toxicity, several organs as well as haematological parameters are potential targets of toxicity for BPA. Within this HOC, no human studies were available, while ten clusters with relevant endpoints were identified in animal studies: body weight, liver effects, kidney effects, lung effects, thyroid effects, parathyroid effects, pituitary gland effects, adrenal gland effects, bone marrow effects and effects on haematological parameters. Overall, none of the evaluated clusters of effects in this HOC was considered Very Likely or Likely. In each of the evaluated clusters, effects were noted at least in one exposure period, but there was inconsistency among the available studies and, therefore, these effects were judged as As Likely As Not (ALAN). Mode of action (MoA) studies suggested oxidative stress as a potential pathogenetic mechanism for adverse effects, but other mechanisms may be operational as well.

In the 2015 EFSA opinion on BPA (EFSA CEF Panel, 2015) it was stated that based on human studies, there were indications that BPA may be linked to immunological outcomes, although a causal link could not be established. In addition, studies in animals lent support to the possibility of effects on the immune system. An effect of concern was, in particular, increased Immunoglobulin E (IgE) in allergic lung inflammation, but like the human studies, the animal studies suffered from shortcomings, for which reason the CEF Panel did not take these effects forward for the risk characterisation. In a later statement in 2016, after evaluating two additional studies of effects of exposure to BPA on the immune system, in which potential allergic conditions were investigated, the CEF Panel confirmed its position that the studies available at that time suggested effects on the immune system, but that the studies were not sufficiently robust to take them forward for risk characterisation (EFSA CEF Panel, 2016). The newly available literature data confirmed that the immune system is a target of toxicity for BPA. Within the HOC Immunotoxicity, one relevant cluster of endpoints was identified in the human studies: asthma/allergy, including data from the exposure periods pregnancy and childhood. In the animal studies, five clusters of relevant endpoints were identified: innate immunity, cellular immunity, humoral immunity, inflammation and allergic lung inflammation. Based on the human data, a positive association between BPA exposure and asthma/allergy was judged as ALAN. Based on the animal data, the clusters allergic lung inflammation, cellular immunity and inflammation showed effects that were judged as Likely. In the other clusters, effects were also noted, but the data were less consistent, and these effects were judged as ALAN. In the cluster allergic lung inflammation, an effect noted was on the production of specific IgE in response to an allergen, which was deemed as adverse as it is a crucial parameter in inducing allergic reactions in the respiratory tract. Other effects in that cluster supported the likelihood of this effect. The likely effect in the cluster cellular immunity was supported by the consistency of the different endpoints within that cluster. The most sensitive parameter affected by BPA was the increased number of Th17 cells. Although Th17 cells are T cells, and hence were put in the cluster cellular immunity, they play a pivotal role in allergic responses, and therefore the effect on Th17 cells is consistent with the effect on the production of specific IgE. *In vivo* evidence was supported by MoA studies. *In vitro* studies indicated the ability of BPA to induce immune deregulation, possibly leading to an increased susceptibility to develop inflammatory diseases.

In the 2015 EFSA opinion on BPA (EFSA CEF Panel, 2015), in the WoE, a likelihood level of ALAN of metabolic effects of BPA was based on the human and animal evidence. The newly available literature data indicated that BPA may induce adverse metabolic effects. Within the HOC Metabolic effects, five clusters of endpoints were identified in the human studies: obesity, cardiometabolic effects, thyroid effects, type 2 diabetes mellitus (T2DM) and gestational diabetes mellitus, including data from one or more of the exposure periods pregnancy, childhood and adulthood. In the animal studies, eight clusters of relevant endpoints were identified: obesity, fat deposition in the liver, glucose regulation, blood lipids, uric acid, type 1 diabetes mellitus (T1DM), other metabolic hormones and thyroid hormones. Based on the human data, none of the metabolic clusters showed effects that were considered Likely or Very Likely. A positive association between BPA exposure and obesity and T2DM was judged as ALAN, while a positive association between BPA exposure and cardiometabolic effects, thyroid effects and gestational diabetes mellitus was judged as Not Likely. Based on the animal data, no metabolic clusters were considered Very Likely. The cluster uric acid was considered Likely (in the adult exposure period), since increased levels were observed in the liver of mice and in the serum of mice and rats after BPA exposure. The other metabolic endpoints were considered either ALAN (obesity, fat deposition in the liver, glucose regulation, blood lipids and T1DM) or Not Likely (other metabolic hormones and thyroid hormones), in one or more exposure periods. Plausible MoAs of BPA were available for metabolic effects from animal and *in vitro* studies.

In the 2015 EFSA opinion on BPA (EFSA CEF Panel, 2015), a likelihood level of ALAN was assigned to neurological, neurodevelopmental and neuroendocrine effects of BPA in a WoE approach. The newly available literature data supported that the central nervous system is a target of toxicity for BPA. Within the HOC Neurotoxicity and developmental neurotoxicity, the evaluation of the human data considered endpoints from the cluster neurodevelopment. In the animal studies, three clusters of endpoints were identified: neuromorphology, nervous system functionality and behaviour. Based on the human data, it was concluded that an association between BPA exposure and impaired neurodevelopment was Not Likely. Based on the animal data, all three neurotoxicity clusters showed effects that were judged as Likely. In the neuromorphology cluster, Likely effects were found for the endpoints dendritic spine

density of pyramidal cells in hippocampus (CA1 and dentate gyrus areas) after developmental exposure and for the endpoints number of neurons in hippocampus (CA1 and CA3 areas), and dendritic spine density in pyramidal cells in medial part of the prefrontal cortex (PFC) after exposure during the growth phase/young age. In the nervous system functionality cluster, a Likely effect on the endpoint AchE activity during the adult exposure period was identified. In the behaviour cluster, Likely effects were noted for the endpoint anxiety/emotionality during all exposure periods (developmental, growth phase/young age, adult and indirect exposure through the male germline). Furthermore, the endpoint learning/memory showed a Likely influence of BPA from developmental and growth phase/young age exposure, and effects on sensory-motor coordination and salt preference were considered Likely in adults. Several mechanisms of action have been proposed, but the linkage between identified effects of BPA with brain structure, function and development have not been sufficiently explored in the literature to draw conclusions on the MoA.

In the 2015 EFSA opinion on BPA (EFSA CEF Panel, 2015), the CEF Panel concluded that the evidence was not sufficient to infer a causal link between BPA exposure and reproductive and developmental effects in humans. However, it was re-confirmed that BPA is a reproductive toxicant in experimental animal studies at high doses (above a human equivalent dose (HED) of 3.6 mg/kg bw per day), while a likelihood level of ALAN was given to reproductive and developmental effects of BPA in animals at low doses (below HED 3.6 mg/kg bw per day). The newly available literature data indicated that reproduction is a target of toxicity for BPA. Within the HOC Reproductive and developmental toxicity, five relevant clusters of endpoints were identified in the human studies: fetal and post-natal growth, prematurity, pre-eclampsia, male fertility and female fertility, including data from one or more of the exposure periods pregnancy, childhood and adulthood. In the animal studies, three clusters of relevant endpoints were identified: developmental toxicity, female reproductive toxicity and male reproductive toxicity. The clusters included data from one or more of the exposure periods developmental until weaning, developmental until adulthood, growth phase, adult exposure and indirect (germline) exposure. Based on the human data, none of the clusters showed effects that were judged as Likely or Very Likely. An association between maternal BPA exposure and impaired pre- and post-natal growth, shorter duration of gestation or preterm delivery, reduced male fertility and pubertal development when exposed during childhood, was judged as Not Likely. An association between BPA exposure and reduced female fertility and pre-eclampsia during adulthood and pubertal development when exposed during pregnancy was judged as ALAN. Based on the animal data, both female and male reproductive toxicity clusters showed effects that were judged as Likely. In the female reproductive toxicity cluster, there were Likely effects on ovary weight and histology and uterus histology after developmental exposure, on ovary histology after developmental and adult exposure, on implantation rate after growth phase/young age exposure and on ovary histology (follicle counts) after adult exposure. In the male reproductive toxicity cluster, there were Likely effects on epididymis (exfoliated germ cells and inflammation) after developmental exposure (pre-natal and/or post-natal until adult), on testis histology (decreased seminiferous tubule diameter) after growth phase/young age exposure and on sperm (motility, viability and acrosome reaction) after adult exposure. In the developmental toxicity cluster, effects were also noted, but the results were less consistent, and these effects were judged as ALAN (for the endpoints bone development, mammary gland histology, body weight (in the developmental exposure), mammary gland weight and mammary gland histology (in the developmental and adult exposure) as well as body weight and age at first oestrus (in the growth phase/young age exposure period)). Supporting evidence for plausible MoAs of BPA on reproductive toxicity effects was available. They include oestrogen and androgen receptor (AR) interactions and associated downstream and cross-stream effects, including epigenetic changes.

In the 2015 EFSA opinion on BPA (EFSA CEF Panel, 2015), the CEF Panel concluded that there were indications that BPA exposure may be associated with cardiovascular effects; these effects were considered to be ALAN. The newly available literature data investigated the cardiovascular system as a target of toxicity for BPA. Within the human HOC Cardiotoxicity, no case-control or cohort studies were available. Thus, the evidence for an association between BPA exposure and cardiotoxicity in human was considered Inadequate. In the animal studies, six clusters of relevant endpoints were identified: absolute and relative heart weight, incidence of cardiac lesions, cardiac structural changes (as measured by echocardiography), effects on cardiac function (as measured by echocardiography), blood pressure and atherosclerotic lesions. Based on the animal studies, the evidence of BPA effects was judged as

Not Likely in the majority of the cardiotoxicity clusters, and in few clusters as Inadequate, in one or more exposure periods.

In the 2015 EFSA opinion on BPA (EFSA CEF Panel, 2015), the CEF Panel judged BPA effects on mammary gland proliferation as Likely based on results from a subchronic rat study with pre-natal exposure to BPA as well as proliferative and related morphological changes in the mammary gland reported in other studies. The evidence for proliferative changes in prostate and other organs (testis, liver) was evaluated as too weak to reach a definite conclusion. Overall, the findings in mammary gland, prostate and other organs were considered 'insufficient to conclude that there is a link to cancer development in later life' and the likelihood level of 'Unlikely to ALAN' was assigned to carcinogenic effects of BPA. The newly available literature data indicated that in the HOC Carcinogenicity and mammary gland proliferative effects, the following organs were targets of BPA-induced toxicity: mammary gland, prostate and uterus. Within the HOC Carcinogenicity and mammary gland proliferative effects, no human studies were available, while five clusters with relevant endpoints were identified in animal studies: mammary gland weight, mammary gland histology, prostate histology, uterus weight and uterus histology. For histology, four sub-clusters were considered: non-neoplastic changes, pre-neoplastic lesions, neoplastic lesions, proliferation and apoptosis as evaluated by quantitative immunohistochemistry. The cluster mammary gland weight was judged Not Likely. The clusters mammary gland histology, prostate histology and uterus weight showed effects that were not consistently reported in the available studies and, therefore, these effects were judged as ALAN. Also, regarding the subclusters linked to lesions in the mammary gland, inconsistencies were noted: in the developmental until weaning exposure period no increase in pre-neoplastic lesions (Not Likely), but a higher incidence in neoplastic lesions (Likely) was observed. In the developmental to adult exposure period an increase in pre-neoplastic lesions (ALAN) was reported but no increase in neoplastic lesions was detected (Not Likely). Therefore, these effects contributed to the overall judgement ALAN in the cluster mammary gland histology. MoA studies in mammary gland addressing epigenetic effects, changes in gene expression and changes in hormone receptor levels suggested various MoAs of BPA. MoA studies on prostate cancer indicated that BPA can enhance the susceptibility to tumorigenesis in rodents co-treated with very high levels of oestradiol (E2) and testosterone, while developmental and chronic exposure to BPA without additional sex hormones did not demonstrate a direct tumorigenic effect. In the cluster uterus histology, the non-neoplastic changes gland cellular anomalies, squamous metaplasia and cystic endometrial hyperplasia were considered adverse and judged as Likely based on studies with developmental exposure (pre-natal and/or post-natal until weaning) to BPA. Whereas MoA studies on uterine cells suggested various MoAs of BPA possibly involved in the induction of proliferative changes, the results of rodent studies did not demonstrate a tumorigenic activity of BPA.

In the 2015 EFSA opinion on BPA 2015 (EFSA CEF Panel, 2015), the CEF Panel concluded 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 findings in the postlabelling assays *in vitro* and *in vivo* were judged Unlikely to be of concern, given the lack of mutagenicity and clastogenicity of BPA *in vitro* and *in vivo*. Based on the scientific literature re-considered from the 2015 EFSA opinion and the newly published literature and available data from 2013 until 21 July 2021, the CEP Panel concluded that BPA does not induce gene mutations in bacteria, while it induces DNA strand breaks, clastogenic and aneugenic effects in mammalian cells *in vitro.* Oxidative stress related mechanism(s) are possibly implicated in the DNA damaging and clastogenic activity elicited by BPA *in vitro.* There is limited evidence for DNA and chromosomal damaging activities of BPA *in vivo*. The available studies do not provide evidence of aneugenicity of BPA in germ cells *in vivo*. In contrast to consistent *in vitro* positive findings, the *in vivo* findings in several studies with either high or with limited reliability were inconsistent. The CEP Panel concluded that there is no evidence supporting an *in vivo* genotoxic hazard posed by BPA through a direct interaction with DNA. The CEP Panel concluded that it is Unlikely to Very Unlikely that BPA presents a genotoxic hazard, the causes of which include a direct mechanism. Therefore, it was concluded that the balance of evidence allows a health-based guidance value (HBGV) to be established.

Benchmark dose (BMD) analyses for dose-response modelling were performed for the endpoints with an assigned likelihood level of Likely or Very Likely. A cut-off value of 10 for the ratio between the lowest non-zero dose and the BMD lower confidence interval (BMDL) was set. Studies where the ratio

was higher or equal to 10 were considered not adequate to be evaluated by the BMD approach, but were considered in the uncertainty analysis. Studies reporting effects that were judged as ALAN were also not subjected to BMD analysis, but were included in the uncertainty analysis. BMD analyses were performed using the BPA administered doses without conversion to HED. HED converted values were used subsequently to compare the different modelling outcomes. Taken all available information into consideration, the CEP Panel identified BPA effects on more than one HOC, and the increased number of Th17 cells in the immune system was considered the critical effect. After conversion of the doses to HED, the CEP Panel identified the lowest BMDL value of 0.93 ng/kg bw per day for the increasing effect of BPA on Th17 cells in mice and used this as RP for the risk assessment of BPA. The CEP Panel did not apply the uncertainty factor (UF) for inter-species variability in toxicokinetics because this was already taken into account by the conversion into HED. The remaining UF of 25 accounting for the inter-species toxicodynamic difference (2.5) and the intra-human variability in toxicokinetics and toxicodynamics (10) was used to derive the TDI.

The uncertainty analysis indicated that it was around 90% probable that no other endpoint was more sensitive than Th17 cells. The Panel therefore concluded that no additional UF was needed and that a HBGV based on the identified RP is justified. Accordingly, the CEP Panel established a TDI for BPA of 0.04 ng/kg bw per day.

The CEP Panel carried out an assessment of the risk to public health related to the presence of BPA in foodstuffs without performing an updated exposure assessment, in accordance with the terms of reference as provided by the EC. Therefore, the CEP Panel compared the newly derived TDI of 0.04 ng/kg bw per day with the dietary exposure estimates for BPA from the 2015 EFSA opinion. The comparison of the dietary exposure estimates with the TDI showed that both the mean and the 95th percentile dietary exposures in all age groups (including all infants and toddler groups) exceeded the TDI by two to four orders of magnitude.

The CEP Panel noted that a TDI based on the second most sensitive endpoint (ovarian follicle counts) would also be exceeded by the dietary exposure by two to three orders of magnitude.

The CEP Panel is aware that the exposure assessment presented in the 2015 EFSA opinion may not fully represent the current dietary exposure. Even considering this uncertainty, since the exceedance was so large, the CEP Panel concluded that there is a health concern from dietary BPA exposure for all age groups of the general population.

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1. Introduction

Bisphenol A (BPA) is used as a monomer in the manufacture of polycarbonates (PC) and epoxy resins and other polymeric materials, as well as for certain paper products (thermal printing). PCs are used in food contact materials such as reusable beverage bottles, infant feeding bottles, tableware (plates and mugs) and storage containers. Epoxy resins are used in protective linings for food and beverage cans and vats.

BPA is authorised for use as a monomer in plastic food contact materials, in accordance with Commission Regulation (EU) No 10/2011/EU[1] on plastic materials and articles intended to come into contact with foodstuffs. The former specific migration limit (SML) of 3 mg/kg was reduced to of 0.6 mg/kg in 2002 and then further reduced to 0.05 mg/kg following EFSA's 2015 opinion (EFSA CEF Panel, 2015). In addition, Commission Implementing Regulations (EU) No 321/2011[2] and (EU) No 2018/213[3] place restrictions on the use of BPA, respectively in the manufacture of PC infant feeding bottles and in varnishes and coatings intended to come into contact with food and amending Regulation (EU) No 10/2011 as regards the use of that substance in plastic food contact materials

1.1. Background and Terms of Reference as provided by the requestor

In 2016, EFSA received a mandate from the European Commission on the 'Re-evaluation of the risks to public health related to the presence of BPA in foodstuffs and protocol for the risk assessment strategy'. The mandate states the following:

> 'In accordance with Article 29(1)(a) of Regulation (EC) No 178/2002[4], the European Commission asks EFSA to:
>
> - establish a protocol detailing the criteria for new study inclusion and for toxicological evidence appraisal for the re-evaluation of BPA, to ensure an efficient and transparent re-assessment of BPA;
> - re-evaluate the risks to public health related to the presence of BPA in foodstuffs.
>
> In particular, the re-evaluation should take into consideration new data available from the results of the US National Toxicology Program (NTP)/Food and Drug Administration (FDA) study due in 2017 as well as all other new available information not previously evaluated by EFSA and which fulfil the criteria laid down in an established protocol. This re-evaluation should seek to clarify the remaining uncertainties concerning the toxicological endpoints of BPA, especially those concerning the mammary gland, reproductive, metabolic, neurobehavioural and immune systems and to establish a full tolerable daily intake (TDI on the basis of the new information available.'

1.2. Interpretation of the Terms of Reference

The first part of the mandate was fulfilled in 2017 with the endorsement by the EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF) and subsequent publication of the BPA hazard assessment protocol (EFSA, 2017a, from this point forwards named as the '2017 protocol'). This document states in detail the new methods and/or the criteria that will be used in the BPA re-evaluation for data collection, study inclusion, evidence appraisal and integration. The draft protocol underwent public consultation, was revised according to the comments received and published together with the technical report on the outcome of the public consultation (EFSA, 2017b).

1 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, pp. 1–89.
2 Commission Implementing Regulation (EU) No 321/2011 of 1 April 2011 amending Regulation (EU) No 10/2011 as regards the restriction of use of Bisphenol A in plastic infant feeding bottles. OJ L87, 2.4.2011, pp. 1–2.
3 Commission Regulation (EU) 2018/213 of 12 February 2018 on the use of bisphenol A in varnishes and coatings intended to come into contact with food and amending Regulation (EU) No 10/2011 as regards the use of that substance in plastic food contact materials OJ L 41, 14.2.2018, pp. 6–12.
4 Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety. OJ L31, 1.2.2002, pp. 1–24.

As anticipated both in the protocol and in the public consultation report and further detailed in the EFSA Executive Director's letter of 14 March 2018 to the Commission, the working group (WG) of independent experts in charge of the re-evaluation of BPA safety will test the new methodology on a selection of papers previously appraised in the 2015 BPA opinion (EFSA CEF Panel, 2015) and to compare the outcomes. This testing phase should ensure that the methodology used for the 2015 BPA opinion and 2016 statement on immunotoxicity (EFSA CEF Panel, 2016) is robust, even though not as structured as the new one.

The second part of the mandate, the full re-evaluation of the safety of BPA, is aimed to assess whether the new scientific evidence (published from 2013 onwards until 15 October 2019 and not previously evaluated by EFSA) still supports the current temporary tolerable daily intake (t-TDI) for BPA of 4 µg/kg bw per day. As specified in the revised protocol, the evaluation will cover:

1) the adverse effects in humans associated with the exposure to BPA via any route;
2) the adverse effects in animals after:
    - oral exposure to BPA at doses equal or below the cut-off of 10 mg/kg bw per day (based on the benchmark dose lower confidence interval ($BMDL_{10}$) used by the EFSA CEF Panel to set the t-TDI in 2015)
    - other exposure routes [subcutaneous (s.c.), intraperitoneal (i.p.), intravenous (i.v.), inhalation and intratesticular] at doses equal or below the cut-off of 10 mg/kg bw per day, when converted to oral dose, taking into account the interspecies kinetics differences (see TK Chapter 3.1.1.5 of the opinion). No cut-off will be applied for dermal[5] studies.

For point 2, when all the doses in one study converted from other routes to oral will result above the oral cut-off of 10 mg/kg bw per day, the study will be excluded from every step of the assessment.

3) the human and animal toxicokinetics of BPA.

The target population of the hazard assessment is the EU general population, including specific vulnerable groups (embryos, fetuses and infants). The target chemical substance is bisphenol A (BPA; chemical formula $C_{15}H_{16}O_2$, CAS No 80-05-7 and EC No 201-245-8). BPA derivatives will not be object of the assessment. Any endpoint will be considered as potentially relevant for the assessment and a similar categorisation system of Health Outcome Categories used in the EFSA opinion of 2015 (EFSA CEF Panel, 2015) will be used in the new review as follows: General toxicity (e.g. liver and kidney), Reproductive and developmental, Neurotoxicity and neurodevelopmental toxicity, Immunotoxicity, Metabolic effects, Cardiotoxicity, Carcinogenicity and mammary gland proliferative effects, and Genotoxicity. In addition, toxicokinetic aspects of BPA will be examined. In case newly identified endpoints do not belong to any of the above, a new appropriate category will be added.

1.3. Additional information

1.3.1. BPA uses

BPA is an organic chemical used in the manufacture of PC plastics, epoxy resins and other polymeric materials. PC is used for manufacturing food and liquid containers, such as tableware (plates and mugs), microwave ovenware, cookware and reservoirs for water dispensers, as well as for non-food applications, such as toys and pacifiers with PC shields. BPA-based epoxy phenolic resins are used as protective linings for food and beverage cans and as a coating on residential drinking water storage tanks and supply systems.. BPA is also used in the manufacturing of a number of non-food-related applications, e.g. epoxy resin-based paints, medical devices, surface coatings, printing inks, flame retardants, and also for certain paper products (thermal paper e.g. for cash receipts). BPA is also found in clothes mainly those made from polyester and spandex. It may occur due to its use as an intermediate chemical in the manufacturing of antioxidants for textile finishing and in production of dyes.

[5]As there is very little toxicokinetic evidence available on dermal exposure, a conservative approach will be used and no cut-off was established.

1.3.2. Previous EFSA assessments

EFSA has issued several scientific opinions and statements on BPA in 2007, 2008, 2010, 2011, 2015 and in 2016 (EFSA 2007, 2008; EFSA CEF Panel 2010, 2011, 2015, 2016).

In 2006 EFSA published a Panel opinion on BPA safety assessment establishing a TDI for BPA of 0.05 milligram per kilogram (mg/kg) body weight per day, based on the no adverse effect level of 5 mg/kg body weight per day in multigeneration rodent studies and applying an uncertainty factor (UF) of 100 (EFSA, 2007).

In 2008 EFSA published a Panel opinion on the toxicokinetics of BPA, reaffirming the TDI established in 2006, concluding that age-dependent toxicokinetics differences of BPA in animals and humans would have no implication for the assessment of BPA previously carried out by EFSA (EFSA, 2008).

In 2010 the EFSA CEF Panel carried out a comprehensive evaluation of all the recent toxicological data on BPA and concluded that no new scientific evidence had been published since the EFSA opinions of 2006 and 2008 that would call for a revision of the TDI established in 2006 of 50 µg/kg bw per day (EFSA CEF Panel, 2010).

In 2011 EFSA published a Panel statement on BPA (EFSA CEF Panel, 2011) in relation to possible divergences between the conclusions of the EFSA Scientific opinion on BPA of September 2010 and those in the reports on BPA published in September 2011 by the French Agency for Food, Environmental and Occupational Health and Safety (ANSES) (ANSES, 2011). EFSA considered that the information in the ANSES report would not trigger a change of the outcome of the 2010 opinion, but that a more in-depth review of additional data in recent literature could be required, including the then ongoing low-dose studies at the National Center for Toxicological Research/FDA and at National Toxicological Program/National Institute of Environmental Health Sciences, which aimed to address, at least in part, the current uncertainties regarding the potential health effects of BPA.

In its 2015 opinion, the CEF Panel dealt with the assessment of the risk to public health associated with BPA exposure. EFSA established a t-TDI of 4 µg/kg bw per day due to uncertainties on low-dose effects. By comparing this t-TDI with the exposure estimates, the CEF Panel concluded that there is no health concern for any age group from dietary exposure or from aggregated exposure. The CEF Panel noted considerable uncertainty in the exposure estimates for non-dietary sources, while the uncertainty around dietary estimates was relatively low (EFSA CEF Panel, 2015). In 2016, EFSA released a statement on immunotoxicity of BPA following a request from the Dutch authorities for the re-evaluation of the t-TDI as set by EFSA (EFSA CEF Panel, 2016). The CEF Panel concluded that the results of the immunological studies as referred to in the Dutch report were not sufficient to call for a revision of the t-TDI, but that they would be further considered as the part of the studies to be appraised for the full re-evaluation of the safety of BPA.

1.3.3. NTP CLARITY-BPA program

Part of the mandate received by EFSA from the European Commission for the re-evaluation of the safety of BPA included an evaluation of the data coming from the US NTP research programme, called Consortium Linking Academic and Regulatory Insights on BPA toxicity (CLARITY-BPA). In particular, the re-evaluation should take into consideration new data available from the results of the US NTP/FDA study (The CLARITY-BPA Core Study: A Perinatal and Chronic Extended-Dose-Range Study of Bisphenol A in Rats), initially due in 2017, but actually published in October 2018.

The CLARITY-BPA program was developed to study the full range of potential health effects from exposure to BPA. The programme was initiated by National Institute of Environmental Health Science (NIEHS), NTP, and the US FDA.

CLARITY-BPA has two components:

1) Core Study: A two-year guideline-compliant study of potential BPA toxicity in rats.

70) Grantee Studies: Investigational studies conducted by university researchers testing a range of additional endpoints.

The Core Study tested potential BPA toxicity in rodents; findings were published in Camacho et al. (2019), identified, in this opinion, as RefID 11370. It also provided animals and tissues for further study by project grantees. The Grantee Studies used animals raised in the same conditions and exposed to the same doses of BPA as the Core Study, and the researchers were blinded to the doses of BPA administered.

The raw data of the Grantees studies were available on the FDA/NTP website[6] also from 15 October 2018, but the publications of the papers were postponed after this date.

The table below lists each grantee, their institution, links to the data for their study focus area, and links to their published results.

Table 1: List of Grantees.

Principal investigator	Institution	Study focus area and data link	Publications
Scott Belcher	North Carolina State University	Heart	Gear et al., 2017 [RefID 2229]
Nira Ben-Jonathan	University of Cincinnati	Obesity/Adipose Tissue	Raw data
Kim Boekelheide	Brown University	Testis/Sperm	Dere et al., 2018 [RefID 11815]
Jodi Flaws	University of Illinois at Urbana-Champaign	Ovary	Patel et al., 2017 [RefID 5708]
Nestor Gonzalez-Cadavid	University of California, Los Angeles	Penis	Raw data
Andrew Greenberg	Tufts University	Diabetes/Pancreas/Liver	Raw data
Shuk-Mei Ho	University of Cincinnati	Uterus	Leung et al., 2020 [RefID 13789]
Norbert Kaminski	Michigan State University	Immune/Spleen/Thymus	Li J et al., 2018a [RefID 12460] Li J et al., 2018b [RefID 12461]
Heather Patisaul	North Carolina State University	Behaviour/Brain	Witchey et al., 2019 [RefID 13782] Arambula et al., 2018 [RefID 11050] Arambula et al., 2017 [RefID 232] Arambula et al., 2016 [RefID 231] Rebuli et al., 2015 [RefID 6127]
Gail Prins	University of Illinois at Chicago		Prins et al., 2018 RefID 13779]
Cheryl Rosenfeld	University of Missouri	Behaviour/Brain	Cheong et al., 2018 [RefID 11739] Johnson et al., 2016 [RefID 3241]
Ana Soto	Tufts University	Mammary Gland	Montévil et al., 2020 [RefID 13788]

[6] https://cebs.niehs.nih.gov/cebs/program/CLARITY-BPA

Frederick vom Saal	University of Missouri	Urogenital System	Uchtmann et al., 2019 [RefID 13784]
Thomas Zoeller	University of Massachusetts Amherst	Thyroid/Brain	Bansal and Zoeller, 2019 [RefID 13783]

Three grantees studies (from Nira Ben-Jonathan, Nestor Gonzalez-Cadavid and Andrew Greenberg) had not been published in peer-reviewed papers at the time of endorsement of this opinion. Therefore, the materials and methods and the raw data available on the FDA website[7] on these studies were used for the appraisal assessment (see Annex E). The conclusions from the CLARITY-BPA Core Study stated that:

> 'statistical differences between BPA treatment groups, particularly below 25,000 µg/kg bw per day, and the vehicle control group detected by the low-stringency statistical tests applied to histopathology lesions, were not dose responsive, sometimes occurring in only one low or intermediate dose group, and did not demonstrate a clear pattern of consistent responses within or across organs within the stop-dose and continuous-dose arms and sacrifice times. In contrast, the high oestradiol (E2) dose elicited several oestrogenic effects in females in a clearly interpretable and biologically plausible manner. Several observations at 25,000 µg BPA/kg bw per day may be treatment related, including effects mentioned above in the female reproductive tract (ovary, uterus, and vagina) and in the male pituitary' (Camacho et al., 2019 [RefID 11370]).

Both the results from the CLARITY-BPA Core Study and from the Grantees studies were assessed by the CEP Panel and reported in the assessment section of the current opinion.

2. Data and Methodologies

2.1. Data

The evaluation is based on new evidence published from 1 January 2013 until 15 October 2018. The studies published in 2013 and already appraised by EFSA in its 2015 opinion on BPA and in its 2016 statement on immunotoxicity of BPA (EFSA CEF Panel, 2015, 2016) have not been re-assessed in the re-evaluation.

In the '2017 protocol', the proposed ending date was 30 August 2018 according to the initially planned publication date of the BPA NTP CLARITY study report (NTP. CLARITY-BPA. Chemical Effects in Biological Systems (CEBS). Research Triangle Park, NC (USA): National Toxicology Program (NTP), https://cebs.niehs.nih.gov/cebs/program/CLARITY-BPA). However, the BPA NTP CLARITY study report was finally published in September 2018 and the time span for the evidence search was therefore extended until 15 October 2018.

For the genotoxicity evidence, the time span of the evidence search was extended until 21 July 2021.

The methods that were used for data collection through literature searches and call for data are detailed in Annex A (i.e. Bisphenol A (BPA) hazard assessment protocol).

2.2. Literature search

The literature search covered the period 1 January 2013 until 15 October 2018. Publications of the Grantees studies, postponed to this date, were also considered (see details in Chapter 1.3.3). For the genotoxicity evidence, the time span of the evidence search was extended until 21July 2021.

Details on the information sources and the search streams used for retrieving the literature to be assessed can be also found in Chapter 3.2 of Annex A and in Appendix A (Outcome of the call for data).

Table 2: Outcome of the literature searches.

[7] https://cebs.niehs.nih.gov/cebs/program/CLARITY-BPA

Records identified	Through database searching, n = 13,636 Through call for data, n = 7
Title and abstract screening	n = 13,643
Full text screening	n = 3,231
Appraisal	Animal General toxicity, n = 54 Animal Immunotoxicity, n = 42 Animal Carcinogenicity and mammary gland proliferative effects, n = 46 Animal Metabolic effects, n = 82 Animal Neurotoxicity and developmental neurotoxicity, n = 94 Animal Reproductive and developmental toxicity, n = 153 Animal Cardiotoxicity, n = 22 Human Case–control, n = 26 Human Cohort, n = 99
Extraction	Animal studies, n = 298 Human case–control and cohort studies, n = 105
Narrative review	Animal Mode of Action studies, n = 288 *In vitro* Mode of Action studies, n = 310 Human Mode of Action studies, n = 33 Human Mode of Action studies, n = 33 Human Cross-sectional studies, n = 177

2.2.1. Call for data

The call for data was launched on the EFSA website on 9 March 2018 and interested parties were given the opportunity to submit data until 15 October 2018. The purpose of this call for data was to obtain from interested parties and/or stakeholders human and animal hazard studies/data (published, unpublished or newly generated) relevant to the re-evaluation of BPA and its TDI.

The data received by means of this call underwent the same screening for relevance and appraisal procedures planned for studies gathered through bibliographic searches, following the instructions described in the Annex A.

All the details on the references submitted and screened are reported in Appendix A.

2.2.2. Selection of studies

The methods for selecting the studies (screening of titles and abstracts, examining full-test reports for eligibility of studies) are reported in Chapter 4 of Annex A.

2.3. Methodologies

The methods for collecting the data from the included studies, for appraising and weighting the evidence, for performing the hazard characterisation and for analysing the uncertainties in the assessment are described in Chapters 5, 6, 7, 8, 9 and 10 of Annex A and here below in more detail.

2.3.1. BPA Hazard assessment protocol: development and testing

The 2017 protocol's methodology (inclusion/exclusion criteria and critical appraisal) (EFSA, 2017a) was first piloted on a set of studies previously appraised by EFSA (e.g. previously concluded to be of high, medium or low reliability). This early pilot phase was performed to ensure that there is not a significant divergence in the outcome of the study appraisals when using the 'old' methodology (applied to the 2015 EFSA CEF Panel risk assessment of BPA and 2016 CEF Panel statement on BPA immunotoxicity) vs. the 'new' methodology (according to the EFSA '2017 protocol'). The testing phase, its outcome and the resulting refinement of the appraisal methodology has been described in the EFSA Technical report (EFSA, 2019).

The aim of the testing phase was to test the functioning of the internal validity methodology for epidemiological and animal studies, i.e. whether the final tier of internal validity (on a three-level scale, with Tier 1 being the highest quality), automatically assigned on the basis of pre-defined criteria by the program DistillerSR to each study, reflected the internal validity according to expert judgement.

For the epidemiological studies, all of them were allocated to Tier 3, in full accordance with expert judgement, mainly due to low confidence in the BPA exposure assessments based on single spot urine sample measurements. A step-wise approach was proposed for refining the appraisal of epidemiological studies: if the question in the protocol on the confidence in exposure was answered negatively, the paper would be directly allocated to Tier 3 and the evaluation would stop.

For the animal studies, the 2017 internal validity appraisal tool allocated the studies only to Tier 1 or Tier 3. To improve discrimination of studies into three tiers, the appraisal tool was refined by re-defining the key questions and re-calibrating the rules for the automatic tier allocation. Moreover, it was agreed to allow the experts to re-assign a paper to a different tier of internal validity than that automatically assigned by the tool, if an appropriate justification was given.

After revision, the 2019 methodology was re-applied, reaching a percentage of comparability between automatic and expert judgement-based tier allocation equal to 91% when tested on the 40 studies (in 43 out of 47 appraisals).

The testing phase was also performed to assess the comparability of the study appraisal outcomes (i.e. reliability scores vs. internal validity tiers) by the methodology applied in the EFSA outputs of 2015 and 2016 (the '2015 methodology') and the 2019 methodology. It is acknowledged that the 2015 and 2019 methodologies present some differences with respect to the elements considered for assessing the study quality (i.e. reliability vs. internal validity). Nonetheless, the key study used to derive BPA's tolerable daily intake in the 2015 opinion was also considered to be of high quality according to the 2019 methodology. In addition, the outcome of the appraisal of the papers by the 2019 methodology versus the 2015 methodology was overall comparable or more stringent in 92% of the cases (24 out of 26 appraisals). It follows that despite some intrinsic differences, the 2015 methodology previously used by EFSA to appraise the BPA evidence is considered sufficiently robust, even though not as structured as the 2019 methodology.

The amendments to the 2017 protocol and the resulting refined 2019 methodology are fully documented in Annex A.

The 2019 methodology has been implemented for the full re-evaluation of the newly obtained BPA evidence as described below.

2.3.2. Definition of Health Outcome Categories and Clusters

Given the large volume and complexity of the emerging evidence and the observed heterogeneity thereof, a structured and detailed definition and organization of the assessed health outcome categories and relevant clusters was deemed necessary. This approach facilitated the process of effectively and successfully appraising the evidence, the proper alignment of human and animal data clusters and, where possible, the integration of human and animal data. This opinion recognizes the following heath outcome categories: General toxicity, Immunotoxicity, Metabolic effects, Cardiotoxicity, Neurotoxicity and developmental neurotoxicity, Reproductive and developmental toxicity, Carcinogenicity and mammary gland proliferative effects. Within each health outcome category (HOC), clusters were identified that include several toxicologically relevant endpoints that are physiologically or toxicologically related, and that in concert shed light on the likelihood of an effect of BPA exposure in that cluster. The endpoints are measures of an individual parameter that is adverse in itself, i.e. an apical endpoint, or may be involved in the development of an adverse condition, i.e. an intermediate endpoint.

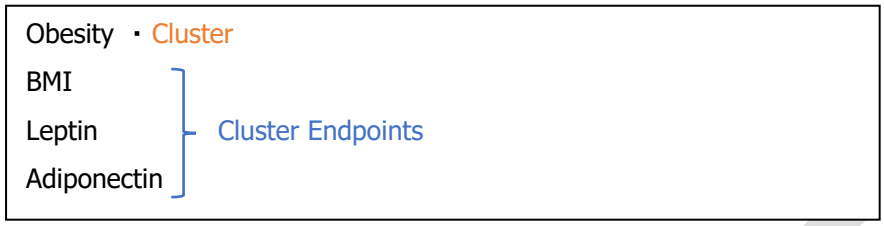
The approach used for HOC Genotoxicity is described in Chapter 2.3.5.

In epidemiological studies, outcome definition and reporting usually follow the current trends in human morbidity and global burden of disease data. For the group of epidemiological studies related to BPA exposure, an approach for the cluster identification similar to that of the International Classification of Diseases (ICD) system[8] was used as a starting point. Introduced by WHO, ICD is a hierarchical classification standard for all clinical and research purposes and incorporates an extensive matrix of diseases, disorders and other related health conditions. The following structured process was used:

[8] https://www.who.int/standards/classifications/classification-of-diseases

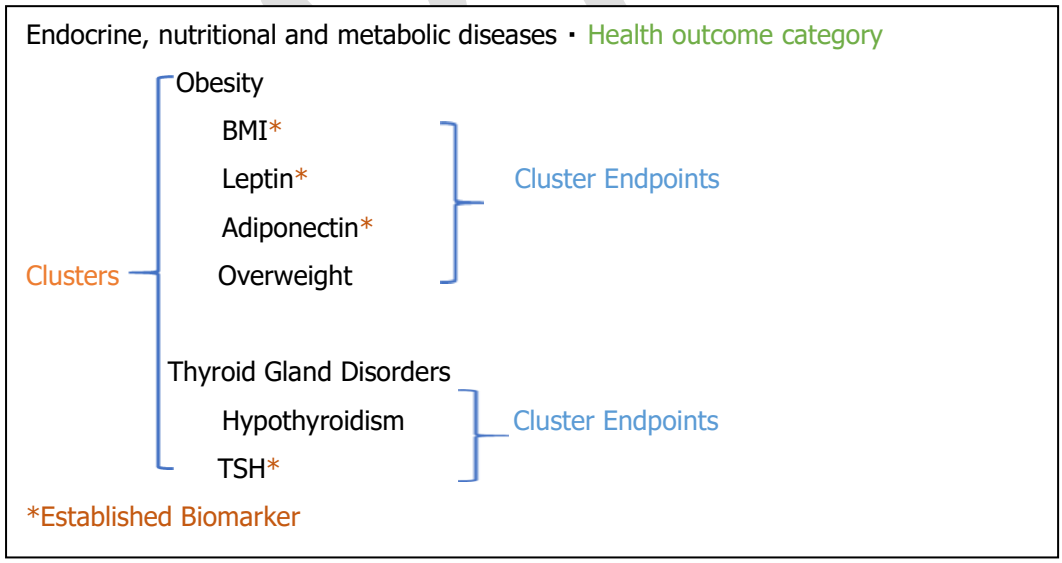
1) Endpoints comprising a distinct disease entity (e.g. obesity) were first identified and included as a core cluster element.

2) Moving downwards in the hierarchy, within each formed cluster all the additionally retrieved endpoints, representing established clinical or other related research measures or biomarkers of the core cluster element [e.g. body mass index (BMI), leptin, adiponectin, etc.], were included as distinct endpoints.



3) Moving upwards in the hierarchy, the chapter in ICD to which this particular disease belonged (e.g. Endocrine, nutritional and metabolic diseases) was identified, and a relevant 'Health outcome category' with the same name was created. These Health Outcome Categories were linked with a Toxicological Effect Category whenever appropriate. Within this group all the identified disease entities were included as separate clusters (e.g. Diabetes Mellitus; Thyroid Gland Disorders; etc.).

4) The Health outcome category tree was then merged.



During this process, it was acknowledged that the approach needed to be customised. First, there are disease entities that – for the purposes of risk assessment – fit better in another HOC compared with the one assigned in ICD-10. For example, gestational diabetes is grouped under 'Diseases related to pregnancy, childbirth and the puerperium' in ICD-10; we chose to include it in the 'Metabolic and related endocrine effects' category because the toxicological pathway linking exposure and endpoint was considered closer to metabolic effects. Second, endpoints from different ICD-10 chapters were grouped

to create a more coherent HOC group based on their common mechanistic backgrounds. For example, under 'Neurodevelopment', attributes such as cognition, psychomotor development, intelligence, etc., were grouped, given that all these endpoints share common mode of action (MoA) elements.

Based on the above, the **HOC, Clusters (C) and Endpoints (E)** were organised as follows:

HOC: General toxicity
- C: Liver toxicity
  - E: ALT
  - E: AST
  - E: gamma-glutamyl transpeptidase (γ-GTP)
- C: Kidney toxicity
  - E: Hyperuricemia

HOC: Effects involving the immune system (Immunotoxicity)
- C: Asthma/allergy
  - E: Acute bronchiolitis
  - E: Acute otitis media
  - E: Allergic diseases
  - E: Asthma
  - E: Atopic dermatitis
  - E: Atopy
  - E: Bacterial colonisation
  - E: Bronchitis
  - E: Chest infections
  - E: Croup
  - E: Eczema
  - E: Forced respiratory volume in 1s (FEV_1)
  - E: Fraction of exhaled nitric oxide (FENO)
  - E: IgE
  - E: PC20
  - E: Pneumonia
  - E: 'Rashes, eczema or hives'
  - E: Rhinitis (allergic)
  - E: Wheeze
- C: Infections other than respiratory tract
  - E: Infectious enteritis
  - E: Urinary tract infections

HOC: Metabolic and related endocrine effects
- C: Obesity
  - E: Adiponectin
  - E: Birth weight (BW)
  - E: Body Fat
  - E: Body mass index (BMI)
  - E: Fat leg
  - E: Fat mass
  - E: Fat mass index (FMI)
  - E: Fat trunk
  - E: Fat trunk/leg ratio
  - E: Leptin
  - E: Obesity
  - E: Obesity (central)
  - E: Overweight
  - E: Rapid growth
  - E: Skinfold thickness
  - E: Subcutaneous adipose tissue (SAT)
  - E: Visceral adipose tissue (VAT)

E: Visceral/Subcutaneous adipose tissue ratio (VAT/SAT)
E: Waist circumference
E: Weight change
E: Weight gain (annual)
C: Cardiometabolic effects
E: Cholesterol (total)
E: High density lipoprotein (HDL)
E: Low-density lipoprotein (LDL)
E: Triglycerides
E: Diastolic blood pressure
E: Pulse Pressure
E: Systolic blood pressure
C: Thyroid
E: Free thyroxine (FT_4)
E: Free triiodothyronine (FT_3)
E: Thyroid stimulating hormone (TSH)
E: Total thyroxine (TT_4)
E: Total triiodothyronine (TT_3)
C: Type 2 diabetes mellitus (T2DM)
E: C-peptide
E: C-peptide index (CPI)
E: Type 2 diabetes mellitus (T2DM)
E: Epidermal growth factor receptor (eGFR)
E: Glucose
E: Homeostasis model assessment of insulin resistance (HOMA-IR)
E: IGF-1
E: Insulin
E: Leptin
C: Gestational diabetes mellitus
E: Gestational diabetes mellitus (GDM)
E: Glucose
E: Impaired glucose tolerance

HOC: Mental and neurological effects (Neurotoxicity)
C: Neurodevelopment
E: Anxiety
E: Behaviour
E: Behaviour (Behaviour Assessment System for Children–2, BASC-2)
E: Behaviour (boys)
E: Behaviour (Behaviour Rating Inventory of Executive Function–Preschool, BRIEF-P)
E: Behaviour (girls)
E: Cognitive development
E: Cortisol
E: Cortisol reactivity
E: Depression
E: Executive function
E: Intellectual ability
E: Psychomotor development
E: Social responsiveness scale-2 (SRS-2)
E: Sociality
E: Sociality – Autistic behaviour (Social Responsiveness Scale)
E: Virtual Morris water maze (VMWM)

HOC: Reproductive and developmental toxicity
C: Fetal and post-natal growth
E: Abdominal circumference
E: Biparietal diameter

E: Birth length
E: Birth weight
E: Chest circumference
E: Femoral length
E: Gestational age at birth
E: Growth rate
E: Head circumference
E: Height
E: Large for gestational age
E: Low birth weight (LBW)
E: Length of gestation
E: Placental weight
E: Ponderal index
E: Small for gestational age (SGA)
E: Weight
E: Weight for length
E: Weight for length (birth)
E: Weight (fetal)
C: Prematurity
E: Gestational age at birth
E: Length of gestation
E: Preterm delivery
C: Pre-eclampsia
E: Pre-eclampsia (onset, severity)
C: Male fertility
E: E2
E: Estrone
E: Fecundability
E: Follicle stimulating hormone (FSH)
E: FSH:Inhibin B ratio
E: Hypo-osmotic swollen
E: Inhibin B
E: LH:Testosterone ratio
E: Live Birth rate
E: Luteinising Hormone (LH)
E: Sex
E: Sperm amplitude head displacement
E: Sperm average path velocity
E: Sperm beat cross frequency
E: Sperm concentration
E: Sperm count
E: Sperm curvilinear velocity
E: Sperm DNA fragmentation index
E: Sperm head acrosome area
E: Sperm head area
E: Sperm head elongation factor
E: Sperm head length
E: Sperm head perimeter
E: Sperm head width
E: Sperm high DNA stainability
E: Sperm linearity
E: Sperm morphology amorphous
E: Sperm morphology bicephalic
E: Sperm morphology coiled tail
E: Sperm morphology cytoplasmatic droplet
E: Sperm morphology megalo head
E: Sperm morphology micro head

837 E: Sperm morphology neck and midpiece abnormalities
838 E: Sperm morphology other tail abnormalities
839 E: Sperm morphology pyriform
840 E: Sperm morphology round
841 E: Sperm morphology strict criteria
842 E: Sperm morphology taper
843 E: Sperm morphology traditional normal
844 E: Sperm motility
845 E: Sperm straight line velocity
846 E: Sperm straightness
847 E: Sperm straw distance [=motility]
848 E: Sperm volume
849 E: Testicular volume
850 E: Testosterone
851 C: Female fertility
852 E: Aneuploid pregnancy
853 E: Antral follicle count
854 E: Clinical pregnancy
855 E: Corpus luteum rescue
856 E: Day-3 FSH
857 E: Early miscarriage
858 E: Embryo quality
859 E: Endometrial wall thickness
860 E: E2
861 E: E2 trigger level
862 E: Fecundability
863 E: Fertilisation rate
864 E: FSH
865 E: Follicular phase length
866 E: Human chorionic gonadotropin
867 E: Implantation
868 E: Infertility
869 E: Live birth rate
870 E: Luteal phase length
871 E: Luteinising hormone (LH)
872 E: Metaphase II (MII) oocytes
873 E: Miscarriage
874 E: Ovarian volume (OV)
875 E: probability of implantation
876 E: Progesterone
877 E: Sex
878 E: Time to implantation
879 E: Total oocytes
880 C: Pubertal/endocrine
881 E: % Mammary fibroglandular volume (%FGV)
882 E: 2D:4D digit ratio
883 E: Androstenedione
884 E: Anogenital distance anus – penis (AGDap)
885 E: Anogenital distance anus – clitoris (AGDac)
886 E: Anogenital distance anus – fourchette (AGDaf)
887 E: Anogenital distance anus – scrotum (AGDas)
888 E: Breast Development
889 E: Breast volume
890 E: Breastfeeding
891 E: Dehydroepiandrosterone (DHEA)
892 E: Dehydroepiandrosterone Sulfate (DHEA-S)
893 E: E2

E: Facial hair growth
E: Free testosterone
E: Genital development
E: Gonadarche
E: Inhibin B
E: Luteinising hormone (LH)
E: Mammary fibroglandular volume (FGV)
E: Menarche onset
E: Perceived insufficient milk supply (PIM)
E: Peripubertal hormone levels
E: Pubarche (boys)
E: Pubarche (girls)
E: Pubertal development scale
E: Pubic Hair
E: Sex hormone-binding globulin (SHBG)
E: Testicular volume
E: Testosterone
E: Thelarche
E: Voice change

HOC: Carcinogenicity
C: Prostate
E: Prostate cancer
C: Lymphoid tissues
E: Lymphoma

The following endpoints:

- myocardial infarction
- coronary artery stenosis
- peripheral artery disease.

were considered key in the 2015 EFSA Scientific opinion on the risks to public health related to the presence of bisphenol A (BPA) in foodstuffs (EFSA CEF Panel, 2015) but new information was not found in the studies considered for the new evaluation.

For each of the above-mentioned endpoints, the clusters were formed taking timing of exposure into consideration. That is, a distinction was made between exposure occurring during pregnancy, childhood and adulthood, as the aetiology of disease (e.g. neurodevelopment, fertility and other endocrine abnormalities) differs between stages of development.

Considering the problems in exposure measurement found in the literature retrieved, i.e. that no study measured BPA exposure in a way that was considered appropriate for assessment, and that the assessment was focused on finding possible adverse associations related to the exposure to BPA, it was decided to bring forward to WoE analysis only those clusters/exposure periods for which at least two studies were available and at least one of those studies reported a statistically significant effect for one of the endpoints measured.

In addition, although not meeting the criteria for inclusion, the cluster on Thyroid effects was also considered in the WoE analysis since maternal and infant thyroid function were considered as relevant in the 2015 EFSA Scientific opinion on the risks to public health related to the presence of bisphenol A (BPA) in foodstuffs.

The outcome of this approach, i.e. the Clusters (C) and Exposure periods (Exp) brought forward to weight of evidence (WoE) analysis, are presented for each HOC separately under Chapter 3.1. Hazard identification.

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

One area of particular interest when reviewing the toxicological profile of BPA is the possible presence of so-called *low-dose effects* (often synonymously referred to as 'doses in the range of human exposure' and/or 'human relevant doses'). Although no clear definition exists, low dose in the case of BPA has been defined as < 5 mg/kg bw per day in EFSA's previous assessment (EFSA CEF Panel, 2015). This value corresponds to the No observed adverse effect level (NOAEL) for changes in body and organ weights identified in two multigeneration reproductive studies in rodents (Tyl et al. 2002, 2006) that formed the basis for the TDI set by EFSA in 2007 (EFSA, 2007) and in 2015.

In the scientific debate, the presence of possible low-dose effects and its consequences for risk assessment has been the subject of some controversy and divergent views. This may have been partly fuelled by lack of studies specifically designed to capture such effects. The growing research interest in this area and the increasing number of studies designed to capture possible occurrence of low-dose effects has the potential to shed more light on this issue. This is particularly the case for BPA, and in this assessment a relatively large number of studies covering this dose range (< 5 mg/kg bw per day) was assessed compared with previous opinions. The CLARITY study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) is one example of such studies.

Another area of debate in chemical risk assessment is the presence of non monotonic dose response curves (NMDR). Although non-monotonicity is a well-known biological phenomenon (Yordanov and Stelling, 2018) the challenge in chemical risk assessment partly relates to the difficulty of objectively identifying the presence of NMDR in toxicological studies that often include too few dose groups for robust statistical evaluation of non-monotonicity. In addition, in cases where effect sizes are modest, higher statistical power (e.g. more animals) would help but it is seldom achieved.

Another important issue that often receives limited attention in the debate is that the presence of non-monotonicity may on its own be of limited importance for risk characterisation if the observed changes are not adverse.

Although methodologies to assess NMDR in toxicological studies have been proposed (Beausoleil et al., 2016; Badding et al., 2019), there is currently no consensus on these methods and different approaches of varying robustness, ranging from visual inspection to fitting any non-linear curve though data, have been applied by different researchers. In 2018, EFSA established a WG whose aim was to assess the impact of NMDRs on EFSA's human health risk assessments (EFSA Scientific Committee, 2021b). The reader is referred to that opinion for more comprehensive review on previous activities by EFSA and other international organisations to address NMDR.

In line with the recommendations of that opinion, the WG on BPA re-evaluation decided to apply the following criteria to assess if indications for an NMDR were present in individual studies or in the overall body of evidence:

Step 1- at single study level:

- The studies should include at least three doses plus the control.
- Two adjacent doses should show an effect in the same direction, but these effects would not need to be statistically significant in both cases. Statistical significance (relative to controls) in one the two doses would be sufficient.
- The biological plausibility of the effect should also be considered.

Step 2- at endpoint cluster level:

- If the effect is not biologically plausible, the effects should be present at the same doses in more than one study (replicability of the results).
- There should be a time and dose-consistent relationship among related effects/endpoints (e.g. intermediate and apical adverse effects).

All studies were evaluated according to these criteria. If a dose-response curve met these criteria, the dose-response would be described as showing 'indications for a NMDR'. If the study authors reported identifying an NMDR in their study evaluated by objective measures that contradicted the WG evaluations (such as curve fitting or other statistical evaluation), more in-depth analyses were performed by the CEP Panel. One study, in particular, prompted detailed assessment for the presence

of NMDR, because the endpoint evaluated (mammary gland development) was considered potentially adverse and the study was appraised as having a low risk of bias (Montévil et al., 2020 [RefID 13788]). The reader is referred to Appendix B (i.e. the Montévil study: Consideration of low-dose effects and non-monotonic dose-response reported in that study) for more details on the analyses performed.

In Table 3 a summary of the studies in which the authors indicated a NMDR or the CEP Panel identified indications for an NMDR is presented.

Table 3: List of studies (RefIDs) with indications for NMDR.

HOC	Indication for NMDR according to study's authors	Indication for NMDR according to WG experts following our criteria
General toxicity		RefIDs 11370, 2614
Metabolic effects	RefIDs 6319, 8375	RefIDs 6319, 8375, 13784, 9247, 12854
Neurotoxicity and developmental neurotoxicity	RefID 9083	RefIDs 9083, 13782, 3462
Cardiotoxicity		RefID 490,
Carcinogenicity and mammary gland prolifdcuative effects	RefIDs 3453, 13788	RefIDs 3453, 3990, 11370, 13788
Reproductive and developmental toxicity	RefIDs 4128, 3453, 13788	RefIDs 4128, 3453, 3990, 13788, 4779, 13099, 1216
Immunotoxicity		RefID 11370

2.3.4. Method for assessing uncertainty

2.3.4.1. Method for uncertainty analysis for hazard characterisation

The purpose of the uncertainty analysis is to assess whether other effects of BPA may potentially occur after exposure to lower doses than the endpoint on which the reference point (RP) is based (i.e. the effect on Th17 cells) and, if so, inform a decision on what size of additional UF would be suitable to take those effects into account. If required, the additional UF together with the UFs for interspecies differences for toxicodynamics and intraspecies differences for both toxicokinetics[9] and toxicodynamics would then determine the overall UF to be used when deriving the TDI.

For this purpose, the main part of the uncertainty analysis focused on five HOCs (Immunotoxicity, Metabolic effects, Neurotoxicity and developmental neurotoxicity, Reproductive and developmental toxicity, and Carcinogenicity and mammary gland proliferative effects) and, within these, on 21 clusters of endpoints which were rated ALAN, Likely or Very Likely in the WoE assessment (see Chapter 3.1). Consideration of a sixth HOC, General toxicity, was deferred to a later stage (see below). Cardiotoxicity was not considered in the uncertainty analysis because the evidence was judged either as Not likely or as Inadequate in the WoE assessment. The uncertainty analysis concluded with an assessment of the overall uncertainty, where clusters rated less than ALAN and other potentially relevant considerations were taken into account (see below).

The uncertainty analysis was conducted in accordance with EFSA's guidance on uncertainty analysis, using a combination of methods appropriate to each step of the assessment as described below (a 'case-specific' uncertainty analysis, EFSA Scientific Committee, 2018a).

[9] The interspecies differences in toxicokinetics are accounted for by the conversion of the doses into human equivalent doses (HED)

Overall approach

The approach taken for the uncertainty analysis is summarised graphically in Figure 1. The lower part of the figure shows the BMDL taken by the CEP Panel as the RP for risk characterisation. An expert knowledge elicitation (EKE) was conducted for each of the 21 clusters, providing a distribution quantifying uncertainty about the estimated[10] lowest BMD for effects in that cluster that occur in animals and are relevant and adverse for humans.

Two examples of such distributions are illustrated graphically in Figure 1.

The distributions for all 21 clusters were then combined by calculation to obtain a distribution quantifying uncertainty about the estimated lowest BMD across all 21 clusters. Quantiles of this distribution were then used to inform consideration of how large an additional UF (if any) is warranted. This is illustrated by the black two-headed arrow Guide for additional UF at the bottom of Figure 1. Assessing the TDI with this additional UF would then take the 21 clusters of effects into account. For example, taking the fifth[th] percentile from the combined distribution (as illustrated by the diamond symbol at the top of Figure 1) might be considered as equivalent to the normal practice of using the lowest BMDL as the RP. However, the final decision on whether and how large an UF is needed also took account of other sources of uncertainty affecting the assessment and the level of conservatism that is appropriate for setting the TDI required.

The following paragraphs describe the methods used at each stage. Further technical details are provided in the annexes referenced below.

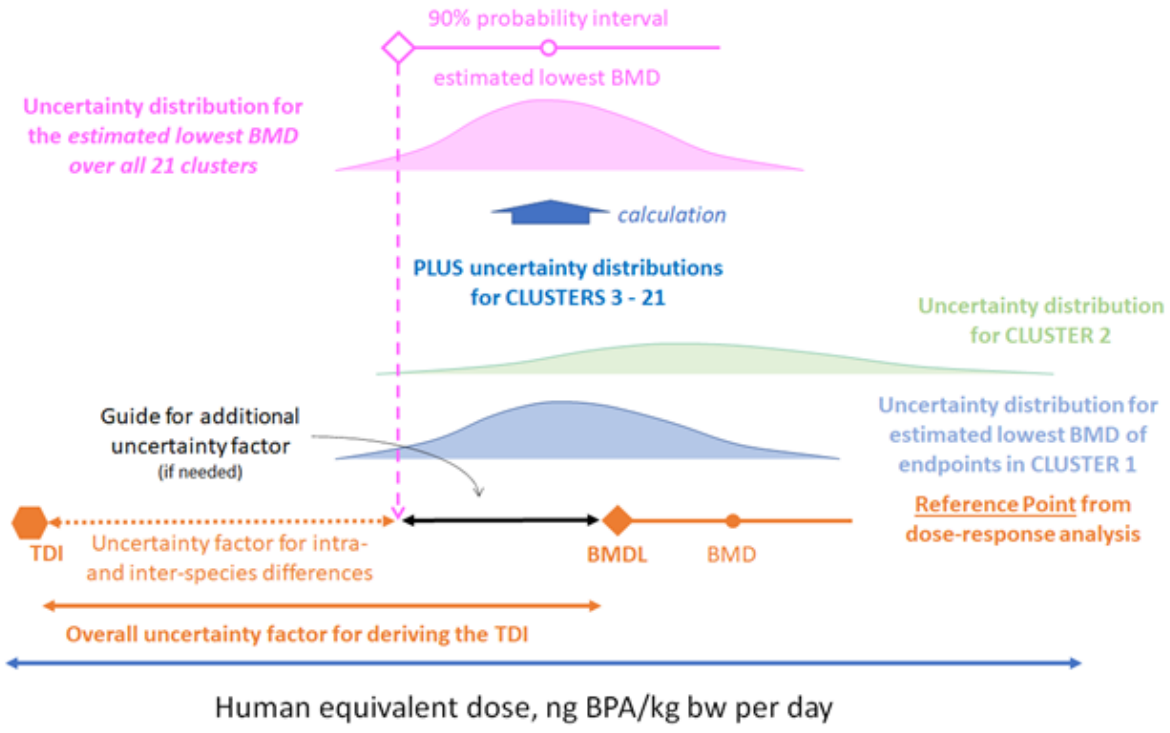


Figure 1: Graphical overview of the approach taken in the uncertainty analysis.

[10] This is referred to as the 'estimated' lowest BMD to reflect that it was assessed by expert judgement, informed by the studies selected from the weight of evidence assessment, and not by a BMD analysis.

Assessment of clusters by expert judgement

Studies to be considered for each cluster were evaluated by two or three experts per cluster, chosen for their expertise on the endpoints in that cluster. An EKE protocol was devised, adapting EFSA's guidance for EKE (EFSA, 2014) to elicit judgements from the experts for two questions per cluster. The short version of Question 1 was 'What is your probability[11] that there is at least one endpoint in the WoE table for this cluster that occurs in animals tested with BPA and is relevant and adverse in humans?'. Question 2 was 'If one or more endpoints in the WoE table for this cluster occurs in animals tested with BPA and is both relevant and adverse for humans, what is your prediction for the lowest BMD of those endpoints, expressed as HED?'. The experts were also provided with a longer version of each question and a list of supporting definitions (see Annex J) to ensure that each question was well-defined and interpreted consistently by the experts, as required by EFSA's EKE guidance.

Experts were asked to provide an approximate probability (i.e. a lower and upper probability, EFSA Scientific Committee, 2018a) in response to Question 1 and a probability distribution for Question 2. It was anticipated that in some cases a multimodal distribution might be needed to take into account that the lowest BMD might come from one of several endpoints within the cluster. To allow for this, the distributions were elicited using the roulette method (EFSA, 2014), in which experts build a histogram for the distribution representing their uncertainty.

For each cluster, the experts based their assessment on the studies rated as Tier 1 or 2 in the WoE assessment (see Annex A, Chapters 6-8). Each expert was provided with an Excel template to record their judgements, together with a summary of the evidence and reasoning they considered for each question. A copy of the Excel template is provided in Annex J. The experts were introduced to the two types of probability judgement involved and to the Excel template and a training exercise was conducted using a relevant example for each type of question. Advice on how to make probability judgements and operate the template was included during the training and also in the template file.

The experts were advised, when making their judgements, to consider all relevant evidence and reasoning of which they were aware, and all identifiable sources of uncertainty. For Question 2, experts were advised to consider the results of BMD analysis when available, as well as no observed adverse effect levels (NOAELs) and lowest observed adverse effect levels (LOAELs), and to take into account the magnitude of effects at the LOAEL and the relevant benchmark response (BMR) for each endpoint. Guidance was provided on how to convert doses reported from animal studies to HEDs and a table of factors for this purpose was provided (Table 1, in Chapter 3.1.1.4–HED factors). Experts worked independently on their judgements over a period of 2 to 3 weeks, during which time additional advice and support was available when needed.

Review and revision of cluster assessments

The experts' judgements and reasoning were reviewed and discussed in a series of half-day web meetings, one for each HOC, where each cluster and question was considered in turn. Each meeting was attended by the experts who made the judgements being reviewed, plus a facilitator and two rapporteurs. For each question, the experts were invited to discuss their judgements and reasoning, which were displayed in a summary file on screen. When discussing Question 2, key studies influencing their judgements were identified and NOAELs, LOAELs and BMDLs/BMDs/BMDUs from those studies were added to a graph displaying the experts' distributions for Question 2, to assist the experts in their discussion. At the end of the discussion of each question, the experts were invited to review their personal judgements and reasoning and to revise them if they wished, in the light of the discussion. The discussion was conducted in a similar manner to that used in the 'Sheffield' EKE method (EFSA, 2014), except that no attempt was made to reach a consensus between experts, partly due to time

[11] The question asks the experts for 'your probability' expressed as a percentage, because these are subjective probabilities which represent the judgement of each expert (EFSA Scientific Committee, 2018a).

limitations and partly because it was considered useful to take account of differences between experts later in the process (see below).

Calculations to derive a distribution for the estimated lowest BMD over the 21 clusters

The experts' revised judgements were combined using the R software (R Core Team, 2021), in a series of steps, described below. The R code that was written for this purpose was reviewed independently and is available at the link provided in Annex K.

In the first step, parametric distributions were fitted to the judgements for Question 2 for each expert in each cluster, using the fitting methods and criteria described in Annex K. The set of parametric distributions comprised normal, t, beta, skewed normal and skewed t plus mixtures of non-skewed normal or t-distributions to provide an appropriate fit for clusters where the experts' roulette histograms were flat or bimodal.

Second, for each cluster and expert, the probability from Question 1 was combined with the distribution from Question 2 by multiplication. The former is the expert's probability that there is at least one endpoint in the cluster that occurs in animals and is relevant and adverse for humans; the latter is their distribution for the estimated lowest BMD in that cluster if there is at least one such endpoint (i.e., conditional on Question 1). Multiplying these provides a cumulative probability function (Cpf) for the estimated lowest BMD of effects that occur in animals and are relevant and adverse for humans. This was repeated for each expert in each cluster, resulting in 2 or 3 cpfs per cluster. As their probability for Question 1 was expressed as a range, each cpf had a lower and upper bound on its curve. The lower and upper bound of the cpf resulted from multiplying the distribution for Question 2 by, respectively, the lower and upper bound of the probability for Question 1.

The third step combined the cpfs for all 21 clusters by a probability calculation to produce the cpf for the estimated lowest BMD across all selected clusters for endpoints that occur in animals and are relevant and adverse for humans, assuming that the judgements for different clusters are independent. The calculation used the same principles that apply when calculating the probability of obtaining 'heads' at least once when tossing a coin two times, and is described in text and equations in Annex K. Quantiles from the cpf for the estimated lowest BMD across clusters are the output required to inform consideration of the need for an additional UF for deriving the TDI (as illustrated by the pink diamond in Figure 1).

As mentioned above, the second step produced 2-3 cpfs per cluster, each with a lower and upper bound. These reflect imprecision in the experts' judgements (the ranges of probabilities provided for Question 1) and differences between experts assessing the same cluster, which are both part of the overall uncertainty. To take account of this, the lower and upper bounds for the 2 or 3 cpfs for each cluster were combined by enveloping, i.e., taking the minimum and maximum cumulative probability at each dose. This reduces the 2-3 cpfs to a single cpf for each cluster, with lower and upper bounds reflecting the imprecision of the Question 1 probabilities and the differences between experts. The calculation for step 3 was then performed twice: once with the lower bound of the cpf for each cluster, producing a lower bound for the cpf for the estimated lowest BMD across clusters; and once with the upper bound of the cpf for each cluster, producing an upper bound for the cpf for the estimated lowest BMD across clusters.

The multiplication in step 1 and probability calculations in step 3 above are based on assuming independence between the cpfs for different clusters. The potential impact of deviations from independence were explored by sensitivity analysis (below).

Sensitivity analysis

A sensitivity analysis was conducted to identify which clusters had most influence on the cpf for the estimated lowest BMD across all clusters, so that these clusters could be subjected to further review (see below). A second sensitivity analysis was conducted to compare the cpf obtained when the

parametric distributions were fitted to the experts' judgements for Question 2 with the cpf obtained when non-parametric distributions were fitted by linear interpolation between judgements for each expert. These two sensitivity analyses were repeated twice later in the assessment, to check whether the results changed when the experts' judgements were revised. A third sensitivity analysis was conducted later in the assessment, to examine the potential impact of deviations from independence of judgements between selected clusters. The sensitivity analyses were performed in R, using code included in Annex KJ, and the results were used to inform the review and discussion of the main calculation results and assessment of overall uncertainty (see below).

Expert review and revision of calculation results

The results of the calculations and sensitivity analysis were reviewed and discussed in a web meeting attended by all the experts who conducted the cluster assessments, plus other members of the WG conducting the BPA assessment and the same facilitator and two rapporteurs as before. This was followed by a facilitated discussion of the cluster with most influence on the cpf for the estimated lowest BMD across all clusters. The two experts who had assessed this cluster described the reasoning for their judgements on Questions 1 and 2. This was followed by an extensive and detailed discussion by the whole group, after which the same two experts (who had specialist expertise on this cluster) revised their judgements in the light of the discussion. Their revised judgements were then used to repeat the calculations and sensitivity analysis and produce revised outputs.

In the same meeting, the WG considered the HOC General toxicity. Comparing the summarised data for General toxicity to the elicited distributions from the other health categories, the experts agreed that it was not necessary to elicit judgements for the General toxicity clusters.

Elicitation with additional experts for the most influential cluster

In the light of the range of opinions expressed when discussing the most influential cluster, it was agreed to elicit judgements on Question 2 for this cluster from all the experts in the WG. This was done at the next meeting. To inform those judgements, the meeting started with a presentation on the biology, mechanisms and health consequences for this cluster of endpoints, discussion of the choice of BMR for the key endpoint in this cluster, review of the dose-response studies available for this cluster and discussion of uncertainties affecting those studies. This was followed by a repetition of the EKE training previously provided, for the benefit of those who had not participated in earlier judgements. The experts were then asked to consider their plausible limits for Question 2 and a brief discussion was held to agree on consensus lower and upper limits that covered the range of their individual limits. The experts were next asked to work separately to make their judgements on the distribution within the consensus limits, using the roulette method and the Excel template that was described above. The judgements were collected and displayed in a table for discussion, together with parametric and empirical (histogram) distributions fitted to each individual's judgements. The reasons for the range of opinion were then explored, by a structured discussion and listing of the evidence and reasoning for the lower and upper ends of the range covered by the experts' individual distributions. No attempt was made to elicit a consensus distribution, because the differences between experts were large and it was important to examine their impact on calculation of the cpf for the estimated lowest BMD across clusters (see below). Instead, the experts were invited to review and revise their individual distributions in the light of the discussion, if they wished. The calculations described earlier were then repeated, using each expert's fitted distribution in turn, to show how the different individual judgements for the most influential cluster affected the cpf for the estimated lowest BMD across all clusters.

Consideration of additional uncertainties and dependencies

The final step of an uncertainty analysis should be assessment of overall uncertainty, combining the results of earlier steps with any additional uncertainties that are not yet quantified (EFSA Scientific Committee, 2018a). For this purpose, the results of sensitivity analysis for selected dependencies between clusters were presented, and a structured discussion was held to elicit a list of additional sources of uncertainty. Considering these, the WG judged that the dependencies and additional

uncertainties would not alter the assessment of overall uncertainty provided already by the range of distributions resulting from calculations using the judgements of different experts for the most influential cluster. The WG therefore agreed to use that range of distributions as the basis for assessing overall uncertainty about the estimated lowest BMD across all clusters. It was also agreed to explore options for integrating or averaging the range of judgements to assist the WG in developing consensus conclusions on the overall uncertainty. The outcome of this was presented and discussed at the next meeting.

Averaging cpfs of different experts for the same cluster

Revised calculations were conducted to explore the effect of aggregating the cpfs of different experts for each cluster by averaging before calculating the cpf of the estimated lowest BMD across clusters. As there is no basis for reducing the approximate probability (range of probabilities) for Question 1 to a precise probability (e.g. the midpoint), the averaging was repeated with the lower and upper bounds of the approximate probability for Question 1.

Within each cluster, the cpfs of different experts for the same cluster were aggregated by taking the unweighted linear pool, which gives equal weight to each expert. The average cpfs for all the clusters were then combined in the same way as in previous calculations (see above). This produced a cpf for the estimated lowest BMD across all clusters, which again had a lower and upper bound reflecting the combined effect of the differences between lower and upper bounds of the approximate probabilities for Question 1 for all clusters. Sensitivity analysis was conducted to show how the lower and upper bounds of the cpfs would change if the WG were to agree on a precise consensus probability for Question 1 for the most influential cluster.

The WG members were asked whether they would agree on a consensus range or precise probability for Question 1 for the most influential cluster. To inform discussion of this, the reasoning of the two experts with specialist knowledge of this cluster were displayed. Next, the WG experts were asked whether they accepted the results of averaging across experts in each cluster as their consensus assessment for the combined cpf for all clusters, or whether they preferred to report multiple cpfs reflecting their differing individual distributions for Question 2 for the most influential cluster. The WG experts' responses were then used in a final repetition of the calculations, producing a lower and upper bound for the consensus cpf for the estimated lowest BMD across all clusters.

Sensitivity analysis for consensus distribution

The sensitivity analyses were repeated using the consensus probability of 66% for Question 1 on allergic lung inflammation and the averaged individual distributions for each cluster. The purpose of this was to check whether the final consensus cpf for the estimated lowest BMD across all clusters was more sensitive to the method of distribution fitting and possible dependencies between clusters than was the case in earlier sensitivity analysis.

Additional UF for deriving the TDI

In the final step in the uncertainty analysis, the WG reviewed the results of the preceding steps and a consensus was sought on whether, when deriving the TDI, an additional UF is needed to allow for uncertainty about whether effects on other endpoints may potentially occur after exposure to lower doses than the endpoint on which the RP was based. To inform this discussion, tabulated percentiles of the cpf for the estimated lowest BMD across all clusters were displayed, based on the lower and upper consensus bounds of the cpf that resulted from the decisions made by the WG in the preceding step. This was accompanied by a parallel table, showing the ratio of the RP (BMDL) to each percentile of the consensus lower and upper bounds of the cpf. The WG was also reminded of the wider range of cpfs that results when individual judgements were not averaged. A final discussion was then conducted to seek consensus on whether an additional UF was needed and, if so, how large it should be.

2.3.4.2. Method for uncertainty analysis for genotoxicity

The purpose of the uncertainty analysis for genotoxicity was to assess the degree of certainty for the conclusion on whether BPA presents a genotoxic hazard by a direct mechanism (direct interaction with DNA), taking into account the available evidence and also the associated uncertainties. This overall question was divided into two sub-questions, which were assessed by three WG members with specialist expertise in genotoxicity assessment. The three experts were asked to express their judgement about each question in the form of an approximate probability (i.e., a lower and upper probability), and to summarise the evidence and reasoning on which their judgement was based.

The experts' judgements were elicited by a structured procedure based on EFSA's Guidance on expert knowledge elicitation (EFSA, 2014), adapted to the context of the genotoxicity assessment. The specific questions to be addressed by the three experts were defined as follows:

- Sub-question 1: What is your probability (%)[12] that there is a genotoxic hazard in humans from BPA?
- Sub-question 2: If there would be a genotoxic hazard in humans from BPA, what is your probability that its causes include a direct mechanism?

The word 'include' in sub-question 2 was introduced to accommodate the possibility that both direct and indirect mechanisms could operate together.

The experts were provided with guidance on how to assess and express their probability judgements for the two questions. They were asked to consider all the data they had reviewed for the genotoxicity assessment, including results from *in vitro* studies and animal models, taking into account their relevance to humans; the available human data were considered not relevant (see Annex L).

The three experts first worked on the questions independently, based on the evidence they had already reviewed and evaluated for the opinion, and recorded their probabilities and the reasoning for their judgements in an excel template similar to that which was used for Question 1 in the uncertainty analysis for non-genotoxic endpoints (see preceding section and Annex M). This was followed by a facilitated meeting, where the three experts presented their judgements and reasoning and discussed them together with the WG Chair. After the meeting, the three experts were invited to review and, if they wished, revise their judgements and reasoning in the light of the discussion.

Each expert's revised probabilities for the two sub-questions were multiplied to provide a probability for the overall question. This is appropriate because the second question is conditional on the first. The first sub-question provides a probability for BPA presenting a genotoxic hazard; the second question provides a conditional probability that, *if* BPA presents a genotoxic hazard, there is a direct mechanism. So the product of these is a probability that both are true: that BPA does present a genotoxic hazard and that there is a direct mechanism. Because the experts' probabilities were approximate (ranges), the calculation is done by interval arithmetic (see Annex B.7 in EFSA Scientific Committee, 2018b) and the resulting probabilities are also approximate.

The three experts presented and discussed their revised judgements and reasoning in a facilitated meeting with the full WG. The WG discussed the results of the calculations combining the experts' probabilities for the two questions and expressed the conclusion of the WG both as a probability range and using verbal likelihood terms from the approximate probability scale, which is recommended by EFSA (Table 2 in EFSA Scientific Committee, 2018a) for harmonised use in EFSA assessments. Finally, the WG discussed the implications of their conclusion for whether a TDI could be set for BPA or whether a Margin of Exposure approach was required.

[12] The question asks the experts for 'your probability' expressed as a percentage, because these are subjective probabilities which represent the judgement of each expert (EFSA Scientific Committee, 2018a).

2.3.5. Method for assessing genotoxicity

The evaluation of data quality for hazard/risk assessment includes the evaluation of reliability and relevance (Klimisch et al., 1997; OECD, 2005; ECHA, 2011; EFSA Scientific Committee, 2017c; EFSA Scientific Committee, 2021a).

In the assessment of genotoxicity studies, the data quality has been evaluated based on reliability and relevance. Reliability has been assessed using a scoring system based on criteria published by Klimisch et al. (1997).
In a second step, the relevance (high, limited, low) of the study results was assessed based on reliability of the study and other aspects, e.g. genetic endpoint, purity of test substance, route of administration and status of validation of the assay.

Genotoxicity studies evaluated as of high or limited relevance have been considered in a WoE approach. Genotoxicity studies evaluated as of low relevance have not been further considered in the assessment.

The different steps of the evaluation are described in the following sections.

2.3.5.1. Evaluation of reliability of results of genotoxicity studies – general considerations

Reliability is defined as "evaluating the inherent quality of a test report or publication relating to preferably standardized methodology and the way that the experimental procedure and results are described to give evidence of the clarity and plausibility of the findings" (Klimisch et al., 1997).

In assigning the reliability score, the compliance with the Organization for European Economic Cooperation and Development (OECD) Test Guidelines (TGs) or standardized methodology and the completeness of the reporting as detailed below were considered.

The reliability scores were:

1) reliable without restriction
2) reliable with restrictions
3) insufficient reliability
4) reliability cannot be evaluated
5) reliability not evaluated, since the study is not relevant and/or not required for the risk assessment (in case the study is reported for reasons of transparency only).

These reliability scores were defined as follows (Klimisch et al., 1997):

1) Reliable without restriction
   "This includes studies or data from the literature or reports which were carried out or generated according to generally valid and/or internationally accepted testing guidelines (preferably performed according to Good Laboratory Practice (GLP)) or in which the test parameters documented are based on a specific (national) testing guideline (preferably performed according to GLP) or in which all parameters described are closely related/comparable to a guideline method."

2) Reliable with restrictions
   "This includes studies or data from the literature or reports (mostly not performed according to GLP), in which the test parameters documented do not totally comply with the specific

testing guideline, but are sufficient to accept the data or in which investigations are described which cannot be subsumed under a testing guideline, but which are nevertheless well documented and scientifically acceptable."

3) Insufficient reliability[13]

"This includes studies or data from the literature/reports in which there are interferences between the measuring system and the test substance or in which organisms/test systems were used which are not relevant in relation to the exposure (…) or which were carried out or generated according to a method which is not acceptable, the documentation of which is not sufficient for an assessment and which is not convincing for an expert judgment."

4) Reliability cannot be evaluated[14]

"This includes studies or data from the literature, that do not give sufficient experimental details and that are only listed in short abstracts or secondary literature (books, reviews, etc.)."

In case a study is reported for reasons of transparency only, a further score (5) may be required.

5) Reliability not evaluated
The study is not relevant and/or not useful for the risk assessment. The following references (Klimisch et al., 1997; OECD, 2005; ECHA, 2011; EFSA Scientific Committee, 2011, EFSA Scientific Committee, 2017c; EFSA Scientific Committee, 2021a) may be consulted for more details and examples.

Each reliability box in the summary tables (see Annex L) started with the reliability score, followed by comments justifying the score. This is equally applicable for *in vitro* as *in vivo* studies.

2.3.5.2. Evaluation of relevance of results of genotoxicity studies - -general considerations

The relevance of the study (high, limited or low) is based both on its reliability and on the relevance of the test results.

The relevance of the test results was mainly, but not exclusively, based on:

- Genetic endpoint (high relevance for gene mutations, structural and numerical chromosomal alterations as well as results obtained in an *in vivo* comet assay, which belongs to the assays recommended by the EFSA Scientific Committee (2011) for the follow-up of a positive *in vitro* result; lower relevance for other genotoxic effects). Other test systems although potentially considered of limited or low relevance may provide useful supporting information.
- Route of administration (e.g. oral vs. intravenous, intraperitoneal injection, subcutaneous injection, inhalation exposure) in case of *in vivo* studies.
- Status of validation (e.g. for which an OECD TG exists or is in the course of development, internationally recommended protocol, validation at national level only, no validation).
- Reliability and relevance of the test system/test design irrespectively of whether a study has been conducted in compliance with GLP or not.

[13] Klimisch et al. (1997) used the term "Not reliable", however, "Insufficient reliability" was considered more appropriate.
[14] Klimisch et al. (1997) used the term "Not assignable", however, "Reliability cannot be evaluated" was considered more appropriate.

- Information on BPA purity grade and/or the supplier. If only the supplier was available, the company's website was consulted to retrieve the purity grade, or the authors were contacted to ask for it. If none of the two information were reported or obtained, the relevance was considered low and the study was excluded from the WoE assessment.

Studies for which the relevance of the result was judged to be low were not considered further.

2.3.5.3. Presentation of the study evaluations

All evaluated studies were summarised, either in a narrative form (Appendix E) or in tables (reported in Annex L), to structure the outcome of the evaluations in a transparent way and to provide a possibility to consider the relevance of study results in a weight-of-evidence approach. Remarks were included in the column "Reliability" and assigned relevance to the test results to justify the judgments. Minor and/or major deviations from OECD TGs were reported in column "Reliability" (e.g. lack of positive control, inappropriate exposure conditions, limited reporting etc.).

In these tables, the studies were grouped based on genetic endpoints or test systems and chronologically within these groups. The results were evaluated and presented as positive, negative, equivocal or inconclusive. If considered relevant for the interpretation of the genotoxicity endpoints, non-genotoxicity endpoints (e.g. reactive oxygen species (ROS) production) were reported in a narrative way only, but the results were not classified as "positive" or "negative".

2.3.5.4. WoE approach

The WoE approach applied to the evaluation of genotoxicity data is based on EFSA Scientific Committee recommendations (EFSA Scientific Committee, 2011, 2017b,c). As recommended by the EFSA Scientific Committee (EFSA Scientific Committee, 2011, 2017b), a documented WoE approach for the evaluation and interpretation of genotoxicity data' has been applied, taking into account not only the quality and availability of the data on genotoxicity itself, but also all other relevant data that may be available. These include data on MoA and on toxicokinetics when available. The main steps of the WoE approach applied in the genotoxicity assessment of BPA are described below.

Assembling of the evidence into lines of evidence of similar type

In a first step, the CEP Panel evaluated all available *in vitro* and *in vivo* studies addressing the three main endpoints of genotoxicity: gene mutations, structural and numerical chromosomal aberrations (CA) in addition to DNA damage endpoint (evaluated by Comet assay). The study results addressing each of these endpoints were grouped into lines of evidence. Only the studies of high and limited relevance were included.

Studies investigating the BPA MoA were considered, e.g. DNA oxidation, ROS (when genotoxicity was also investigated in the same study), DNA binding, interference with proteins involved in chromosome segregation during cell division, modulation of expression of genes involved in DNA repair or in chromosome segregation and markers of DNA double strand breaks (DSBs) (e.g. γH2AX). Evidence from the mechanistic studies may support the lines of evidence for the genotoxicity endpoints.

Weighting of the evidence

A quantitative method to weight the evidence was not considered appropriate due to the quantity and heterogeneity of the evidence to be integrated. A qualitative method based on expert judgment was applied. All studies evaluated for reliability and relevance (as described above) were listed in tables (Annex L). The evaluation of the studies of high and limited relevance was described in the opinion, including the conclusion for each line of evidence. The consistency of the evidence was assessed and presented in the opinion.

Integrating all the evidence

The lines of evidence of the above genotoxicity endpoints were assessed separately. To elucidate the MoA of BPA, mechanistic studies were considered.

Integrating evidence from the MoA with lines of evidence from genotoxicity endpoints allows a reduction in the uncertainty on the potential genotoxicity. In case genotoxic effects were observed, evidence from the MoA may allow clarification if the genotoxicity is due to a direct or indirect mechanism.

3. Assessment

3.1. Hazard identification

3.1.1. Toxicokinetics and metabolism

3.1.1.1. Outcomes of the 2015 BPA opinion

Species-stage and life-stage dependent differences in the toxicokinetic profile of BPA must be considered when comparing toxicokinetic data from different species.
BPA-glucuronidation 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 lesser extent. 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 (2007) reported oxidative BPA metabolites to also occur in mice.
The oral systemic bioavailability of unconjugated BPA in adults is 2.8% in rats, 0.45% in mice and 0.9% in monkeys, based on oral versus intravenous (i.v.) toxicokinetic data. Lower concentrations of unconjugated BPA and BPA conjugates were measured in the amniotic fluid of rats and of monkeys in comparison with the serum levels. In early pregnancy, exposure of the fetus at the same dose (in µg/kg body weight of the pregnant animal) might be higher than in later pregnancy because of the development of metabolising enzymes in the liver of the fetus 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 (BPA-G) comprises approximately 80% of the total BPA concentration. Pup exposure via lactation is low, i.e. approximately 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 polymorphically expressed in humans.

The default intraspecies UFs used to derive a health-based guidance value (HBGV) are considered sufficient to account for possible differences in rates of metabolism of BPA between human individuals. Data from toxicokinetic studies in various laboratory animal species provide the basis for internal dose conversion metrics for neonatal-to-adult stages and for different routes of exposure. Moreover, physiologically-based pharmacokinetic (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 the application of the human equivalent dose (HED) concept for providing HEDs for points of departure derived from critical 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 simulated 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 absorption.

The CEF Panel did not consider skin metabolism, as the conjugation enzymes involved in the metabolism of BPA are expressed at a very low level. 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.

3.1.1.2. New data on BPA toxicokinetics

Publications on BPA toxicokinetics (published from 2013 to 2018, and not considered in the 2015 opinion) were assessed in a narrative way and are reported here.

Animal data

Mice

The group of deCatanzaro (Pollock et al., 2014 [RefID 5870], 2017a [RefID 5869], 2017b [RefID 5871]; Borman et al., 2017 [RefID 655]; Pollock et al., 2018) performed a series of studies in which they investigated the effect of triclosan, diethylhexyl phthalate, butyl paraben, propylparaben and tetrabromobisphenol A, with and without concurrent triclosan, as well as a mixture of triclosan, tetrabromobisphenol A, butyl paraben, propylparaben and diethylhexyl phthalate on the serum and tissue concentrations of ^{14}C-BPA, which were given concomitantly, in female and male mice. The co-administration of the substances influenced the tissue distribution of ^{14}C-BPA by increasing the radioactivity of the one single measured serum sample, taken 1 h after administration, and of some tissue levels.
As no investigation was performed to elucidate the underlying mechanism for the higher radioactivity, the data are of limited interest for this risk assessment of BPA.

Draganov et al. (2015) [RefID 1689] investigated the kinetics of BPA in neonatal mice on post-natal day (PND) three following single-dose oral or subcutaneous (s.c.) administration. The study was divided in three parts: (i) a mass-balance part to confirm the mode of administration, (ii) a pharmacokinetic part, in which total radioactivity was measured in female PND3 rats, and (iii) a metabolic profiling part, in which two groups of rats received ^{3}H-BPA either orally or subcutaneously. Blood from five rats per time point was collected and their plasma was pooled for analysis of total radioactivity and analysis of BPA, BPA-G and BPA-sulfate (BPA-S), after high-performance liquid chromatography (HPLC) separation and fractionated measurements. The method was validated by liquid chromatography-tandem mass spectrometry (LC-MS/MS) confirmation of the radioactive peaks. The authors evaluated several techniques for administration and found a technique with a recovery of 92.3 ± 3.36% (oral) and 88.2 ± 2.9% (by s.c. administration) being sufficiently precise for performing part (ii) and (iii) of the study. The AUC of plasma concentration–time profile of the total radioactivity was 1879 ng/g × h following oral and 1576 ng/g × h following s.c. administration of 400 µg/kg bw ^{3}H-BPA. The AUC for BPA, BPA-G and BPA-S was 15 ng/g × h, 1065 ng/g × h and 33.8 ng/g × h after oral administration and 49.8 ng/g × h, 913 ng/g × h, 31.2 ng/g × h after s.c. injection, indicating first pass metabolism by the oral route of administration. The AUC of non-conjugated BPA in this study of 15 ng/g × h (or 65.8 nM × h) with a dose of 400 µg/kg bw compared well with the AUC of 26 nM × h reported by Doerge et al. (2011) for a dose of 100 µg/kg bw given to neonatal mice on PND3.

Pollock and deCatanzaro (2014) [RefID 5867], investigated the distribution of BPA into several organs in mice with special focus on the uterus. In one experiment, performed in 4-month-old female mice, doses of 0.5 µg/kg, 5 µg/kg or 50 µg/kg ^{14}C-BPA were given by the oral route and the distribution of the radioactivity was measured. In another experiment, 50 µg/kg ^{14}C-BPA was administered by the oral route to 4-month-old female mice either once, or dosing for 7 or 28 days, and the distribution of radioactivity in several organs was measured. In all the experiments, ^{14}C-BPA measurements were done 1 h after the dosing. Radioactivity was measurable in the organs investigated and also in the serum following all doses with the exception of 5 µg/kg bw. In this dose group, no radioactivity could be measured in the olfactory bulb, cerebellum, frontal cortex and hypothalamus. Following repeated dosing (50 µg/kg ^{14}C-BPA), the concentration in the uterus was higher than in heart, lung, muscle and adipose tissue after 28 but not after 7 days treatment; in this experiment concentration in the ovary was also higher than in heart, lung, muscle and adipose tissue following 28 days treatment. This study does not contribute to an interspecies extrapolation of the kinetics.

Rat

Pollock and deCatanzaro (2014) [RefID 5867] also investigated the distribution of BPA into several organs in rats with special focus on the uterus. In the first experiment, the distribution of ^{14}C-BPA (dose 50 µg/kg bw by the oral route) was studied in cycling female rats either without pre-treatment or with pre-treatment with ICI 182.780, an oestrogen antagonist, or E2. In the second experiment, the distribution of ^{14}C-BPA (dose 50 µg/kg bw by the oral route) was studied in inseminated female rats.

In the third experiment, the concentration of the parent compound (aglycone) versus the total BPA in the rat uterus was the focus of the study following 50 µg/kg BPA on the oral route. In all the experiments, ^{14}C-BPA measurements were done 1 h after the dosing. Radioactivity was measurable in the organs investigated, in the serum, following all doses with the exception of 5 µg BPA /kg bw. In this dose group, no radioactivity could be measured in the olfactory bulb, cerebellum, frontal cortex and hypothalamus. In oestrus cycling rats, in inseminated rats (for every of the three doses), the concentration of radioactivity in the uterus was higher compared with that in heart, lung, muscle, adipose tissue and ovary. Following repeated dosing in mice (50 µg/kg bw of ^{14}C-BPA), concentration in the uterus was higher than in heart, lung, muscle and adipose tissue after 28 but not after 7 days treatment; in this experiment concentration in the ovary was also higher than in heart, lung, muscle and adipose tissue following 28 days treatment. In uterine samples from cycling rats, the concentration of the aglycone was 71.9 ± 3.4% of the total BPA concentration. As it is known that more than 90% of the total BPA in the plasma of rats is conjugated BPA (e.g. Churchwell et al., 2014), it is most probable that the higher concentration of aglycone found in this study is due to deconjugation of the BPA conjugates during sample preparation. This study does not contribute to an interspecies extrapolation of the kinetics.

The study of Kazemi et al. (2016a) [RefID 11132] was performed in three groups of male rats that received 5, 25 and 125 µg/kg bw per day of BPA, by gavage, for 35 consecutive days. The number of rats per group was not given. Three other groups of rats received nonylphenol (doses 5, 25, and 125 µg/kg bw per day also for 35 days). The results of this part of the study will not be reported here. At the end of the study, 2 mL blood samples were taken and BPA was measured in the serum. Specimens of the testes were also obtained and after processing the concentration of BPA was measured. The analytical method used HPLC separation and fluorescence detection. Neither limit of detection (LOD) nor limit of quantification (LOQ) for the method are given. The authors report concentrations of 0.03 µg/mL, 0.3 µg/mL and 1.13 µg/mL BPA in serum and 0.3 µg/mL, 0.82 µg/mL and 3.59 µg/mL BPA in the testes with doses of 5, 25, 125 µg/kg bw per day. The concentrations are several orders of magnitude higher than those reported from other authors when using specific methods (use of labelled BPA and LC/MS/MS) (Churchwell et al., 2014). The analytical method used does not give a BPA-specific signal and therefore, the results of this study do not contribute to the knowledge on BPA kinetics.

In the course of a study, aimed at investigating the effect of increasing doses of BPA (0.5, 5, 50 and 250 mg/kg bw per day) on the development of bile duct proliferation by Jeong et al. (2017) [RefID 3133], toxicokinetic studies were carried out. Plasma samples were taken on day 1 (PND6) and day 91 of the study at 0.25, 0.5, 1, 2, 4, 8 and 24 h after dosing by gavage and BPA was determined using a specific and sensitive method. AUC and Cmax were determined. On day 91, the plasma levels following 0.5 mg/kg bw and 5 mg/kg bw were below the level of quantification (1.68 ppb= 1.68 µg/L = 7.4 nM). The AUCs were 26 times (50 mg/kg bw) and 125 times (250 mg/kg bw) higher on day 1 (PND6) than on day 91, consistent with an early life (PND6) lower expression of conjugation enzymes of BPA. A marked supra-linear increase of AUC with dose indicated saturation of metabolism in PND6 animals at such high doses.

Other animals

The study of Collet et al. (2015) [RefID 1268] aimed to predict the clearance in humans by allometric scaling based on concentration–time data after intravenous administration of 5 mg/kg bw BPA to mice, rats, dogs, piglets, ewes and horses. From the data, plasma clearance was calculated and resulted in 0.00078 (mice), 0.019 (rats), 0.31 (dogs), 1.3 (piglets), 1.6 (ewes) and 6.2 (horses) L/min, values close to the hepatic blood flow in the respective species. The human clearance was estimated to be 1.79 (95th prediction interval 0.36–8.83) L/min, which exceeded the human liver blood flow indicating a possible clearance in the gut wall.

In the study of Gauderat et al. (2016) [RefID 2219], fetal sheep at the end of pregnancy were exposed to an intravenous (iv) administration of both BPA and BPA-G at the same molar dose (21.9 µmol/kg; corresponding to 5 mg/kg bw BPA and to 8.86 g/kg bw BPA-G). After BPA administration, BPA disappeared from the plasma with a half-life of 0.31 h and BPA-G and BPA-S were found in the plasma, BPA-G being the main metabolite. Both BPA-G and BPA-S had a half-life longer than the parent compound. After BPA-G administration, its half-life was 28 h and not only BPA-G was detected in the plasma of the fetal sheep but also BPA, however, in very low concentrations (200-fold to 8000-fold less than BPA-G), indicating that BPA-G could be de-glucuronidated in the fetal sheep. In the maternal

plasma BPA, BPA-G and BPA-S were found following i.v. administration of BPA to the fetal sheep, whereas BPA-G was the only molecule determined in the maternal plasma after BPA-G administration to the fetal sheep. This indicates that BPA, BPA-G and BPA-S are transported from the fetal blood via the placenta into the maternal blood. The ratios between fetal and maternal plasma concentrations indicate that BPA and PBA-S are better transported than BPA-G.

BPA kinetics was studied in the adult sheep, 2 weeks after termination of the pregnancy. After BPA administration, BPA disappeared from the plasma with a half-life of 1.6 h; BPA-G was the main metabolite with a half-life of 0.84 h. BPA-S was also formed and disappeared with a half-life similar to that of BPA.

Repeated dosing of BPA-G (five doses) produced similar low BPA levels than after single dosing. Similarly, low BPA levels could also be measured in the amniotic fluid after five doses of BPA-G to pregnant sheep.

For the extrapolation to the human situation, it should be considered that the study was performed in sheep at the end of pregnancy. In the human fetus, extremely low expression of glucuronidation enzymes has been reported up to 20 weeks; until this time enzyme protein levels were not detectable (Divakaran et al., 2014 [RefID 1631]; Coughtrie, 2015). At birth, the levels were 10% of the levels expressed in older infants and in adults. In the human fetus, BPA can only be glucuronidated to a very small extent; the process described in the fetal sheep of releasing a small extent of BPA from BPA-G may also take place in humans but the resulting BPA concentration would be low.

In the study of Guignard et al. (2016) [RefID 2450], four ewes received a dose of 100 mg/kg bw BPA by nasogastric gavage and 13 days apart of 10 mg/kg bw BPA through food pellets. Blood was taken from the jugular vein in short intervals in the first 2 h and then every second hour until 10 h post dosing as well as 24 h after dosing. The first blood sample was taken 1.8 min after ingesting the pellets and 4.8 min after nasogastric feeding. BPA and BPA-G in serum was estimated by LC-MS with a low inter- and intraday variation (<15%) and a LOQ of 1 ng/mL for BPA and 5 ng/mL for BPA-G. It is remarkable that in the first 2 h the serum concentration following pellet ingestion was equal or even higher than following nasogastric feeding despite the fact that the dose was 10-fold lower. The AUC-BPA/dose was 2-fold to 4.5-fold lower following nasogastric feeding than following pellet ingestion. This finding together with the higher ratio of BPA-G BPA following nasogastric feeding indicate high first pass metabolism on this mode of administration, whereas administration by food pellets with long-time contact to the buccal membranes indicate absorption of BPA directly into the systemic circulation. Therefore, the systemic availability of BPA administered by food pellets in ewes was higher than by nasogastric feeding [relative bioavailability of nasogastric feeding vs food pellets was 31 ± 5%; mean ± standard error mean (SEM)]. The authors compared the AUCs after i.v. administration to calculate absolute bioavailability with the AUCs following nasogastric tubing and following administration by food pellets. The absolute bioavailability was calculated to amount to 0.8 ± 0.2% and 2.7 ± 0.3% (mean ± SEM) by nasogastric feeding and by food pellets, respectively.

In the study of Guignard et al. (2017) [RefID 2451], the authors compared the relative systemic availability following oral (by pellets) versus s.c. administration of BPA at a dose of 10 mg/kg bw and 5 mg/kg bw, respectively, in four ewes (50–70 kg). The serum concentrations of BPA, BPA-G and BPA-S were measured by a reliable method [LC-MS with a low interday and intraday variation (<15%) and a LOQ of 1 ng/mL for BPA and 5 ng/mL for BPA-G]. When comparing the AUCs, the relative systemic availability of the oral dose was 3.3 ± 0.3%. The AUCs of the BPA-G were similar after adjustment for dose. The ratio of the AUC-BPA-G/AUC-BPA was 25 higher following oral compared with s.c. administration due to the high first pass effect. In the second part of the study, doses of 0.5 µg/kg bw, 50 µg/kg bw and 5000 µg/kg bw by s.c. administration were continuously administered throughout the pregnancy. The serum BPA profile on gestation day (GD) 62 was in accordance with the predicted values following a dose of 5000 µg/kg bw using a bi-compartmental model parametrised with parameters from the first part of the study with 5 mg/kg bw s.c. BPA. Two weeks before the expected term a caesarean section was performed. Maternal and fetal serum from the jugular vein, cord blood and amniotic fluid were obtained at delivery. Concentrations could not be measured for the 0.5 µg/kg bw dose and not in all animals in the medium dose of 50 µg/kg bw. In cord blood, fetal blood and amniotic fluid, the BPA-G and BPA-S concentrations were much higher than BPA ones (100- to 1000-fold and 2- to 50-fold, respectively). The BPA concentration in the fetal serum was 2–5-fold lower than

the concentration in cord blood and in the amniotic fluid. In the maternal blood, the concentration of BPA was 7.5-fold lower than the concentration of BPA-G and 4-fold higher than the concentration of BPA-S, the findings being compatible with escaping first pass metabolism by the s.c. administration in the ewe, whereas by delivery to the fetal circulation via cord vessel BPA undergoes first pass. BPA in the amniotic fluid is due to renal elimination of BPA-G and de-glucuronidation as was demonstrated in the earlier study of Gauderat et al. (2016) [RefID 2219].

Human data

Ten male adult volunteers were exposed to 30 µg/kg bw deuterated BPA (d6-BPA) dissolved in tomato soup, via the oral route (Teeguarden et al., 2015b [RefID 7155]). Blood samples and urine samples were collected at frequent intervals up to 24 h. BPA, total BPA, BPA-G and BPA-S were determined in serum and in urine using a specific and sensitive LC-ES/MS/MS analytical method. The plasma BPA half-life for distribution and terminal elimination were 0.87 ± 0.3 h (mean ± SEM) and 5.5 ± 0.5 h (mean ± SEM), respectively. C_{max} of aglycone was 0.43 ± 0.14 nM (SEM). AUC of aglycone was 2.5 ± 0.4 nM × h (SEM). The administered dose was excreted to 104 ± 2.6 % (SEM) as total BPA in the urine within 24 h. Concerning the conjugates, further metabolites, such as a mixed BPA-glucuronide/sulfate bis-conjugate and BPA-bis-sulfate, have been identified.

In the study of Thayer et al. (2015) [RefID 7183], 14 adults (six men and eight women) received 100 µg/kg bw deuterated BPA (d6-BPA), applied to a cookie, by the oral route. Blood and urine samples were taken at frequent intervals and aglycone and total BPA were determined in serum and in urine using a specific and sensitive LC-ES/MS/MS analytical method, BPA-G and BPA-S conjugates were determined in urine. The plasma BPA half-life for distribution and terminal elimination were 1.2 ± 0.32 h and 5.6 ± 1.2 h, respectively. No significant difference was found between the terminal half-life of total BPA (6.4 ± 2.0 h) and that of BPA. C_{max} of the aglycone was 6.5 ± 3.2 nM, 0.39 ± 0.17% of the total BPA concentration of 1711 ± 495 nM. AUC of aglycone accounted to be 23 ± 6.2 nM × h, 0.56 ± 0.16% of the AUC of total BPA. Within 48 h, 95 ± 7.1% of the administered dose was excreted as total BPA in the urine and 0.11 ± 0.19% of the total BPA was BPA aglycone. Concerning the conjugates, BPA-G was the main metabolite with 87 ± 6.9% in the urine, whereas BPA-monosulfate accounted for 3 ± 2.3%. There was some indication that further conjugates occur in humans, such as a mixed BPA-glucuronide/sulfate bis-conjugate and BPA-bis-sulfate.

PBPK models

A previously developed PBPK model for BPA in adult rhesus monkeys (Fisher et al., 2011) was modified to characterise the pharmacokinetics of BPA and its phase II conjugates in adult humans following oral ingestion (Yang X et al., 2015). By the oral route, metabolism is taking place in the enterocytes lining the gastrointestinal tract and in the liver. Therefore, data from *in vitro* studies on BPA metabolism in the liver and in the small intestine were used and the PBPK model was parameterised using oral pharmacokinetic data with deuterated BPA (d6-BPA) delivered in cookies to adult humans after overnight fasting. The availability of the serum concentration–time course of unconjugated d6-BPA offered direct empirical evidence for the calibration of BPA model parameters.

The recalibrated PBPK adult human model for BPA was then evaluated against published human pharmacokinetic studies with BPA (Teeguarden et al., 2015b [RefID 7155]; Thayer et al., 2015 [RefID 7183]). A hypothesis of decreased oral uptake was needed to account for the reduced peak levels observed in adult humans, where d6-BPA was delivered in soup and food was provided before BPA ingestion, suggesting the potential impact of dosing vehicles and/or fasting on BPA disposition. With the incorporation of Monte Carlo analysis, the recalibrated adult human model was used to address the interindividual variability in the internal dose metrics of BPA for the US general population. Model-predicted peak BPA serum levels were in the range of pM, with 95% of human variability falling within an order of magnitude.

This recalibrated PBPK model for BPA in adult humans provides a scientific basis for assessing human exposure to BPA that can serve to minimise uncertainties incurred during extrapolations across doses and species.

In the Teeguarden et al. (2015b) [RefID 7155] study, the Yang et al. (2015b) model was used to address the question whether buccal absorption has taken place in this study in which serum

concentrations of BPA were measured following intake of deuterated BPA in tomato soup (see description of the Teeguarden et al., 2015b [RefID 7155] study above). The authors were of the opinion that the results of the pharmacokinetic model simulations together with a faster appearance half-life of the metabolite d6-BPA-G compared with d6-BPA (0.29 h vs 0.45 h) were evidence against a meaningful absorption of BPA in humans through any non-metabolising tissue following BPA administration in tomato soup on the oral route.

Karrer et al. (2018) [RefID 12289] adjusted the model developed by Yang et al. (2015b) by modifying the maximal velocity of the glucuronidation in the small intestine. This was scaled up to the body weight for comparing the pharmacokinetic behaviour of BPA with that of bisphenol S (BPS), bisphenol F (BPF) and bisphenol AF (BPAF). The model was extended by including dermal exposure and the Thayer et al. (2015) [RefID 7183] data were used to calibrate the model. In the context of this assessment, it is noted that the predicted AUC 0–24 h for a dose of 30 µg/kg bw (the dose used by Teeguarden et al., 2015b [RefID 7155]), was 4.15 (2.91–5.15) nM × h, whereas the experimental value, measured by Teeguarden et al. (2015b) [RefID 7155], was 2.5 (1.4–5.7) nM × h.

3.1.1.3. Summary of the new toxicokinetic outcomes

Several studies in animals and in humans were published after the closing date for literature review in the previous EFSA opinion on BPA (EFSA CEF Panel, 2015).

Most studies in mice and rats do not report new findings that may contribute to a better understanding of the kinetics of BPA, since total radioactivity was measured without separation of parent compound and metabolies.Two studies (Draganov et al. (2015) [RefID 1689] and Jeong et al. (2017) [RefID 3133]) confirmed earlier findings in mice (Doerge et al., 2011) and rats on the immature metabolism of neonatal mice and rats (Doerge et al., 2010).

Collet et al. (2015) [RefID 1268] investigated the concentration–time data and the derived clearance after intravenous administration to several species (mouse, rat, dog, piglet, ewe, horse). As the data have been obtained after intravenous administration, they may be used to estimate the absolute bioavailability of BPA but are not suitable for deriving the HEDFs in the context of this assessment in which only effects observed after oral administration are considered.

In the Gauderat et al. (2016) [RefID 2219] study, fetal sheep at the end of pregnancy received an intravenous administration of BPA and also BPA-G. After BPA-G administration, not only BPA-G was detected in the plasma of the fetal sheep, but also BPA, though in very low concentrations (200-fold to 8000-fold less than BPA-G), indicating that BPA-G could be partially de-glucuronidated in the fetal sheep. It was also shown that glucuronidation takes place in the fetal sheep with only a small fraction being transported through the placenta to the ewe. For the extrapolation to the human situation it should be considered that in the human fetus extremely low expression of glucuronidation enzymes was reported (Divakaran et al., 2014 [RefID 1631]; Coughtrie, 2015). At birth, the levels were 10% of the levels expressed in infants with a higher age and in adults. Hence, in the human fetus, BPA could be glucuronidated to only a very small extent; the process described in the fetal sheep of producing BPA from BPA-G may take place, but the resulting BPA concentrations would be very small.

Guignard et al. (2016) [RefID 2450] described different systemic availability of BPA when given by nasogastric tubing or as pellets to four ewes. They calculated the absolute bioavailability as 0.8 ± 0.2% and 2.7 ± 0.3% (mean ± SEM) by nasogastric tubing and by food pellets, respectively, by comparing the results of this study with those following intravenous administration. The difference in bioavailability suggests that BPA, when administered as pellets and masticated by the ewes, can be absorbed through the buccal mucosa, taken up in the systemic circulation and therefore escape pre-systemic elimination. This leads to a higher systemic bioavailability compared with direct administration in the gastrointestinal tract.

Two studies in human volunteers (Teeguarden et al., 2015b [RefID 7155] and Thayer et al., 2015 [RefID 7183]) were available after the publication of the 2015 EFSA opinion. In the study of Thayer et al., BPA was applied to a cookie and after drying chewed down (no time frame given), whereas in the

study of Teeguarden et al. (2015b) [RefID 7155], BPA was added to tomato soup which was eaten within 11 min. In both studies a highly sensitive and specific method was used for the measurements of BPA and its conjugated metabolites. It is noted that the dose-corrected AUC of BPA (aglucon) is 2.8 fold higher in the study of Thayer et al. (2015) [RefID 7183] compared with the dose-corrected AUC of BPA (aglucon) in the study of Teeguarden et al., 2015b [RefID 7155]).

3.1.1.4. Human equivalent dose (HED)

In the previous EFSA opinion on BPA (EFSA CEF Panel, 2015), the extrapolation from the RP to the TDI was performed using an approach by which the toxicokinetic standard subfactor for the interspecies extrapolation was substituted by a BPA-specific HEDF. The HEDF is calculated by dividing the AUCs of unmetabolized (parent) BPA of animals (e.g. mice or rats) by the AUCs of humans (AUC animal/AUC human), both AUC values being corrected for the dose (AUC [corrected for dose] animal/AUC [corrected for dose] human).

The concentrations used for the calculation of the AUCs are those in the blood of the systemic circulation that is the relevant metric for the exposure of organs/tissues others than the liver. They are measured after orally administered BPA is absorbed (100%, Völkel et al., 2002) and pre-systemically metabolised in the enterocytes of the gut wall and the liver cells. Because of the high metabolic capacity of the enterocytes and the liver cells, most of the BPA is metabolised. The amount of unmetabolized BPA released into the circulation, the systemic availability, is roughly between 0.45% (mice) and 6% (rats) of the oral dose, leading to concentrations in the systemic circulation of below 1 nM after a dose of 100 µg/kg bw. Human data on i.v. administration, which are needed to calculate the systemic availability, are lacking, because toxicokinetic data are available only following oral administration.

Concerning the liver, the situation is different from other organs/tissues. Before reaching the liver, BPA is metabolised only by gut enterocytes. Therefore, the concentration entering the liver is higher than that leaving the liver into the systemic circulation. However, for effects in the liver, the liver concentration and the related AUC is the relevant metric. No data were available on the proportion of the pre-systemic elimination of BPA from enterocytes in the gut wall and from the liver cells. This would be needed for parameterising a PBPK model allowing the prediction of the concentration in the liver and the related AUC. In the absence of such data, the AUC in the peripheral blood was used also for the liver to substitute the toxicokinetic part of the UF by an HEDF. For all species this is a conservative assumption, because the concentration in the liver and the related AUC are higher than in the systemic circulation, i.e. when using the AUCs in the systemic circulation, effects are assumed to occur at lower concentrations then in reality.

The uncertainty related to this procedure is due to differences in the extent of pre-systemic enterocyte metabolism between species, the liver blood flow and the liver weight, whereas it can be assumed that the partitioning from blood to liver tissue does not differ between species to a marked extent. The CEP Panel considered that uncertainty might be in the range of a factor of 0.1 to 2.

At the time the 2015 EFSA opinion (EFSA CEF Panel, 2015) was written, results from the human studies performed by Thayer et al. (2015) [RefID 7183] and Teeguarden et al. (2015a [RefID 9938], 2015b [RefID 7155]) were not available and, therefore, the AUC for humans was estimated using a PBPK model (Yang X et al., 2013). The AUC predicted using this model for human adults was 3.6 nM × h for a dose of 100 µg/kg bw or 0.036 nM × h/1 µg/kg bw, which is 6.4 fold and 2.3 fold lower than the mean values of the experimental results by Thayer et al. (2015) [RefID 7183] and by Teeguarden et al. (2015b) [RefID 7155], respectively. The AUC estimated using the model of Yang et al. (2013) for the human infant (>3 months up to 12 months) was predicted to be 3.0 nM × h for a dose of 100 µg/kg bw or 0.03 nM × h/1 µg/kg bw. No experimental data are available for this age group.

The CEP Panel noted the difference by a factor of 2.8 in the means of the dose-corrected AUC between the study of Thayer et al. (2015) [RefID 7183] and that of Teeguarden et al. (2015b) [RefID 7155], which might be explained by three hypotheses:

1) The findings represent the variability in the population, as the AUC-range predicted by Karrer et al. (2018) [RefID 12289] using the corrected Yang et al. (2015) model, calibrated by the data of Thayer et al. (2015) [RefID 7183], overlaps with the range of AUC in the study of Teeguarden et al. (2015b) [RefID 7155].

2) The findings are due to saturation of the metabolism in the enterocytes, as the intracellular concentrations in the upper part of the small intestine might exceed the Km for glucuronidation when the dose is 100 µg/kg bw, whereas for 30 µg/kg bw the concentration is below the Km for glucuronidation.

3) The findings are due to the difference in the application of BPA with only a small contact time to the mucosa of the mouth when eating the tomato soup and a longer contact time and, therefore, higher absorption through the mucosa of the mouth, whereby the absorbed BPA escapes first pass. This hypothesis is supported by the findings in ewes of Guignard et al. (2016) [RefID 2450], who described different systemic availability of BPA when given by nasogastric tubing (no contact to buccal mucosa) or as pellets (extensive contact to buccal mucosa). It is supported by observations in humans for nitroglycerol with a systemic availability of <1% when a solution was administered orally and swallowed *versus* approximately 30% when applied sublingual (Noonan and Benet, 1985, 1986).

Hypothesis (1) was rejected because the data of the two studies are statistically significantly different ($p < 0.05$; Mann–Whitney *U*-test). In addition, in the study of Thayer et al., the participants had different genotypes for glucuronidation and, hence, the difference could not be explained by an involuntary selection of participating subjects all having the slow metabolising genotypes.

Hypothesis (2) was also discarded, because a linear relationship between doses (between 0.5 and 100 µg/kg bw) and simulated AUCs was demonstrated using the PBPK model described by Karrer et al. (2018) [RefID 12289] (see Appendix B).

Concerning hypothesis (3), the finding in the study on ewes (Guignard et al., 2016 [RefID 2450]) indicates that some absorption in the mouth can occur, escaping the first pass metabolism and leading to a higher AUC. This might be an explanation for the higher AUC in the Thayer et al. (2015) [RefID 7183] study with respect to the Teeguarden et al. (2015b) [RefID 7155] study. It should be noted that in the former opinion on BPA (EFSA CEF Panel, 2015), the sublingual absorption was discussed and this exposure route was considered negligible in the context of a chronic exposure by food, where the BPA concentration is <0.1 mg/kg food, while in the animal experiment (Gayrard et al., 2013 [RefID 2223]), the BPA concentration in solution was three orders of magnitude higher. In the current hypothesis, the difference of the AUCs between the Thayer et al. (2015) [RefID 7183] and the Teeguarden et al. (2015b) [RefID 7155] studies is less than one order of magnitude (the factor is 2.8) and the CEP Panel considers that the BPA absorption in the mouth is plausible and the most probable explanation for the difference in the concentration and AUCs found in the two studies.

The CEP Panel considered that both study designs, [Teeguarden et al. (2015b) [RefID 7155], Thayer et al. (2015) [RefID 7183]] represent realistic behaviour when humans are eating food. To take this consideration into account when selecting the AUC for calculating the HEDF, the CEP Panel decided to select a value representing the central tendency of the data (e.g. mean, median etc.) combining the data of both studies. Because of the small number of participants in the two studies, the median value was chosen. The CEP Panel noted the difference of the resulting AUC-value of 15.7 nM x h per 100 µg/kg bw compared to the AUC-value of 3.6 nM x h per 100 µg/kg bw used in the former evaluation (EFSA CEF Panel, 2015). This difference is due to the fact that the human AUC used in 2015 was the result of PBPK modelling, whereas repeated measures after a single dose in humans were used to estimate the AUC in the current assessment. The mean observed AUC per 100 µg/kg bw in the experimental study of Teeguarden et al. (2015b) [RefID 7155] was close to the result of PBPK modelling. However, the AUC of 15.7 nM x h per 100 µg/kg bw is the median value of two studies in which the observed lowest and the highest AUC, both dose-corrected, differ by a factor of 7.

The CEP Panel also considered that the kinetic metric to be selected is the AUC. Therefore, HEDFs were calculated using the median AUCs from animal studies. The resulting HEDF-values are 0.0155 for mice and 0.1656 for rat (see Table 4).

Table 4: Human equivalent dose factor (HEDF), comparing AUCs for doses of 100 µg/kg bw.

Species (oral route)	AUC (nM × h)	HEDF (AUC animal/AUC human)

Human (Thayer et al., 2015 [RefID 7183] and Teeguarden et al., 2015b [RefID 7155]) (median)	15.7	
Mouse(n=1)(*) (Doerge et al., 2011)	0.244	0.0155
Rat (mean) (Doerge et al., 2010)	2.6	0.1656
Ewe – gavage (mean) (Guignard et al., 2016 [RefID 2450])	1.88	0.1197
Ewe – diet (mean) (Guignard et al., 2016 [RefID 2450])	6.84	0.4357
Rhesus monkey (mean) (Doerge et al., 2010)	1.5	0.095
Dog (mean) (Gayrard et al., 2013 [RefID 2223])	2.19	0.1395

(*) Every time point concentration measurement was done from pooled blood of n= 12 mice and one AUC was derived.

3.1.1.5. Conversion factors from BPA in feed/drinking water to mg/kg bw per day during gestation/lactation period

The doses reported in the animal studies in drinking water (mg/L) or feed (mg /kg) were converted to mg/kg bw per day. The factors used to convert the BPA doses given in feed or drinking water to mg/kg bw per day were retrieved from the EFSA guidance on selected default values to be used by the EFSA Scientific Committee, Scientific Panels and Units in the absence of actual measured data (EFSA Scientific Committee, 2012).

When a dose is given before gestation as well as during gestation (e.g. exposure started 2 weeks before mating until PND21), the chronic conversion factor was always used for premating, mating, gestation and lactation periods on dams.

According to the EFSA Scientific Committee (2012), the conversion factors from drinking water to body weight for female mice were 0.191 for subacute (4 weeks) and 0.164 for subchronic (13 weeks) studies. In the study by Pacchierotti et al. (2008, reported in the Chapter 3.1.9 on Genotoxicity HI) female mice were treated for 7 weeks. By linear interpolation for 7 weeks a conversion factor of 0.182 (rounded to 0.18) could be used.

The conversion factors used are reported in Table 5.

Table 5: Conversion factors from BPA in feed/drinking water to mg/kg bw per day during gestation/lactation period.

Species	**Conversion factor from drinking water**			**Conversion factor from feed**
	Chronic	**Subchronic**	**Subacute**	**Chronic**
Mice	0.09	0.15	0.18	0.15
Rat	0.05	Not applicable	Not applicable	0.05
Hamster	0.05	Not applicable	Not applicable	0.05
Rabbit	0.03	Not applicable	Not applicable	0.03
Rhesus monkey	Not applicable			Not applicable
Marmoset monkey	Not applicable			Not applicable

3.1.1.6. Converting factors from doses given by intravenous, intraperitoneal, subcutaneous, inhalation or intratesticular route to doses given by oral route

The exposure routes other than oral (i.e. intravenous, intraperitoneal, subcutaneous, inhalation, intratesticular) were converted to oral doses taking into account the toxicokinetic species-specific factors reported in Table 6.

Dosing by gavage (intragastric) is similar to oral dosing and no conversion is needed. For rabbits, hamsters and gerbils data were not available; therefore, the doses eliciting adverse effects when given by a non-oral route could not be converted to an oral route and could not be used as the basis for a TDI, although the information could support findings in other species.

Table 6: Converting factors from doses given by intravenous, intraperitoneal, subcutaneous or inhalation route to doses given by oral route

Species	Systemic availability (bioavailability) (%)			Multiplication factor (converting factor) from i.v./s.c./inhalation internal dose to oral dose	Multiplication factor (converting factor) from i.p. internal dose to oral dose
	oral	i.v./s.c.	i.p. (50% of the dose to be calculated as if given by the oral route+ 50% of the dose if given by the i.v./s.c route))***		
Rat	2.8	100	51.4	35.7	18.35
Mouse	0.45	100	50.22	222.2	111.6
Sheep	0.8*–2.7**	100	50.4–51.35	125–37	63–19.1
Monkey	0.9	100	50.45	111.1	56.1

Administration by * nasogastric tubing; ** pellets; ***See Lukas et al. (1971) for details of calculation.

In the study of Chen L et al. (2018) [RefID 11712] BPA was given by intratesticular injection. Hence, the calculations were performed to derive the oral dose which would have resulted in the same concentration in the testes, as resulting from the injection into the testes. The following physiological and distributional data were considered: weight of a testis, equal distribution in the testis, partition coefficient (PC) (predicted from the algorithm of Schmitt, 2008).

Table 7: Calculations of the oral doses from the intratesticular doses (i.e. for Chen L et al., 2018 [RefID 11712]), considering the data reported in Ariyaratne et al. (2000), Doerge et al. (2010) and Pilari et al. (2017).

Dose (pmol)	Weight of a testis (g)	Conc. in a testis (nM)	Conc. in blood (nM)		Blood (nM)	Dose (µg/kg bw)	PC testis/blood*	Conc in blood (nM) for test. conc.	Dose (µg/kg bw)	Dose in mg/kg bw
100	1.6 ± 0.02	1.6	62.5 (=100/1.6)	0.38 ± 0.19	0.38	100	3	20.83 (=62.5/3)	5481.6 (=100/0.38 *20.83)	5.5
1000			625 (=1000/1.6)					208.3 (=625/3)	54816 (=100/0.38 *208.3)	54.8

* Ratio concentration in testis/concentration in blood (partition coefficient, PC) = ~3 (Yoo et al., 2000)

The oral dose needed would be 5.5 mg/kg bw or 54.8 mg/kg bw to produce a concentration in the testes after injection of 100 pmol or 1000 pmol.

3.1.2. General toxicity

3.1.2.1. Epidemiological studies

Two human studies were available addressing effects of BPA on General toxicity: Lee S et al. (2018) [RefID 12428] on liver toxicity and Hu et al. (2019) [RefID 12151] on kidney toxicity.

However, the criteria to bring forward to (WoE analysis the clusters/exposure periods were not met, i.e. there were no clusters/exposure periods for which at least two studies were available with at least one reporting a statistically significant adverse association for one of the endpoints measured.

3.1.2.2. Animal studies

For the HOC General toxicity, a total of 54 studies was appraised by the CEP Panel. The details of the appraisals (internal and external validity) are reported in Annex E.

The endpoints for each study identified as relevant are reported in Annex F. BPA effects in liver and kidney (liver and kidney weight) were already key in the 2015 EFSA opinion (EFSA CEF Panel, 2015, Section 3.2.5). For more details see Annex A, Section 2.5.

Identification of clusters of relevant endpoints

From the newly available BPA literature, data on body weight, liver and kidney toxicity were retrieved along with effects in lung, thyroid, parathyroid, pituitary gland, adrenal gland and bone marrow. Changes in organ weights, histology or clinical chemistry potentially associated with toxicity in these organs were considered as relevant endpoints under the respective clusters, i.e. liver effects, kidney effects, lung effects, thyroid effects, parathyroid effects, pituitary gland effects, adrenal gland effects and bone marrow effects. An additional cluster encompasses haematological effects.

Body weight effects

Based on the newly evaluated studies, body weight was identified as a relevant endpoint. To evaluate compound-related effects on the growth of rodents, body weight data are commonly collected in toxicology studies (Hoffman et al., 2002). For this endpoint, a substantial decrease (typically ≥ 10%) or a substantial increase (typically ≥20%) in body weight or body weight gain were considered adverse. In this evaluation, body weight was regarded as a transversal endpoints and changes in this endpoint were assessed in the HOC Metabolic effects.

Liver effects

Based on the newly evaluated studies the following relevant endpoints were grouped into the cluster Liver effects:

- absolute and relative liver weight;
- histological changes including cystic and other hepatic degeneration, congestion, inflammation, necrosis, steatosis, angiectasis, hepato-diaphragmatic nodule, and apoptosis;
- clinical chemistry parameters, i.e. liver enzymes [alkaline phosphatase (ALP), alanine transferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH)], total protein, total bile acids, bilirubin, activated partial thromboplastin time (aPTT), albumin, globulin, albumin/globulin (ALB/GLO) ratio.

Increases in liver to body weight ratio were considered more relevant than absolute weight changes for the evaluation of the WoE (Bailey et al., 2004). Among various causes for liver weight increases, hepatocellular hypertrophy is a common response following xenobiotics administration in toxicity studies and may be linked to an adaptive enzyme induction in the absence of adverse effects in histopathology or clinical chemistry parameters related to liver toxicity (Hall et al., 2012). Several xenobiotics are known as enzyme inducers and lead to liver enlargement associated with hypertrophy and/or transient hyperplasia (Maronpot et al., 2010). Other histological changes characterised by glycogen or lipid accumulation (fatty change or steatosis) may be induced by physiological or pathological conditions that can influence metabolic processes (Thoolen et al., 2010). Hepatodiaphragmatic nodule is a congenital lesion, usually interpreted as 'background lesion'. However, a treatment related effect cannot be excluded in the progeny of animals exposed during pregnancy (Greaves, 2007). Angiectasis is an age-related lesion but it can also be caused by chemicals. Cystic degeneration is another age-related lesion, whose pathogenesis is not fully understood. Fatty change or steatosis may be linked to single cell necrosis. Cell degeneration and apoptosis may be triggered by either physiological or pathological processes induced by xenobiotics or several diseases. Necrosis may also be associated with inflammatory responses in the liver (Greaves, 2007). It is also well known that

changes in albumin and increases in serum levels of liver enzymes such as ALT, AST and LDH are indicators of liver damage (Hall et al., 2012). Other clinical chemistry changes in fat metabolism, e.g. triglyceride and cholesterol levels, were attributed to the HOC Metabolic effects.

Kidney effects

The following relevant endpoints were retrieved for the cluster Kidney effects:

- absolute and relative kidney weight
- histological changes such as mineralisation, hyperplasia, renal tubule cysts, nephropathy
- clinical chemistry parameters, e.g. urinary volume, blood urea nitrogen (BUN), urea and creatinine in serum.

For kidney weight changes, absolute and preferentially relative weights were considered, however it was noted that the relationship to body weight (or brain weight) is not strictly proportional (Bailey et al., 2004). Increases in kidney weight may be associated with either adaptive or pathological processes particularly in the presence of relevant histological alterations (Greaves, 2007). Mineralisation is encountered frequently in rodent toxicity studies. Simple tubule hyperplasia can be chemically induced and may be a consequence of single cell degeneration with compensatory regeneration (Frazier et al., 2012).

Xenobiotics may induce inflammatory reactions and lead to a damage of glomerular epithelial cells. Functional changes associated with glomerular lesions include loss of plasma albumin, urea and creatinine (Greaves, 2007).

Lung effects

BPA effects in lungs were only retrieved from the new literature not evaluated in the former EFSA opinions (EFSA 2007; EFSA CEF Panel, 2010, 2015). The following relevant endpoint was identified:

- absolute and relative weight.

Changes in lung weight can be associated to several pathologic conditions. Increased lung weight can be due to the pathological accumulation of fluids within the airways (e.g. haemorrhage, oedema, catarrhal exudate) or to the thickening of the interstitial tissue due to infiltration of inflammatory (e.g. lymphocytes. macrophages, eosinophils) or reactive cells (e.g. fibroblasts). Other lesions such as granuloma and tumours can also induce weight gain of the lung. The efficacy of lung weight as an indicator of lung injury in rat inhalation studies has been assessed by Wahlström et al. (2013), in which study the weight change in lungs as biomarker for predicting lung histopathology was over 80%.

Thyroid effects

BPA effects on thyroid gland were taken from the NTP Clarity Report (2018)/Camacho et al. (2019) [RefID 11370] and the following histological changes were identified as relevant endpoints:

- hyperplasia (C-cell, follicular cells, focal);
- ultimobranchial cysts.

The thyroid gland of rodents (and especially of male rats) is highly sensitive to derangement by xenobiotics and physiologic perturbations, predisposing to a higher incidence of proliferative lesions (e.g. hyperplasia and adenomas of follicular cells) in response to chronic TSH stimulation than in the human thyroid (Brändli-Baiocco et al., 2018).

Parathyroid effects, pituitary gland effects, adrenal gland effects

BPA effects on these organs were taken from the NTP Clarity Report (2018)/Camacho et al. (2019) [RefID 11370] and the following histological changes were identified as relevant endpoints:

- in the parathyroid: hyperplasia;
- in the pituitary gland: pars distalis hyperplasia, pars distalis cysts;
- in the adrenal gland: cortex hypertrophy, cytoplasmic vacuolisation, medulla hyperplasia.

Endocrine organs are often affected in toxicological studies in which proliferative changes are observed. Endocrine organ hypertrophy and hyperplasia occur in humans and experimental animals such as rodents either spontaneously during ageing or following long-term treatment, e.g. with hormonally active substances. Proliferative changes can be reversible or may eventually develop into neoplastic lesions (Greaves, 2007).

Bone marrow effects

BPA effects on bone marrow were taken from the NTP Clarity Report (2018)/Camacho et al. (2019) [RefID 11370] and the following histological changes were identified as relevant endpoints:

- hypocellularity;
- myeloid cell hyperplasia.

As the bone marrow is a site of intense cell multiplication and maturation, it can be influenced by chemicals that affect specific haematopoietic cell types or cellular proliferation/differentiation (Reagan et al., 2011). Bone marrow should be examined in context with toxicology studies, as the histomorphology may reflect changes resulting from moribund condition, body weight loss, or decreased body weight gain, which could be mistakenly concluded to be test article related (Reagan et al., 2011). Concerning the method of investigation, it should be noted that histopathological assessment of the bone marrow tissue is qualitative, whereas cytology is quantitative and is required for a correct assessment of haematopoietic cell differentiation and maturation (Willard-Mack et al., 2019).

Haematological effects

- The following relevant endpoints were retrieved: haemoglobin, mean corpuscular haemoglobin (MCH), eosinophils, platelets, packed cell volume.

The interaction of a toxic compound or its metabolites with cellular constituents may lead to changes in haematological parameters. Such changes in experimental animals have a high predictive value for human toxicity (Arika et al., 2016). Considering the natural variations of haematological parameters in animals, the adversity of any changes related to BPA treatment have to be evaluated against the background of historical control data (de Kort et al., 2020).

WoE of the clusters of relevant endpoints

The main information extracted from the studies addressing relevant endpoints in the HOC General toxicity are summarised in Annex G. The outcome of the weight of the evidence is described in the text below and presented in a tabulated format in Annex H.

The clusters of the effects of BPA on General toxicity considered for this assessment were the following:

- body weight effects;
- liver effects;
- kidney effects;
- lung effects;
- thyroid gland effects;
- parathyroid gland effects;
- pituitary gland effects;
- adrenal gland effects;
- bone marrow effects;
- effects on haematological parameters.

Body weight effect

The assessment of body weight for each exposure period is described in detail in the Metabolic effects' category (Chapter 3.1.4.2).

Kidney effects

Developmental exposure (pre-natal and/or post-natal until weaning)

For this exposure period, one Tier 1 study in rats (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]), one Tier 2 (Esplugas et al., 2018 [RefID 11900]) and one Tier 3 study in mice (Patel et al., 2013 [RefID 5697]) were identified.

In the rat study there was no change in kidney weights after 1 year of age in male and female rats treated at oral doses of 2.5, 25, 250, 2500 and 25000 µg/kg bw per day (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]). In a mouse study (Patel et al., 2013 [RefID 5697]) there was no change in kidney weights. Therefore, an effect of BPA on kidney weight during developmental exposure was judged as Not Likely.

In the rat study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) several histological effects in kidney were reported. An increase (trend, Poly-3 test) in transitional epithelium hyperplasia was observed in males at 2 years but not in females. In females a significantly increased incidence of renal tubule cysts was found at 2.5 µg/kg bw per day (Poly-3 test) along with a non-statistically significant increase at an adjacent dose of 25 µg/kg bw per day after 2 years (=NMDR). At the same time (2 years) the incidence of nephropathy increased in females at the highest dose of 25000 µg/kg bw per day (only in secondary test) but not in males; no such effect was seen at 1 year in both sexes. No effect on mineralisation was reported in kidneys of both sexes or time points (1 or 2 years). Regarding the clinical chemistry effects (i.e. on the levels of creatinine and BUN) in the mouse study (Esplugas et al., 2018 [RefID 11900]) no effects were found on creatinine, but an increase of urea levels was observed in males at a single subcutaneous dose of 25 µg/kg bw per day (which is equivalent to an oral dose of 5555 µg/kg bw per day); females were not tested in this study. These data on clinical chemistry parameters were considered as limited evidence of kidney effects and therefore graded as ALAN.

Considering the absence of effects on kidney weight (Not Likely) and on clinical chemistry parameters only observed in mice (ALAN), the CEP Panel assigned a likelihood level of ALAN to the kidney effects of BPA in the developmental exposure period, based on the histological findings in the rat study. Therefore, none of the endpoints was taken forward for BMD analysis. However, they were considered in the uncertainty analysis (see Appendix D).

Developmental and adult exposure (pre-natal and post-natal in pups until adulthood)

For this exposure period the following studies were identified: two Tier 1 studies, i.e. one in rats (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) and one in mice (Chatsantiprapa et al., 2016 [RefID 9822]) and two Tier 3 studies, one in rats (Jeong et al., 2017 [RefID 3133]) and one in mice (Patel et al., 2013 [RefID 5697]).

In the Tier 1 studies there was no change in kidney weights in rats after 1 year of treatment at oral doses from 2.5 to 25000 µg/kg bw per day (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) and in mice at 9 and 13 weeks of age in male and females treated daily for 9 weeks, respectively (Chatsantiprapa et al., 2016 [RefID 9822]). In contrast, in the Tier 3 rat study there was a dose-related increase in relative weight in males (but not in females) at the high oral dose, i.e. 250000 µg/kg bw per day, treated from PND6 for 90 days (Jeong et al., 2017 [RefID 3133]). On the contrary, a decrease in kidney weights was observed in the Tier 3 mouse study in males treated with 5 µg/kg bw per day from GD11.5 up to 4 months of age (Patel et al., 2013 [RefID 5697]). The effect of BPA on kidney weight during this exposure period was judged as Not Likely.

In the absence of weight changes several histological findings were observed in the Tier 1 rat study (NTP). In females an increased dose trend of mineralisation was observed along with increased incidence of renal tubule cysts at the lowest dose of 2.5 µg/kg bw per day and nephropathy at the doses of 25 and 2500 µg/kg bw per day after 1 year of treatment. After 2 years a significantly increased incidence and severity of nephropathy was reported in females treated with the lowest dose of 2.5 µg/kg bw per day and a significantly increased incidence of this lesion was also observed at the highest dose of 25000 µg/kg bw per day. In males of the same study hyperplasia of the transitional epithelium

was increased at the dose of 25 µg/kg bw per day and renal tubule cysts were increased at doses of 250 and 2500 µg/kg bw per day (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370], Summary Table) after 2 years. Based on these findings, the effect of BPA on kidney histological changes during this exposure period was judged as ALAN.

No effects were observed on the levels creatinine and BUN in this rat study while a dose-dependent decreases in creatinine was reported in the Tier 3 study in rats (Jeong et al., 2017 [RefID 3133]) at 500, 5000, 50000 and 250000 µg/kg bw per day in males (but not in females) without a change in BUN levels. Therefore, the effect of BPA on these parameters during this exposure period was judged as Not Likely.

Considering the absence of BPA effects on kidney weight in mice and rats (except for an increase in male rats at very high doses) and limited effects on clinical chemistry parameters in rats, the CEP Panel assigned a likelihood level of ALAN to the kidney effects of BPA in the developmental and adult exposure period, based on the histological findings in a Tier 1 study in rats. Therefore, none of the endpoints was taken forward for BMD analysis. However, they were considered in the uncertainty analysis (see Appendix D).

Growth phase/young age exposure

No studies were available for this exposure period.

Adult exposure (after puberty)

For this exposure period the following studies were identified: three Tier 2 studies, i.e. one in mice (Dong et al., 2013 [RefID 1676]) and two in rats (Poormoosavi et al., 2018 [RefID 12913]; Ola-Davies et al., 2018 [RefID 12837]) and three Tier 3 studies, i.e. one in mice (Liang et al., 2018 [RefID 12508]), one in rats (Elobeid et al., 2015 [RefID 1803]) and one in monkeys (Vijaykumar et al., 2017 [RefID 7477]).

No changes in relative kidney weight were found in the two mouse studies, neither in the Tier 2 study in females (Dong et al., 2013 [RefID 1676]) at doses of 1.8 or 0.18 µg/kg bw per day nor in the Tier 3 study in gonadectomised males (Liang et al., 2018 [RefID 12508]) treated s.c. with testosterone propionate and BPA at 40 to 4000 µg/kg bw per day (converted to oral doses of 8888 to 888800 µg/kg bw per day). No changes in kidney weight were found also in the Tier 2 rat study (Ola-Davies et al., 2018 [RefID 12837]). However, in the other Tier 2 rat study (Poormoosavi et al., 2018 [RefID 12913]) there was an increase of absolute kidney weight along with an increase in serum creatinine and urea levels in males (females not tested) treated with a high single dose of 10,000 µg/kg bw per day for 8 weeks. Based on these findings, the effect of BPA on kidney weight was judged as ALAN.

Similarly, an increase of creatinine and BUN were also observed in the Tier 2 rat study (Ola-Davies et al., 2018 [RefID 12837]) in males at 10000 µg/kg bw per day and in the Tier 3 rat study (Elobeid et al., 2015 [RefID 1803]) in males (females not tested) at the two highest doses of 10000 and 50000 µg/kg bw per day but not at lower doses of 100 and 1000 µg/kg bw per day. In a Tier 3 study (Vijaykumar et al., 2017 [RefID 7477]) with male monkeys (females not tested) there were no changes in the creatinine and serum urea levels after treatment with 2.5, 12.5 and 25 µg/kg bw per day for 70 days. The effect of BPA on these parameters during this exposure period was judged as ALAN.

Overall, dose-related effects on kidney weight and clinical parameters during adult exposure were observed in male rats only at BPA doses at or above 10000 µg/kg bw per day, however not in gonadectomised male mice treated with testosterone.

The CEP Panel assigned a likelihood level of ALAN to the kidney effects of BPA in the adult exposure period, mainly based on the effects on kidney weight and clinical chemistry parameters in male rats at high doses. No histological data were available for adult exposure. Therefore, none of the endpoints was taken forward for BMD analysis. However, they were considered in the uncertainty analysis (see Appendix D).

Indirect (germline) exposure

No studies were available for this exposure period.

Overall cluster selection of the endpoints/studies for BMD analysis for kidney effects

Overall, the CEP Panel assigned a likelihood level of ALAN to kidney effects following BPA treatment at different life stages from *in utero* to adulthood.

The CEP Panel considered that the evidence from the studies available did not show a Likely or Very Likely effect of BPA in any exposure period, therefore none of the endpoints was taken forward for BMD analysis.

Liver effects

Developmental exposure (pre-natal and/or post-natal until weaning)

For this exposure period the following studies were identified: seven Tier 1 studies, i.e. one in mice (van Esterik et al., 2014 [RefID 7393]) and six studies in rats (Jiang et al., 2014 [RefID 3190]; Lejonklou et al., 2017 [RefID 3975]; Xia et al., 2014 [RefID 8103]; NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]; Dunder et al., 2018 [RefID 11866]; Greenberg, 2018 (NTP Grantee study) [RefID 13785]); four Tier 2 studies, i.e. three in mice (Eckstrum et al., 2018 [RefID 11874]; Esplugas et al., 2018 [RefID 11900]; Meng Z et al., 2018 [RefID 12708]) and one in rats (Quan et al., 2017 [RefID 6025]); one Tier 3 study in rats (Sadowski et al., 2014a [RefID 6361]).

No changes in liver weight were reported in three studies in mice up to doses of 3000 µg/kg bw per day (van Esterik et al., 2014 [RefID 7393]; Eckstrum et al., 2018 [RefID 11874]; Meng Z et al., 2018 [RefID 12708]) and in six studies in rats up to doses of 50000 µg/kg bw per day (Jiang et al., 2014 [RefID 3190]; Lejonklou et al., 2017 [RefID 3975]; Sadowski et al., 2014a [RefID 6361]; Xia et al., 2014 [RefID 8103]; Dunder et al., 2018 [RefID 11866] and Greenberg (2018) (NTP Grantee study) [RefID 13785]). In one rat study, liver weight decreased in males (females not tested) at 1000 and 100000 µg/kg bw per day but without dose–response (Quan et al., 2016 [RefID 6024]) and there was a trend indicating an increase of liver weight in male rats treated with doses between 2.5 and 25000 µg/kg bw per day but not in female rats (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]). Considering that the liver weight was not statistically increased at any dose compared with controls in the latter study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]), an inverse effect was reported in the study by Quan et al. (2016) [RefID 6024] and in most studies in rats and mice the relative liver weight was unchanged, the CEP Panel judged the effect as Not Likely.

Several histological findings in liver were reported in rat studies. An increase in steatosis was only observed in one study in male rats (females not tested) treated with a single dose of 40 µg/kg bw per day at weeks 15 and 26 but not at week 3 (semiquantitative data; Jiang et al., 2014 [RefID 3190]). Three other rat studies reported no steatosis at doses up to 50000 µg/kg bw per day (Lejonklou et al., 2017 [RefID 3975]; in adult females and males (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]; Dunder et al., 2018 [RefID 11866]). In addition, in the NTP study increases in cystic degeneration (at 2500 and 25000 µg/kg bw per day) and mononuclear cell infiltration (symptom of inflammation) in liver (only at 2.5 and 25000 µg/kg bw per day) were observed in female rats but not in males. An increase of focal inflammation was also observed in a Tier 3 study (Joeng et al., 2017 [RefID 3133]). In the NTP study there was also an increase of angiectasis in male but not in female rats at 25 µg/kg bw per day but without dose–response. An increase in hepatocyte apoptosis was reported in male rats (females not tested) at a single dose of 50 µg/kg bw per day after 15 and 21 weeks but not at 3 weeks (Xia et al., 2014 [RefID 8103]). Considering the histological observations in female or male rat liver which were not always consistent among the reported studies, e.g. on steatosis, the CEP Panel judged these effects as ALAN.

Clinical chemistry parameters potentially associated with liver toxicity were observed in some studies. Two rat studies reported moderate increases in ALT (Jiang et al., 2014 [RefID 3190]; Xia et al., 2014 [RefID 8103]: both from the same laboratory) for which a likelihood of ALAN was given. However, neither this effect nor other increases of liver enzyme activities (i.e. AST or ALP) were observed in the NTP study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]). A moderate increase in ALP was observed in males at a single subcutaneous dose of 25 µg/kg bw per day (which is equivalent to an oral dose of 5555 µg/kg bw per day); females were not tested in this study (Esplugas et al., 2018 [RefID 11900]). A decrease in the levels of total bile acid and total protein in male but not in female rats was reported in the NTP study only at one dose of 25 µg/kg bw per day (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]).

The CEP Panel assigned a likelihood level of ALAN to the liver effects of BPA in the developmental exposure period, based on several histological findings and changes in some clinical parameters. Therefore, none of the endpoints was taken forward for BMD analysis. However, they were considered in the uncertainty analysis (see Appendix D).

Developmental and adult (pre-natal and post-natal in pups until adulthood)

For this exposure period the following studies were identified: three Tier 1 studies, i.e. one in mice (Chatsantiprapa et al., 2016 [RefID 9822]) and two studies in rats (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]; Greenberg, 2018 (NTP Grantee study) [RefID 13785]); one Tier 2 study in mice (Ke et al., 2016 [RefID 3447]) and two Tier 3 studies, i.e. one in mice (Biasiotto et al., 2016 [RefID 575]) and one in rats (Jeong et al., 2017 [RefID 3133]).

No changes in liver weight were reported in one study in female mice (Chatsantiprapa et al., 2016 [RefID 9822]) and three studies in male mice treated either pre-natally plus post-natally with BPA doses of 0.05 up to 500 µg/kg bw per day until week 9 (Chatsantiprapa et al., 2016 [RefID 9822]) and week 23 (Biasiotto et al., 2016 [RefID 575]) or post-natally with a single dose of 0.5 µg/kg bw per day until 8 weeks (Ke et al., 2016 [RefID 3447]). However, there was an increase in absolute and relative liver weight in the latter study in older males treated for 10 months. In juvenile female and male rats treated post-natally for 90 days with 500 to 250000 µg/kg bw per day, no effect on relative liver weight was reported (Jeong et al., 2017 [RefID 3133]). No effect on absolute liver weight was also reported in female rats in the Tier 1 study (Greenberg, 2018 (NTP Grantee study) [RefID 13785]). In contrast, in a rat study with pre-natal and post-natal exposure until 1 year to 2.5 up to 25000 µg/kg bw per day females showed a trend of increased liver weight while males showed a significant decrease only at the lowest dose (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]). Considering that the liver weights in the subacute/subchronic mice and rat studies were unchanged and only effects without a clear dose–response (trend in female rats, only lowest dose in male rats or in a single doses study in male mice) were observed after pre-natal and/or post-natal exposure up to 1 year, the CEP Panel judged the effect as ALAN.

Several histological changes in liver were observed in one rat study after pre-natal and post-natal exposure (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]). Angiectasis was reported for females at the highest dose (25000 µg/kg bw per day) and for males at the lowest dose (2.5 µg/kg bw per day) after 2 years (Camacho, but not in NTP final, [RefID 11370]). No effect on cystic degeneration was found after one or 2 years in rats at any dose (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]). In males an increase in the incidence of hepatodiaphragmatic nodules was observed at 2500 µg/kg bw per day after 1 year (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) but is difficult to explain why this effect was not present after 2 years considering that the lesion is congenital and irreversible. In addition, mononuclear cell infiltration at all doses except for 25 µg/kg bw per day and steatosis (fatty change) was reported at 25 µg/kg bw per day in males after 1 year. However, no effect on steatosis was reported in female rats in the Tier 1 study (Greenberg, 2018, NTP Grantee study [RefID 13785]). The CEP Panel noted that the histological changes in liver were only observed in males and/or only at one dose (angiectasis, hepatodiaphragmatic nodules, steatosis). Therefore, the effects were judged as ALAN.

Changes in clinical chemistry parameters potentially associated with liver toxicity were observed in rat studies. A decrease in the ALB/GLO ratio was observed at high doses in juvenile female and male rats after 90 days of treatment while the albumin level was not changed (Jeong et al., 2017 [RefID 3133], Tier 3). In a rat study with pre-natal and post-natal treatment up to 1 year (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370], Tier 1), there was a trend for slightly lower albumin levels in males but not in females; there was no effect on total protein levels in both sexes. A small increase in ALP levels in females was observed in the rat studies at intermediate doses after 1 year (statistically significant only at 250 µg/kg bw per day) (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) and after 90 days of treatment at high dose of 250000 µg/kg bw per day (Jeong et al., 2017 [RefID 3133]). In both studies no changes were found in ALP levels in males and in ALT levels for females and males. A decrease in AST levels reported only in males in the subchronic study (Jeong et al., 2017 [RefID 3133]) was not considered of toxicological relevance and was not observed in a Tier 1 rat study at 1 year (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]). No effects on liver enzyme levels (ALT, AST) were observed in male mice (Ke et al., 2016 [RefID 3447]). In addition, there

were increased bilirubin levels in the subchronic rat study in both sexes mainly at high doses up to 250000 µg/kg bw per day (Jeong et al., 2017 [RefID 3133]) and a trend for lower total bile acid levels in males in the rat study with pre-natal and post-natal treatment up to 1 year but not in females (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]). Based on the inconsistent findings related to liver enzymes and bilirubin levels after pre- and post-natal exposure the CEP Panel judged clinical chemistry effects in liver as ALAN.

The CEP Panel assigned a likelihood level of ALAN to the liver effects of BPA in the development and adult exposure period, mainly based on several histological findings and some clinical chemistry changes. Therefore, none of the endpoints was taken forward for BMD analysis. However, they were considered in the uncertainty analysis (see Appendix D).

Growth phase/young age exposure

No studies were available for this exposure period.

Adult exposure (after puberty)

For this exposure period the following studies were identified: Two Tier 1 studies in rats (Ding et al., 2016 [RefID 1621]; Vahdati Hassani et al., 2017 [RefID 2614]), eight Tier 2 studies, i.e. four in rats (Amraoui et al., 2018 [RefID 11503]; Mahmoudi et al., 2018 [RefID 12656]; Özaydin et al., 2018b [RefID 12854]; Poormoosavi et al., 2018 [RefID 12913]) and four in mice (Dong et al., 2013 [RefID 1676]; Lin Y et al., 2018 [RefID 4338]; Lv Q et al., 2017 [RefID 4697]; Marmugi et al., 2014 [RefID 4884]) and three Tier 3 studies, i.e. one in rat (Kazemi et al., 2017 [RefID 3441]), one in mice (Liang et al., 2018 [RefID 12508]) and one in monkeys (Vijaykumar et al., 2017 [RefID 7477]). All these studies were performed in male animals, except for one study in female mice (Dong et al., 2013 [RefID 1676]).

Increases in liver weight were reported in three Tier 2 studies with rats at a single high dose of 10000 µg/kg bw per day (Amraoui et al., 2018 [RefID 11503]; Mahmoudi et al., 2018 [RefID 12656]; Poormoosavi et al., 2018 [RefID 12913]) and one Tier 2 study in mice at 50 µg/kg bw per day (Lin Y et al., 2018 [RefID 4338]). In contrast, liver weights were unchanged in rat studies at doses between 50–50000 µg/kg bw per day, i.e. in two Tier 1 studies (at 50 µg/kg bw per day after 35 weeks (Ding et al., 2016 [RefID 1621]) or up to 50000 µg/kg bw per day after 30 day (Vahdati Hassani et al., 2017 [RefID 2614]), one Tier 2 study (up to 500 µg/kg bw per day; Özaydin et al., 2018b [RefID 12854]) and in mouse studies at doses between 50–5000 µg/kg bw per day, i.e. three Tier 2 studies (one in females (Dong et al., 2013 [RefID 1676]) and two in males (Lv Q et al., 2017 [RefID 4697]; Marmugi et al., 2014 [RefID 4884]) and one Tier 3 mouse study with subcutaneous treatment in mice (Liang et al., 2018 [RefID 12508]). Considering that the results in these studies were not consistent and observed with different BPA doses and exposure periods the CEP Panel judged increases in liver weight ALAN.

Histological changes in liver were observed in two Tier 2 rat studies at 10000 µg/kg bw per day, i.e. congestion, degeneration, inflammation, necrosis (Mahmoudi et al., 2018 [RefID 12656]) and steatosis (Özaydin et al., 2018b [RefID 12854]). In a Tier 2 rat study with doses up to 500 µg/kg bw per day no histological changes were reported (Özaydin et al., 2018b [RefID 12854]). Based on the findings in two single-dose studies the CEP Panel judged the histological changes in liver as ALAN.

Changes in clinical chemistry parameters potentially associated with liver toxicity were observed in several rat studies. A decrease in the albumin level was reported in a Tier 2 rat study at 10000 µg/kg bw per day (Amraoui et al., 2018 [RefID 11503]), while no change in albumin, globulin or ALP was observed in a Tier 3 monkey study up to 25 µg/kg bw per day (Vijaykumar et al., 2017 [RefID 7477]). Opposite effects on ALP levels were reported in two rat studies, i.e. an increase in a Tier 2 study at 10000 µg/kg bw per day (Poormoosavi et al., 2018 [RefID 12913]) and a decrease in a Tier 3 study at not clear doses (inconsistent reporting in the publication) (Kazemi et al., 2017 [RefID 3441]). The likelihood of effect given for ALP was judged as ALAN.

Similarly, ALT levels increased moderately in three rat studies (two Tier 2 studies, i.e. Mahmoudi et al., 2018 [RefID 12656], Amraoui et al., 2018 [RefID 11503] and one Tier 1 study, Vahdati Hassani et al., 2017 [RefID 2614]) and markedly in one study (Poormoosavi et al., 2018 [RefID 12913]) while no change in ALT were reported in a Tier 3 rat study at not clear doses (inconsistent reporting in the

publication) (Kazemi et al., 2017 [RefID 3441]). In a Tier 2 mouse study no changes in ALT were found at a single dose of 5000 µg/kg bw per day (Marmugi et al., 2014 [RefID 4884]).

AST levels increased moderately in three rat studies (two Tier 2 studies, i.e. Mahmoudi et al., 2018 [RefID 12656], Amraoui et al., 2018 [RefID 11503] and one Tier 1 study, Vahdati Hassani et al., 2017 [RefID 2614]) and markedly in one study (Poormoosavi et al., 2018 [RefID 12913]) while a decrease in AST was reported in a Tier 3 rat study at not clear doses (inconsistent reporting in the publication) (Kazemi et al., 2017 [RefID 3441]). In a Tier 2 mouse study no changes in AST were found at a single dose of 5000 µg/kg bw per day (Marmugi et al., 2014 [RefID 4884]).

An increase in LDH levels were observed in two rat studies, one with a single dose of 10000 µg/kg bw per day (Mahmoudi et al., 2018 [RefID 12656], Tier 2) and one at 500 µg/kg bw per day but not at higher doses (Vahdati Hassani et al., 2017 [RefID 2614], Tier 1).

Also, bilirubin levels increased in two Tier 2 rat studies at 10000 µg/kg bw per day (Amraoui et al., 2018 [RefID 11503] and Poormoosavi et al., 2018 [RefID 12913]) while no change was observed in Tier 3 a monkey study up to a dose of 25 µg/kg bw per day (Vijaykumar et al., 2017 [RefID 7477]). The CEP Panel judged the changes in liver enzymes and bilirubin in male rats as ALAN.

The CEP Panel assigned a likelihood level of ALAN to the Liver effects of BPA in the adult exposure period, based on several histological findings and changes in some clinical parameters. Therefore, none of the endpoints was taken forward for BMD analysis. However, they were considered in the uncertainty analysis (see Appendix D).

Indirect (germline) exposure

No studies were available for this exposure period.

Overall cluster selection of the endpoints/studies for BMD analysis for liver effects

Overall, dose-related effects on liver weight and clinical parameters during adult exposure were observed in male rats mainly at BPA doses at or above 10000 µg/kg bw per day. An increased liver weight was also reported in females after developmental and adult exposure, but not in male rats and in females or males exposed only during developmental exposure.

In summary, the liver effects following BPA treatment at different life stages from *in utero* to adulthood were judged as ALAN based on histological findings in female and/or male rats and increased liver weight and changes in liver enzymes after adult exposure in male rats. The overall likelihood across all exposure periods, i.e. the highest likelihood given in the cluster liver effects, was ALAN.

The CEP Panel considered that the information from the studies available did not show a Likely or Very Likely effect of BPA exposure on the liver and did not take the findings forward for BMD analysis.

Lung effects

Developmental exposure (pre-natal and/or post-natal until weaning)

For this exposure period only one Tier 2 study in rats (Quan et al., 2017 [RefID 6025]) was identified. In this study an increase in relative lung weight was observed in male offspring (females not tested) following pre-natal exposure to a very high dose, i.e. 100000 µg/kg bw per day but not at lower doses (at or below 10000 µg/kg bw per day).

The CEP Panel assigned a likelihood level of ALAN to the Lung effects of BPA in the developmental exposure period. Therefore, this endpoint (lung weight was not taken forward for BMD analysis. However, it was considered in the uncertainty analysis (see Appendix D).

Developmental and adult exposure (pre-natal and post-natal in pups until adulthood)

For this exposure period three studies were identified: One Tier 3 study in rats (Jeong et al., 2017 [RefID 3133]) and one Tier 1 study (Patel et al., 2015b [RefID 5698]) and one Tier 2 single-dose study in mice (Kasneci et al., 2017 [RefID 3399]). The increase of relative lung weight in the rat study at a very high dose of 250000 µg/kg bw per day in males but not in females was not considered as sufficient

evidence for a lung effect in the absence of supporting data from the mouse studies at a single low dose.

Therefore, the CEP Panel assigned a likelihood level of Not Likely to the Lung effects of BPA in the developmental and adult exposure period. Therefore, this endpoint was not taken forward for BMD analysis.

Growth phase/young age exposure

No studies were available for this exposure period.

Adult exposure (after puberty)

No studies were available for this exposure period.

Indirect (germline) exposure

No studies were available for this exposure period.

Overall cluster selection of the endpoints/studies for BMD analysis for lung effects

Overall, in the absence of data on lung weight following only adult exposure, the CEP Panel assigned a likelihood level of ALAN (based on BPA effects at high doses in male rats) and Not Likely to the Lung effects of BPA in the exposure periods developmental and developmental and adult, respectively. The overall likelihood across all exposure periods, i.e. the highest likelihood given in the cluster lung effects, was ALAN.

The CEP Panel considered that the evidence from the studies available did not show a Likely or Very Likely effect of BPA in any exposure period, therefore none of the endpoints was taken forward for BMD analysis.

Thyroid gland effects

Developmental exposure (pre-natal and/or post-natal until weaning)

For effects on thyroid gland histology following developmental exposure, only one Tier 1 study in Sprague–Dawley rats was identified (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370])

In this study, C-cell hyperplasia was observed in females at 2.5 µg/kg bw per day on PND365, but not at higher doses or at PND730 or in males. No effect on follicular-cell hyperplasia was seen. An increase of ultimobranchial cysts was observed in females at 250 µg and 2500 µg/kg bw per day (NMDR) on PND730 but not on PND365 or in males.

Based on these results, the CEP Panel considered the non-neoplastic changes in thyroid gland in the developmental exposure period ALAN. Therefore, none of the endpoints was taken forward for BMD analysis. However, they were considered in the uncertainty analysis (see Appendix D).

Developmental and adult exposure (pre-natal and post-natal in pups until adulthood)

For effects on thyroid gland histology following developmental and adult exposure, only one Tier 1 study in Sprague–Dawley rats was identified (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]).

In this study, C-cell hyperplasia was seen in males on PND730 (dose trend and significant at 2500 µg/kg bw per day), but not on PND365 or in females. Follicular-cell hyperplasia was seen in females at 2.5 on PND730 and in males at 25 µg/kg bw/day and non-significant at 250 µg/kg bw per day on PND730. No effect on ultimobranchial cysts was observed.

Based on these results, the CEP Panel considered the non-neoplastic changes in thyroid gland in the developmental and adult exposure period ALAN. Therefore, none of the endpoints was taken forward for BMD analysis. However, they were considered in the uncertainty analysis (see Appendix D).

Growth phase/young age exposure

No studies were available for this exposure period

Adult exposure (after puberty)

For effects on thyroid gland histology following adult exposure, only one Tier 1 study in rats was identified (Zhang J et al., 2017 [RefID 8770]).

In this study, no effect on hyperplasia (focal) was observed.

Based on these results, the CEP Panel considered the non-neoplastic changes in thyroid gland in the adult exposure period as Not Likely. Therefore, none of the endpoints was taken forward for BMD analysis.

Indirect (germline) exposure

No studies were available for this exposure period.

Overall cluster selection of the endpoints/studies for BMD analysis for thyroid gland effects

Overall, the CEP Panel assigned a likelihood level of ALAN to the thyroid gland effects of BPA in the developmental and developmental and adult exposure periods, based on an inconsistent increase in C-cell and follicular-cell hyperplastic changes in rats, and Not Likely for the adult exposure period. The overall likelihood across all exposure periods, i.e. the highest likelihood given in the cluster thyroid gland effects, was ALAN.

The CEP Panel considered that the evidence from the studies available did not show a Likely or Very Likely effect of BPA in any exposure period, therefore none of the endpoints was taken forward for BMD analysis.

Parathyroid effects

Developmental exposure (pre-natal and/or post-natal until weaning)

For this exposure period only one Tier 1 study in rats was identified (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]). The histological examination showed only a trend for an increase of hyperplasia in males but not in females. The CEP Panel assigned a likelihood level of ALAN to the Parathyroid effects of BPA in the developmental exposure period. Therefore, this endpoint was not taken forward for BMD analysis. However, it was considered in the uncertainty analysis (see Appendix D).

Developmental and adult exposure (pre-natal and post-natal in pups until adulthood)

For this exposure period only one Tier 1 study in rats was identified (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]). The histological examination showed only a trend for an increase of hyperplasia in males but not in females. The CEP Panel assigned a likelihood level of ALAN to the Parathyroid effects of BPA in the developmental and adult exposure period. Therefore, this endpoint was not taken forward for BMD analysis. However, it was considered in the uncertainty analysis (see Appendix D).

Growth phase/young age exposure

No studies were available for this exposure period.

Adult exposure (after puberty)

No studies were available for this exposure period.

Indirect (germline) exposure

No studies were available for this exposure period.

Overall cluster selection of the endpoints/studies for BMD analysis for parathyroid effects

Overall, considering for both exposure periods a hyperplastic change in male (but not in female) rats, the CEP Panel assigned an overall likelihood level of ALAN to the Parathyroid effects of BPA.

The CEP Panel considered that the evidence from the studies available did not show a Likely or Very Likely effect of BPA in any exposure period, therefore none of the endpoints was taken forward for BMD analysis.

Pituitary gland effects

Developmental exposure (pre-natal and/or post-natal until weaning)

For this exposure period two Tier 1 studies in rats were identified (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]; Mandrup et al., 2016 [RefID 4831]).

The histological examination showed an increase of hyperplasia in the pars distalis in females at doses of 2.5 and 25 µg/kg bw per day and in males at a 250 µg/kg bw per day in one study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]), but no effects for this endpoint were detected in females in the other study (Mandrup et al., 2016 [RefID 4831]).

The CEP Panel assigned a likelihood level of ALAN to the Pituitary gland effect of BPA (hyperplasia) in the developmental exposure period. Therefore, this endpoint was not taken forward for BMD analysis. However, it was considered in the uncertainty analysis (see Appendix D).

Developmental and adult exposure (pre-natal and post-natal in pups until adulthood)

For this exposure period only one Tier 1 study in rats was identified (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]). The histological examination showed only a trend for an increase of hyperplasia in the pars distalis in males but not in females. There was no change in the incidences of cysts in males and females.

The CEP Panel assigned a likelihood level of ALAN to the Pituitary gland effects of BPA in the developmental and adult exposure period. Therefore, none of the endpoints was taken forward for BMD analysis. However, they were considered in the uncertainty analysis (see Appendix D).

Growth phase/young age exposure

No studies were available for this exposure period.

Adult exposure (after puberty)

No studies were available for this exposure period.

Indirect (germline) exposure

No studies were available for this exposure period.

Overall cluster selection of the endpoints/studies for BMD analysis for pituitary gland effects

Overall, in the absence of data following only adult exposure, the CEP Panel assigned an overall likelihood level of ALAN to the Pituitary gland effects of BPA.

The CEP Panel considered that the evidence from the studies available did not show a Likely or Very Likely effect of BPA in any exposure period, therefore none of the endpoints was taken forward for BMD analysis.

Adrenal gland effects

Developmental exposure (pre-natal and/or post-natal until weaning)

For this exposure period only one Tier 1 study in rats was identified (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]). In females a cortex hypertrophy was observed at the highest dose only (i.e. 25,000 µg/kg bw per day). In males a trend for medulla hyperplasia was reported. No change in cytoplasmic vacuolisation was found in males or females.

The CEP Panel assigned a likelihood level of ALAN to the Adrenal gland effects of BPA in the developmental exposure period. Therefore, none of the endpoints was taken forward for BMD analysis. However, they were considered in the uncertainty analysis (see Appendix D).

Developmental and adult exposure (pre-natal and post-natal in pups until adulthood)

For this exposure period only one Tier 1 study in rats was identified (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]). No effects on the adrenal gland were observed in this exposure period.

The CEP Panel assigned a likelihood level of Not Likely to the Adrenal gland effects of BPA in the developmental and adult exposure period. Therefore, none of the endpoints was taken forward for BMD analysis.

Growth phase/young age exposure

No studies were available for this exposure period.

Adult exposure (after puberty)

No studies were available for this exposure period.

Indirect (germline) exposure

No studies were available for this exposure period.

Overall cluster selection of the endpoints/studies for BMD analysis for adrenal gland effects

Overall, in the absence of data following only adult exposure and considering the inconsistent effects reported following developmental exposure, the CEP Panel assigned a likelihood level of ALAN and Not Likely to the Adrenal gland effects of BPA in the developmental and developmental and adult exposure periods, respectively. The overall likelihood across all exposure periods, i.e. the highest likelihood given in the cluster adrenal gland effects, was ALAN.

The CEP Panel considered that the evidence from the studies available did not show a Likely or Very Likely effect of BPA in any exposure period, therefore none of the endpoints was taken forward for BMD analysis.

Bone marrow effects

Developmental exposure (pre-natal and/or post-natal until weaning)

For this exposure period only one Tier 1 study in rats was identified (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]). In this study no effects were reported for females and only effects at single doses (without dose–response) were observed in males for hypocellularity and myeloid hyperplasia.

The CEP Panel assigned a likelihood level of Not Likely to the Bone marrow effects of BPA in the developmental exposure period. Therefore, none of the endpoints was taken forward for BMD analysis.

Developmental and adult exposure (pre-natal and post-natal in pups until adulthood)

For this exposure period only one Tier 1 study in rats was identified (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]). No effects on bone marrow were observed in this exposure period except for an increase in hypocellularity in females at the highest dose.

The CEP Panel assigned a likelihood level of ALAN to the Bone marrow effects of BPA in the developmental and adult exposure period. Therefore, none of the endpoints was taken forward for BMD analysis. However, they were considered in the uncertainty analysis (see Appendix D).

Growth phase/young age exposure

No studies were available for this exposure period.

Adult exposure (after puberty)

No studies were available for this exposure period.

Indirect (germline) exposure

No studies were available for this exposure period.

Overall cluster selection of the endpoints/studies for BMD analysis for bone marrow effects

Overall, considering the scattered findings in the two available exposure periods, the CEP Panel assigned a likelihood level of Not Likely and ALAN to the bone marrow effects of BPA in the developmental and developmental and adult exposure periods, respectively. The overall likelihood across all exposure periods, i.e. the highest likelihood given in the cluster bone marrow effects, was ALAN.

The CEP Panel considered that the evidence from the studies available did not show a Likely or Very Likely effect of BPA in any exposure period, therefore none of the endpoints was taken forward for BMD analysis.

Haematological effects

Developmental exposure (pre-natal and/or post-natal until weaning)

For this exposure period only one Tier 1 study in rats was identified (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]). In this study no effects were reported for females and for males except for a trend for an increase of MCH in females but not in males. Therefore, the CEP Panel assigned a likelihood level of Not Likely to the haematological effects of BPA in the developmental exposure period, and none of the endpoints was taken forward for BMD analysis.

Developmental and adult exposure (pre-natal and post-natal in pups until adulthood)

For this exposure period one Tier 1 study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) and one Tier 3 study (Jeong et al., 2017 [RefID 3133]) in rats were identified.

In the NTP study [RefID 11370], for eosinophils some decreases in males (%) and in females were reported without dose–response. Regarding haemoglobin trends for an increase were observed in males and females and the increase in males was also significant at the highest dose. A trend for an increase in packed cell volume was reported only for males. Opposite trends in females (increase, also significant at the highest dose) and males (decrease) were found for platelets.

Based mainly on the findings in the Tier 1 study the CEP Panel assigned a likelihood level of ALAN to the Haematological effects of BPA in the developmental and adult exposure period. Therefore, none of the endpoints was taken forward for BMD analysis. However, they were considered in the uncertainty analysis (see Appendix D).

Growth phase/young age exposure
No studies were available for this exposure period.

Adult exposure (after puberty)

For this exposure period only one Tier 3 study in monkeys was identified (Vijaykumar et al., 2017 [RefID 7477]).

No changes in haematological parameters were observed in this study at doses up to 25 µg/kg bw per day.

The CEP Panel considered that the evidence was Inadequate to judge the likelihood level of the Haematological effects of BPA in the adult exposure period, so none of the endpoints was taken forward for BMD analysis.

Indirect (germline) exposure

No studies were available for this exposure period.

Overall cluster selection of the endpoints/studies for BMD analysis for haematological effects

Overall, the CEP Panel assigned an overall likelihood level of ALAN across all three exposure periods, i.e. the highest likelihood given in the cluster haematological effects.

The CEP Panel considered that the evidence from the studies available did not show a Likely or Very Likely effect of BPA in any exposure period, therefore none of the endpoints was taken forward for BMD analysis.

3.1.2.3. Integration of likelihoods from human and animal studies

There was no human evidence available for this category. Thus, the overall likelihood of effects of BPA was based on the animal evidence.

Table 8 presents the likelihoods of effect for each cluster and the overall likelihood in the animal stream in General toxicity.

Table 8: Overall likelihood from the animal studies for General toxicity.

Human stream	Animal stream		Integrated likelihood
Cluster: Body weight	**Cluster: Body weight**		
Not applicable	Developmental exposure (pre-natal and/or post-natal until weaning)	ALAN	
	Developmental and adult exposure (pre-natal and/or post-natal in pups until adulthood)	ALAN	
	Growth phase/young age exposure	ALAN	
	Adult exposure (after puberty)	Not Likely	
	Indirect (germline) exposure	Not Likely	
	Overall likelihood	*ALAN*	*ALAN*
Cluster: Kidney effects	**Cluster: Kidney effects**		
Not applicable	Developmental exposure (pre-natal and/or post-natal until weaning)	ALAN	
	Developmental and adult exposure (pre-natal and/or post-natal in pups until adulthood)	ALAN	
	Adult exposure (after puberty)	ALAN	
	Overall likelihood	*ALAN*	*ALAN*
Cluster: Liver effects	**Cluster: Liver effects**		
Not applicable	Developmental exposure (pre-natal and/or post-natal until weaning)	ALAN	
	Developmental and adult exposure (pre-natal and/or post-natal in pups until adulthood)	ALAN	
	Adult exposure (after puberty)	ALAN	
	Overall likelihood	*ALAN*	*ALAN*
Cluster: Lung effects	**Cluster: Lung effects**		
Not applicable	Developmental exposure (pre-natal and/or post-natal until weaning)	ALAN	
	Developmental and adult exposure (pre-natal and/or post-natal in pups until adulthood)	Not Likely	
	Overall likelihood	*ALAN*	*ALAN*
Cluster: Thyroid gland effects	**Cluster: Thyroid gland effects**		

Not applicable	Developmental exposure (pre-natal and/or post-natal until weaning)	ALAN	
	Developmental and adult exposure (pre-natal and/or post-natal in pups until adulthood)	ALAN	
	Adult exposure (after puberty)	Not Likely	
	Overall likelihood	*ALAN*	*ALAN*
Cluster: Parathyroid effects	**Cluster: Parathyroid effects**		
Not applicable	Developmental exposure (pre-natal and/or post-natal until weaning)	ALAN	
	Developmental and adult exposure (pre-natal and/or post-natal in pups until adulthood)	ALAN	
	Overall likelihood	*ALAN*	*ALAN*
Cluster: Pituitary gland effects	**Cluster: Pituitary gland effects**		
Not applicable	Developmental exposure (pre-natal and/or post-natal until weaning)	ALAN	
	Developmental and adult exposure (pre-natal and/or post-natal in pups until adulthood)	ALAN	
	Overall likelihood	*ALAN*	*ALAN*
Cluster: Adrenal gland effects	**Cluster: Adrenal gland effects**		
Not applicable	Developmental exposure (pre-natal and/or post-natal until weaning)	ALAN	
	Developmental and adult exposure (pre-natal and/or post-natal in pups until adulthood)	Not Likely	
	Overall likelihood	*ALAN*	*ALAN*
Cluster: Bone marrow effects	**Cluster: Bone marrow effects**		
Not applicable	Developmental exposure (pre-natal and/or post-natal until weaning)	Not Likely	
	Developmental and adult exposure (pre-natal and/or post-natal in pups until adulthood)	ALAN	
	Overall likelihood	*ALAN*	*ALAN*
Cluster: Haematological parameters	**Cluster: Haematological parameters**		
Not applicable	Developmental exposure (pre-natal and/or post-natal until weaning)	Not Likely	
	Developmental and adult exposure (pre-natal and/or post-natal in pups until adulthood)	ALAN	
	Adult exposure (after puberty)	Inadequate evidence	
	Overall likelihood	*ALAN*	*ALAN*

3.1.2.4. *In vitro* and mechanistic studies

Regarding scoring of likelihood of effects in the WoE for the HOC General toxicity, no relevant clusters were available from the human studies, and no cluster was scored Very Likely or Likely for any exposure periods in the animals. Most clusters in the animal studies were scored ALAN, some Not Likely and one Inadequate evidence. In the following paragraphs, MoA studies for the ALAN clusters among the HOC

General toxicity are considered. The MoA studies on body weight are described in Chapter 3.1.4.4, under the HOC Metabolic effects.

Kidney effects

Among the HOC General toxicity, the cluster Kidney effects was considered. Based on animal studies, the CEP Panel assigned a likelihood level of ALAN to the kidney effects of BPA in the developmental and developmental and adult exposure periods, mainly based on the histological findings (transitional epithelium hyperplasia, renal tubule cysts, nephropathy) in a rat study; and in the adult exposure period mainly based on the effects on kidney weight and clinical chemistry parameters in male rats at high doses.

No evidence was available regarding the MoA of BPA for this effect in the human studies. Three MoA studies were considered, two within the mammalian stream and one in the *in vitro* stream. These studies focused on oxidative stress. Elobeid et al. (2015) [RefID 1803] demonstrated that BPA exposure induced nephrotoxicity through oxidative stress by altering the apoptotic pathway involved. Esplugas et al. (2018) [RefID 11900] showed that gene expression of CYP1A2 was downregulated in mice exposed to BPA. The authors hypothesised that CYP-mediated modifications of BPA may give rise to the formation of unstable reactive intermediates, as well as radical fragments leading to oxidative damage.

In conclusion, data obtained from the few MoA studies indicated oxidative stress as a potential pathogenic mechanism for the weak evidence of kidney damage observed in animal studies.

Liver effects

Among the HOC General toxicity, the cluster Liver effects was considered. Based on animal studies, the CEP Panel assigned a likelihood level of ALAN to the liver effects of BPA in the developmental, developmental and adult and adult exposure periods, mainly based on liver weight and several histological observations and changes in some clinical chemistry parameters.

Epigenetic effects via hepatic DNA methylation were studied in several rodent studies with peri-/post-natal exposure to BPA. Using liver tissue samples from Agouti mouse offspring exposed via their mothers (fed diets with 50 µg/kg or 50000 µg/kg equivalent to 7.5 or 7500 µg/kg bw per day) during gestation and lactation, non-monotonic changes in DNA methylation were observed in genome-wide analyses (Kim JH et al., 2014 [RefID 3521]). Epigenetically dysregulated pathways related to metabolism and stimulus response were identified by gene set enrichment testing.

Several studies report a link between DNA hypomethylation of lipogenic genes and hepatic lipid accumulation (steatosis). Anderson et al. (2017) [RefID 187] studied four genes in livers of adult female mouse offspring from dams treated with 0.05, 50 or 50000 µg BPA/kg diet or control dams and concluded that the DNA methylation in these genes had an impact on pathways related to body weight, body fat phenotypes and energy expenditure. Long-term exposure to BPA (0.5 µg/kg bw per day; via drinking water) was studied in male mice treated from birth up to 10 months resulting in hepatic accumulation of triglycerides and cholesterol (Ke et al., 2016 [RefID 3447]). These outcomes were associated with increased expression levels of key genes involved in lipid synthesis along with altered levels of DNA methylation and expression of genes for critical transcription factors (Srebf1 and Srebf2). The *in vivo* results were supported by knockdown experiments in the murine Hepa1–6 cell line (Ke et al., 2016 [RefID 3447]). Similarly, hepatic lipid accumulation was associated with hypomethylation of lipogenic genes and increased transcription factor Nrf2 signalling (binding to Srebp-1c promoter) in mouse offspring (at week 39) following BPA exposure via dams (25 µg/kg bw per day, i.p. from GD8 to PND16) and drinking water (until PND35) (Shimpi et al., 2017 [RefID 6685]). Sex-specific modulation of hepatic DNA methylation and gene expression were studied in offspring of Sprague Dawley (SD) rats exposed to BPA via dams (100 µg/kg bw per day from GD6 to PND21) (Strakovsky et al., 2015 [RefID 6914]). At PND1, BPA altered methylation and transcription factor binding within the Cpt 1a gene was observed in males but not in females suggesting epigenetic changes in the male hepatic β-oxidation capacity. Males at PND110 had microvesicular steatosis particularly when fed with a high-fat diet after weaning (Strakovsky et al., 2015 [RefID 6914]). In contrast, no changes in DNA methylation were found in livers of female mouse offspring (at 23 weeks) exposed to BPA via dams (3, 10, 30, 100, 300, 1000, and 3000 µg/kg bw per day; from 2 weeks before mating through gestation and lactation) and

fed a high-fat diet starting from week 17 to the end of the experiment (van Esterik et al., 2014 [RefID 7494]).

Diabetes was studied in rat offspring exposed via dams (50 µg/kg bw per day; throughout gestation and lactation) (Ma et al., 2013 [RefID 4748]). BPA-induced insulin resistance observed at week 21 was preceded by hypermethylation and a reduction in the expression of hepatic glucokinase at week 3, possibly indicating an impact on glucose metabolism and an increased risk of developing insulin resistance and type 2 diabetes mellitus. Perreault et al. (2013) [RefID 5797] reported that male mice treated with an acute oral dose of 50 µg/kg bw or the same dose daily over 2 weeks had a reduced glucokinase activity.

Metabolomics based approaches were used in numerous studies addressing BPA-induced alterations of hepatic metabolite profiles, changes in carbohydrate and lipid metabolism and related gene expressions in the liver. Metabolomic profiling of rat urine was performed by Chen MJ et al. (2014) [RefID 1054] following gavage treatment with BPA at 50 or 50000 µg/kg bw per day for 8 weeks. Increases in biotin and riboflavin excretion and in the formation of the hepatic methyl donor S-adenosylmethionine along with enhanced expression of hepatic methionine adenosyltransferases (*Mat1a*, *Mat2a*) were observed dose dependently while increased levels of methylated products (e.g. 3-methylhistidine) in rat urine were only reported for the high dose, indicating protein catabolism. Another metabolome study was performed in male mouse offspring exposed to low BPA doses (0.025, 0.25, or 25 µg BPA/kg bw per day, from GD8 to PND16) (Cabaton et al., 2013 [RefID 776]). Metabolite profiles in livers at PND21 revealed increased levels of taurine (TAU), glutamate (GLU) and glutathione along with decreased levels of lactate, glucose, glycogen, phosphatidylcholine and glycerophosphocholine at the highest dose tested. Dose-dependent and time-dependent metabolic changes in rat livers from offspring exposed perinatally to BPA (subcutaneous doses: 0.25, 2.5, 25, or 250 µg/kg bw per day) were studied by Tremblay-Franco et al. (2015) [RefID 7285] at PND21, 50, 90, 140 and 200). Changes often showed no clear dose–response and were for some metabolites also observed at the low BPA doses, e.g. for increases in phosphorylcholine and choline.

Metabolic changes related to carbohydrate and lipid metabolism were addressed in several *in vivo* studies. Plasma lipid profiles, liver transcriptomic analysis and gene expression were performed in adult male mice exposed to BPA (5, 50, 500 and 5000 µg/kg bw per day) via drinking water for 8 months (Marmugi et al., 2014 [RefID 4884]). The increased plasma glucose (but not insulin) levels at the two highest doses and enhanced cholesterol levels (not dose dependent) along with a hepatic overexpression of genes related to cholesterol biosynthesis indicate BPA-induced hyperglycaemia, glucose intolerance, hypercholesterolemia and increased cholesterol biosynthesis. In a study on male rat offspring perinatally exposed to BPA at 40 µg/kg bw per day (Jiang et al., 2014 [RefID 3190]) changes were observed in mitochondrial fatty acid metabolism at 3 weeks. Reduced ATP production at weeks 15 and 26 was reported along with increased ROS generation, upregulation of genes related to lipogenesis and fatty accumulation/steatosis in liver. In contrast to these findings, long-term exposure of male rats (fed a standard or a high-fat diet) to BPA (50 µg/kg bw per day) had no effect on total cholesterol or total triglycerides and the known mechanisms (Peroxisome proliferator-activated receptors (PPARs) and Sterol regulatory element-binding proteins (SREBPs)) involved in the regulation of hepatic lipid metabolism (Ding et al., 2013 [RefID 1621]). Differential effects of BPA and other bisphenols (100 µg/kg bw per day) on lipid accumulation and glucose homeostasis in adult female mouse offspring (exposed from GD7 to PND21) at PND35 were reported by Meng Z et al. (2018) [RefID 12708]. BPA treatment resulted in an increased relative content of two out of 13 studied fatty acids (i.e. C20:0 and C20:1n9) and affected genes involved in the fatty acid transport. García-Arévalo et al. (2014) [RefID 2193] studied liver triglyceride accumulation and glucose homeostasis in adult male mouse offspring exposed to BPA via dams (subcutaneously injected with 10 µg/kg bw per day from GD9 to GD16). BPA increased the hepatic triglyceride content and *Pparγ* mRNA expression in livers of mice fed a regular diet but not in those on a high-fat diet, while mRNA expression of *Cd36* involved in fatty acid uptake was decreased by BPA with both diets. Liver histology of Watanabe Heritable Hyper-Lipidemic (WHHL) rabbits treated with 400 µg BPA/kg bw per day for 12 weeks revealed mild steatosis and inflammatory cell infiltration in the liver (Fang et al. 2014, RefID 1914). The authors also report on BPA-induced increases of mRNA expression of genes related to the ER pathway, to lipid metabolism and to liver inflammation. Reddivari et al. (2017) [RefID 6131] observed liver inflammation in rabbit

offspring exposed to BPA (200 µg/kg bw per day) from GD15 to PND7 and discussed an association with changes in the gut microbiota and gut metabolites, e.g. reduced short chain fatty acid levels.

Analysing male fetal mouse livers following BPA exposure (50 µg or 50000 µg/kg bw per day until GD18.5) Susiarjo et al. (2017) [RefID 7023] identified increased bile acid and tryptophan levels at the higher dose. Elevated tryptophan levels were also observed in the maternal livers and the authors discussed a potential link to a disturbed glucose metabolism and gestational diabetes. Galyon et al. (2017) [RefID 2131] studied the hepatic insulin signalling in rat offspring exposed to BPA during gestation and lactation via drinking water consumption by dams (at 239 ± 8 µg/kg bw per day and at 466±33 µg/kg bw per day, respectively). This BPA treatment resulted in impaired glucose tolerance (at 6 weeks and 6 months) and altered protein levels of components of insulin signalling (at 3 weeks) in male, but not in female offspring.

Mechanisms leading to dysregulated hepatic lipid metabolism were further investigated *in vitro*. Lin Y et al. (2017) [RefID 4338] reported a BPA-induced increase in serum and hepatic triglyceride via an enhanced expression of Srebf1 and lipogenesis-related genes in male post-weaning mice treated with 50 µg BPA/kg bw per day for 90 days. These effects were also observed in BPA (>100 nM, 48 h)-treated human HepG2 cells and were mediated by a downregulation of microRNA, miR-192. Cabaton et al. (2018) [RefID 11650] reported on metabolomic changes in HepG2 induced by BPA (1 pM, 1nM, 1µM). The endogenous metabolite variations were concentration-specific, indicating significant effects on several amino acids (at all BPA concentrations) and on reduced glutathione (GSH) at the lowest BPA concentration. The authors concluded that BPA modulated major metabolic routes involved in cellular functions and detoxification processes. In another study (Hélies-Toussaint et al., 2014 [RefID 2676]), HepG2 cells were treated with BPA concentrations between 1 fM and 1 µM for 4 days but only 1pM BPA increased the accumulation of triglyceride while glucose uptake was not affected. In contrast to these data, Peyre et al. (2014) [RefID 5812] reported an increase in lipid accumulation in HepG2 cells only at high BPA concentrations (>10 µM; 72 h). Lv Q et al. (2017) [RefID 4697] showed that in livers of male rats fed BPA containing diets (corresponding to 0.5 or 5 µg/kg bw per day), lipid accumulation increased together with the number of Kupffer cells (KC) transformed toward a pro-inflammatory M1 phenotype which may also participate in hepatic lipid deposition *in vivo*. A high concentration of BPA (10 µM) in primary KC cultures also induced this change of KC phenotype and enhanced the levels and the secretion of inflammatory factors. In addition, conditional medium from BPA-treated KC was shown to increase lipid accumulation in HepG2 cells. Using a long-term cell culture model with human hepatoma HepaRG cells (Bucher et al., 2017 [RefID 743]), BPA treatment (0.2–2000 nM) for 3 weeks induced an increase in neutral lipids and triglycerides accumulation at 2 nM; however, the expression of enzymes involved in lipid and carbohydrate homeostasis remained unchanged. Similarly, lipid accumulation in rat hepatoma FaO cells (Grasselli et al., 2013 [RefID 2386]) were observed at high BPA concentrations (≥100 nM; 24 h), interfering with the pathways involved in lipid oxidation and secretion but without an increase in the expression of lipogenic genes. Yang SM et al. (2017) [RefID 8398] reported on the association of lipid accumulation and dysregulated autophagy in mice treated with 5–500 µg BPA/kg bw per day for 8 weeks and further investigated lipid accumulation and the mechanisms involved in the reduced autophagy induction and autophagosome accumulation (via the mammalian target of rapamycin (mTOR) pathway) in HepG2 cells, however *in vitro* only a high BPA concentration (10 µM) was effective.

Several rat studies addressed BPA-induced oxidative stress associated with liver toxicity. Adult male rats treated with a high dose of BPA (10000 µg/kg bw per day) via drinking water over 60 days (Mahmoudi et al., 2018 [RefID 12656]) showed increases in thiobarbituric acid reactive substances (TBARS, a marker of lipid peroxidation), total antioxidant capacity and lipid peroxidation along with decreases in catalase (CAT) and superoxide dismutase activities in the liver. Concomitantly, the hepatic levels of total cholesterol and triglyceride as well as parameters indicating liver damage (ALT, AST, ALP, LDH and TNF-a) were clearly increased. In another study (Vahdati Hassani et al., 2017 [RefID 2614]) male rats treated with 500 µg/kg bw per day for 30 days had elevated serum activities of AST and LDH, increased serum levels of triglyceride, high density lipoprotein (HDL) cholesterol and glucose as well as increased hepatic level of 8-isoprostane and a decreased level of reduced glutathione and also showed periportal inflammation in the liver. At the same dose level, the authors reported increases in elements of mitogen-activated protein kinase (MAPK) pathways, MAPK-activated protein kinase (MAPKAPK) and AKT activation and upregulated microRNA (miR-122) transcript levels in liver. In a follow-up study

Vahdati Hassani et al. (2018) [RefID 11240] observed in livers of male rats treated with the same BPA dose an indication of oxidative stress (increased malondialdehyde (MDA), decreased glutathione levels). In addition, BPA-induced alterations in the expression of (phospho)proteins involved in homocysteine, fatty acid and carbohydrate metabolism and antioxidant defence in liver were reported. In a study by Wei et al. (2014) [RefID 7890] hepatic gene expression of genes and/or proteins were investigated in male rats perinatally exposed to BPA (50 µg/kg bw per day; GD0 to PND21). While BPA induced mild steatosis and altered insulin signalling in livers on a standard diet, BPA worsened the hepatic damage of a high-fat diet (lipid accumulation, inflammation, mild fibrosis; increased activities of AST, ALT, ALP and gamma-glutamyltransferase (GGT)). These findings were associated with indications of an enhanced oxidative stress in liver (mainly in the high-fat diet BPA group) as well as increased mRNA expression of hepatic genes associated with lipogenesis, fatty acid oxidation, and gluconeogenesis. An increased expression of two genes linked to oxidative stress was reported by Kazemi et al. (2016b) [RefID 3442] in adult male rats treated with BPA at 5, 25 or 125 µg/kg bw per day for 35 days: HO-1 (heme catabolism) was induced dose dependently as a monotonic dose response (MDR) while GADD45B (related to cell cycle, apoptosis and DNA repair) showed a NMDR. The same group (Kazemi et al., 2017 [RefID 3441]) reported with the same experimental design a BPA-induced increase of malonaldehyde as an indicator for oxidative stress along with histopathological findings in the liver but surprisingly with a marked decrease in the serum levels of ALP and AST in BPA-treated rats. Thilagavathi et al. (2017) [RefID 9247] investigated the antioxidant status of the liver of adult female rats treated with 10, 50 or 100 µg BPA/kg bw per day for 12 weeks. They observed a dose-dependent decrease of the activities of superoxide dismutase, CAT and glutathione peroxidase (GPx) along with a decreased glutathione content in the liver in all treatment groups. In addition, they reported on a decrease in hepatic cytochrome P450 and cytochrome b5 (phase I detoxification) with a NMDR. Esplugas et al. (2018) [RefID 11900] studied oxidative DNA damage (8-OHdG) in livers of male mice exposed to a single subcutaneous dose of BPA (25 µg/kg bw per day) at PND10. The authors hypothesised that CYP-mediated modifications of BPA may give rise to oxidative damage. They observed a reduced hepatic expression of CYP1A2 but 8-OHdG was not significantly altered in treated mice at 2 months of age.

Using HepG2 cells treated with 100 nM BPA for 72 hours Vidyashankar et al. (2014) [RefID 7469] reported a marked cytotoxicity (56%) along with indications of oxidative stress, i.e. GSH content, reduced activities of antioxidant enzymes (CAT, GPx, superoxide dismutase (SOD)), lipid peroxidation and impaired mitochondrial function, i.e. reduced ATP production and mitochondrial membrane potential. An upregulation of CYP2C9 expression in HepG2 cells was reported with treatment of BPA (10, 100 and 1000 nM) through an ERα-mediated transcriptional activation (Xu JY et al., 2017 [RefID 8203]). This upregulation may alter metabolism in human liver.

Involvement of mitochondria in the induction of BPA-induced apoptosis of liver cells has been studied by Xia et al. (2014) [RefID 8103] in male rat offspring exposed to BPA from G0 to PND21 (50 µg/kg bw per day). The authors observed liver toxicity (increase in serum ALT at week 21) and hepatic apoptosis at week 15 and 21 associated with increased activities of caspase 3 and caspase 9 and an elevated release of cytochrome *c* from mitochondria in hepatocytes suggesting a mitochondrial apoptosis pathway. In addition, *in vitro* experiments with mitochondria isolated from untreated neonatal rat liver and then exposed to BPA further supported the hypothesis that apoptosis is induced by alteration of the mitochondrial ultrastructure and the release of proteins.

In conclusion, many MoA studies addressed several potential mechanisms of BPA in liver and liver cells. It was proposed that epigenetic changes via DNA methylation may have an impact on different signalling pathways related to lipid and carbohydrate metabolism. In addition, oxidative stress may be related to impaired mitochondrial function and liver toxicity.

Lung effects

Among the HOC General toxicity, the cluster Lung effects was considered. Based on animal studies, the CEP Panel assigned a likelihood level of ALAN of BPA effects (increase in lung weight) at high doses in male rats in the exposure periods developmental, developmental and adult, and adult.

No evidence was available regarding the MoA of BPA for this effect in the human studies.

Nine MoA studies were considered, five within the mammalian stream and four in the *in vitro* stream. Most of these studies addressed changes related to allergic/inflammatory responses. An *in vivo* MoA

study in mice (Hijazi et al., 2015 [RefID 2707]) demonstrated that BPA retards fetal lung maturation, as evidenced by diminished alveolar airspace and thickened septa which are both hallmarks of lung immaturity. This immaturity was characterised by aberrant alveolar epithelial type I cell differentiation. The authors concluded that the effects of BPA are likely to be mediated through the glucocorticoid signalling pathway: this point was further investigated in another *in vitro* MoA study carried out by the same authors (Hijazi et al., 2017 [RefID 2708]).

In conclusion, data obtained from MoA studies suggest that BPA can delay fetal lung maturation evaluated through reduced alveolar airspace and thickened septa. Both these findings are related to an increase in the lung weight and can explain the results of the animal studies.

Thyroid gland effects

Among the HOC General toxicity, the cluster Thyroid gland effects was considered.

Based on animal studies, the CEP Panel assigned an overall likelihood level of As Likely As Not (ALAN) for BPA effects following developmental (C-cell hyperplasia, ultimobranchial cysts) and developmental and adult exposure (C-cell hyperplasia, follicular-cell hyperplasia).

No evidence was available regarding the MoA of BPA for this effect in the human studies.

Four MoA studies were considered, two in rodents and two in *in vitro* stream. These studies addressed mainly carcinogenic effects of BPA on the thyroid gland. An animal (rat) study (Zhang J et al., 2017 [RefID 8770]) reported that chronic administration of BPA alone did not increase thyroid carcinoma incidence. However, the study indicated that BPA could enhance the susceptibility to thyroid carcinoma stimulated by N-bis(2-hydroxypropyl)nitrosamine (DHPN) and iodine excess. ERα is probably involved in the proliferation effect of BPA. An *in vitro* study (Zhang YH et al., 2017 [RefID 8883]) reported that BPA mediates oestradiol-like effects by binding to nuclear oestrogen receptors (ERα and ERβ), and also promotes growth by binding to oestrogen membrane receptor (mERα and GPR30) and activating the AKT/mTOR pathway, ultimately altering gene expression to stimulate proliferation of thyroid cancer cells. Porreca et al. (2016) [RefID 5892] investigated the effects of BPA on rat follicular thyroid cell line (FRTL-5). The authors concluded that while BPA does not directly cause genetic damage, it may contribute to thyroid cell damage by impairing DNA repair and efficiency, and, likely, to thyroid carcinogenesis in cooperation with other endogenous or external factors.

In conclusion, data obtained from the few MoA studies available, indicated potential mechanisms that are responsible for an increase in the proliferation of thyroid cells, supporting the limited evidence of hyperplastic changes observed in the *in vivo* animal study. Moreover, it is suggested that BPA could enhance the susceptibility to thyroid carcinoma in combination with other factors.

Parathyroid gland effects

Among the HOC General toxicity, the cluster parathyroid gland effects was considered.

Based on animal studies, the CEP Panel assigned a likelihood level of ALAN of BPA effects in the developmental and developmental and adult exposure periods.

No evidence was available regarding the MoA of BPA for this effect in the human, *in vitro* or animal studies.

Pituitary gland effect

Among the HOC General toxicity, the cluster 'pituitary gland effects' was considered.

Based on animal studies, the CEP Panel assigned a likelihood level of ALAN of BPA effects in the exposure periods developmental and developmental and in adulthood.

No evidence was available regarding the MoA of BPA for this effect in the human, *in vitro* or animal studies.

Adrenal gland effects

Among the HOC General toxicity, the cluster adrenal gland effects was considered.

Based on animal studies, the CEP Panel assigned a likelihood level of ALAN of BPA effects in the developmental exposure period, and Not Likely in the developmental and adult exposure period.

No evidence was available regarding the MoA of BPA for this effect in the human, *in vitro* or animal studies.

Bone marrow effects

Among the HOC General toxicity, the cluster 'bone marrow effects' was considered.

Based on animal studies, the CEP Panel assigned a likelihood level of Not Likely of BPA effects in the developmental exposure period, and of ALAN in the developmental and adult exposure period.

No evidence was available regarding the MoA of BPA for this effect in the human, *in vitro* or animal studies.

Haematological effects

Among the HOC General toxicity, the cluster 'haematological effects' was considered.

Based on animal studies, the CEP Panel assigned a likelihood level of Not Likely of BPA effects in the developmental exposure period, of ALAN in the developmental and adult exposure period, and of Inadequate evidence in adulthood.

No evidence was available regarding the MoA of BPA for this effect in the human, *in vitro* or animal studies.

3.1.2.5. Conclusion on hazard identification for General toxicity of BPA

In the 2015 EFSA opinion on BPA (EFSA CEF Panel, 2015) BPA effects in liver and kidney were judged as Likely based on results from multigeneration studies in mice and rats. Increases in liver and kidney weight were considered relevant systemic effects of BPA (EFSA 2007; EFSA CEF Panel 2010 and 2015) as they were associated in rodents with hepatocellular hypertrophy and nephropathy, respectively. Based on a WoE approach changes in the mean relative kidney weight in a two-generation toxicity study in mice were considered as the most critical effect at low doses. The kidney data were taken forward for a BMD–response modelling resulting in a $BMDL_{10}$ of 8,960 µg/kg bw per day. Using toxicokinetic data the $BMDL_{10}$ value was converted into a HED of 609 µg/kg bw per day which was considered the lowest value to be used for the derivation of a TDI (EFSA CEF Panel, 2015).

The available new information that has been evaluated in the current evaluation is in line with these earlier observations on kidney and liver toxicity. An increase of absolute kidney weight along with changes in clinical biochemistry was reported in male rats (females not tested) treated with a dose of 10000 µg/kg bw per day for 8 weeks during adulthood while no changes in kidney weight were observed from 2.5 to 25000 µg/kg bw per day during the other exposure periods, except for an increase in relative weight in males (but not in females) at 50000 and 250000 µg/kg bw per day treated from PND6 for 90 days. Histological findings in kidney (renal tubule cysts, nephropathy and hyperplasia of the transitional epithelium) at lower doses (2.5 to 2500 µg/kg bw per day) following BPA exposure during developmental life stages until weaning or adulthood were not consistently observed in the evaluated studies and therefore judged as ALAN. Regarding liver weight changes, inconsistent outcomes were reported and therefore this effect was considered as Not Likely (developmental exposure until weaning) or ALAN (developmental and adult and adult exposure). Histological changes in liver (congestion, degeneration, inflammation, necrosis and steatosis) were observed in two rat studies during adult exposure to BPA at 10000 µg/kg bw per day while exposure during the developmental until weaning or adulthood periods resulted in histological findings (angiectasis, cystic degeneration, hepatodiaphragmatic nodules, mononuclear cell infiltration, fatty change) with unclear dose–responses between 2.5 to 2500 µg/kg bw per day. Therefore, the CEP Panel considered the histological effects in female or male rat liver which were not consistently reported in the studies ALAN. Several changes in clinical chemistry parameters potentially associated with liver toxicity (liver enzyme activities, bilirubin levels) following adult exposure to BPA at 10000 mg/kg bw per day indicated likely effects. However, the results from studies with developmental until weaning or adulthood exposure are not consistent regarding these findings and were judged as ALAN.

In three MoA studies on kidney effects, indications for oxidative stress as a potential pathogenetic mechanism for the weak evidence of kidney damage were observed. Similarly, BPA-induced oxidative

stress was suggested to be associated with liver toxicity. Other studies reported evidence of epigenetic effects via hepatic DNA methylation for changes in lipid and carbohydrate metabolism in liver.

Considering the other clusters in this HOC there were only few effects reported in the WoE assessment (effects at very high doses of BPA, i.e. increase of relative lung weight and hypercellularity in bone marrow, inconsistent effects, i.e. hyperplastic changes in thyroid, parathyroid and pituitary gland and hypertrophic changes in adrenal gland, and effects without a clear dose-response (in haematology) that were all judged as ALAN or Not Likely.

Overall, none of the endpoints in the HOC General toxicity were considered Likely or Very Likely and therefore, none of them was taken forward for BMD analysis.

3.1.3. Immunotoxicity

3.1.3.1. Epidemiological studies

For the HOC Immunotoxicity, a total of nine studies was appraised by the CEP Panel. The details of the appraisals (internal validity) are reported in Annex B.

Identification of the clusters to be considered for WoE

On the basis on the approach described in Chapter 2.3.2 'Definition of Health Outcome Categories and Clusters', the following Clusters (C) and Exposure periods (Exp) was brought forward to WoE analysis:

- C: Asthma/allergy
  - Exp: Childhood
  - Exp: Pregnancy

WoE of the relevant clusters

The main information extracted from the studies included in relevant clusters in the HOC Immunotoxicity are summarised in Annex C. The outcome of the weight of the evidence is described in the text below and presented in a tabulated format in Annex D.

Cluster Asthma/allergy

A large number of allergy-related endpoints was studied in the eight longitudinal studies assessing different exposure windows and all using spot urine samples (Donohue et al., 2013 [RefID 10918]; Kim KN et al., 2014 [RefID 3531]; Spanier et al., 2014b [RefID 6862]; Gascon et al., 2015 [RefID 2206]; Wang IJ et al., 2016 [RefID 7635]; Vernet et al., 2017 [RefID 7452]; Zhou AF et al., 2017 [RefID 9013]; Buckley et al., 2018 [RefID 11645]).

The observed heterogeneity for endpoint definitions was considerable, including asthma (n = 3), wheezing (n = 3), chest infections (n = 1), allergic diseases (n = 1), atopy (n = 1), rhinitis (n = 1), atopic dermatitis (n = 1), FEV_1 (n = 1), PC20 (n = 1), IgE (n = 1), and FENO (n = 1). Statistically significant associations in more than one study were observed for asthma and wheezing. In the text below, the assessed longitudinal studies are described in brief. Their detailed description and risk of bias assessment are provided in Annexes C and D.

Exposure during pregnancy

Seven cohort studies (Donohue et al., 2013 [RefID 10918]; Spanier et al., 2014b [RefID 6862]; Gascon et al., 2015 [RefID 2206]; Vernet et al., 2017 [RefID 7452]; Zhou AF et al., 2017 [RefID 9013]; Buckley et al., 2018 [RefID 11645]; Liao et al. (2016) [RefID 4266]) assessed the association between BPA exposure measured in pregnancy and allergy-related endpoints with a cumulative sample size of 2836 participants. The populations under study were of comparable sample size but varied in their characteristics; there were four studies including a European-descent population. BPA exposure was measured via a single spot urine sample in all seven studies and varied between studies. Thus, the currently available longitudinal epidemiological evidence is characterised by a small number of studies,

suboptimal exposure assessment and considerable heterogeneity in the assessed populations, exposure levels and endpoints.

Donohue et al. (2013) [RefID 10918] used a birth cohort to investigate the association between pre-natal BPA exposure and wheeze, asthma and increased FENO in African-American and Dominican children in the USA followed up until the age of 11 years old (n = 568). No statistically significant associations were observed.

Likewise, Spanier et al. (2014b) [RefID 6862] assessed pre-natal BPA exposure in a population of European ancestry (HOME study, n = 398) and parent-reported wheeze (5-year follow-up) and forced expiratory volume in the first second of expiration (FEV_1) at age 4 and 5 years. Statistically significant associations were observed for BPA exposure at 16 weeks gestation and for FEV_1 at 4 years and wheeze at 5 years [Odds Ratio (OR), 95% CI; 1.79, 1.16–2.78], but not for BPA exposure at 26 weeks gestation or mean gestational BPA exposure and wheeze at 5 years or FEV_1 at 5 years.

Gascon et al. (2015) [RefID 2206] in the INMA (INfancia y Medio Ambiente (Environment and Childhood) Project)) birth cohort (n = 657) evaluated whether pre-natal exposure to BPA and phthalates increases the risk of respiratory and allergic outcomes (chest infections, bronchitis, wheeze, eczema, asthma, IgE) in children (7-year follow-up). Pre-natal BPA levels were statistically significantly associated with wheeze [Relative Risk (RR), 1.20; 95% CI, 1.03–1.40], chest infections (RR, 1.15; 95% CI, 1.00–1.32) and bronchitis (RR, 1.18; 95% CI, 1.01–1.37).

Vernet et al. (2017) [RefID 7452] reporting on the EDEN birth cohort (n = 587) assessed the association between pre-natal BPA exposure (among 9 other phenols and 11 other phthalates) and respiratory outcomes related to allergy (FEV_1, asthma, bronchitis, wheezing). No statistically significant associations were observed.

Zhou AF et al. (2017) [RefID 9013] evaluated the association between BPA concentrations collected at delivery and eczema and wheeze in infants at age 6 months (n = 412). BPA was associated with an increased risk of allergic diseases (OR, 1.21; 95% CI, 1.02–1.47).

Buckley et al. (2018) [RefID 11645] in the Mount Sinai Children's Environmental Health Study evaluated the pre-natal exposure to BPA (among other phenol and phthalate biomarkers) and its association to asthma, wheeze and skin atopy at ages 6 and 7 years (n = 164). Overall, no statistically significant association was observed. For asthma, a statistically significant association for boys was observed (diagnosis, OR 3.04, 95% CI, 1.38–6.68; emergency room visits, OR 3.28, 95% CI 1.15–9.34).

Liao et al. (2016) [RefID 4266] in a birth cohort conducted in Taiwan (n = 250) addressed the association between pre-natal BPA exposure (cord blood) and production of TNF-α, IL-6 and IL-10 after stimulating mononuclear cells with Toll-like receptor ligands (TLR1–4 and 7/8), bacteria from nasopharyngeal specimens and the incidence of infections. Significant associations were identified for TLR3-stimulated and TLR4-stimulated TNF-α response as well as for TLR7/8-stimulated IL-6 response, but not for infection or bacterial colonisation during the first year of life.

On the basis of the above, the CEP Panel concluded that the evidence for a positive association between BPA exposure during pregnancy and allergy is ALAN.

Exposure during childhood

Four cohort studies (Donohue et al., 2013 [RefID 10918]; Kim KN et al., 2014 [RefID 3531]; Spanier et al., 2014b [RefID 6862]; Wang IJ et al., 2016 [RefID 7635]) assessed the association between BPA exposure measured in childhood and allergy-related endpoints with a cumulative sample size of 1,546 participants. The populations under study were of comparable sample size but varied in their characteristics; the one study including a European-descent population (Spanier et al., 2014b [RefID 6862]) included 398 children in USA. BPA exposure was measured via a single spot urine sample in all four studies and varied between studies; in the study including individuals of European descent the total BPA geometric mean (µg/g of creatinine) was 2.4 (95% CI, 2.1–2.7; range, 0.5–293.6). Thus, the currently available longitudinal epidemiological evidence is characterised by a small number of studies, suboptimal exposure assessment and considerable heterogeneity in the assessed populations, exposure levels and endpoints.

Donohue et al. (2013) [RefID 10918] implemented a birth cohort to investigate the association between BPA exposure (3, 5 and 7 years) and wheeze, asthma and increased FENO in African-American and Dominican children in the USA followed up until the age of 11 years old (n = 568). No statistically significant associations were observed.

Spanier et al. (2014b) [RefID 6862] is the only study in this group including a population of European ancestry (HOME study, n = 398). Urine samples were collected annually, and parent-reported wheeze was assessed every 6 months for 5 years along with FEV_1 at age 4 and 5 years. No statistically significant associations were observed.

Kim KN et al. (2014) [RefID 3531] included older children (n = 127, age range 7–8 years) in Korea that were assessed three times every 2 years. The association between BPA concentration at 7–8 years of age and wheezing, asthma and PC20 at ages up to 11–12 years were examined. A statistically significant association was observed for wheezing (OR 2.48; 95% CI 1.15–5.31) and asthma (HR 2.13; 95% CI 1.51–3.00) and PC20 (beta 22.33; P = 0.02).

Wang IJ et al. (2016) [RefID 7635] evaluated 453 children in Taiwan (Childhood Environment and Allergic Diseases Study). Urinary BPAG levels at age 3 were statistically significantly associated with asthma at age 6 (OR, 95% CI; 1.27, 1.04–1.55) and IgE.

On the basis of the above, the CEP Panel concluded the evidence for a positive association between BPA exposure during childhood and allergy is ALAN.

Overall conclusions

On the basis of the above, the CEP Panel concluded that the evidence for a positive association between BPA exposure and asthma/allergy is ALAN.

Cross-sectional studies

Six cross-sectional studies investigated the relationship between BPA exposure (maternal and fetal serum, placenta) and immunity-related endpoints (Spanier et al., 2014a [RefID 6861]; Ashley-Martin et al., 2015 [RefID 281]; Savastano et al., 2015 [RefID 6495]; Ferguson et al., 2016a [RefID 1988]; Lin et al., 2018 [RefID 12522]; Youssef et al., 2018 [RefID 13583]). Three of them assessed immunity biomarkers (Ashley-Martin et al., 2015 [RefID 281]; Savastano et al., 2015 [RefID 6495]; Ferguson et al., 2016a [RefID 1988]) and another three studies examined asthma-related endpoints, including the assessment of lung function (Spanier et al., 2014a [RefID 6861]; Lin et al., 2018 [RefID 12522]; Youssef et al., 2018 [RefID 13583]). The immunity biomarkers under study included IgE, thymic stromal lymphopoietin (TSLP), IL-33, IL-1β, IL-6, IL-10, TNF-α, plasma monocyte chemoattractant protein 1 and C-reactive protein (CRP). Across the multiple analyses performed in these studies, statistically significant results were observed for IL-6 in two studies of populations of European ancestry (n = 76, n = 482) (Savastano et al., 2015 [RefID 6495]; Ferguson et al., 2016a [RefID 1988]) and TNFα in a small study (n = 76) (Savastano et al., 2015 [RefID 6495]). All three studies that assessed allergic disorders of the lung included children and yielded statistically significant results. The results from these cross-sectional studies on asthma supports the findings from the longitudinal studies reviewed above.

3.1.3.2. Animal studies

For the HoC Immunotoxicity a total of 42 studies was appraised by the CEP Panel. The details of the appraisals (internal and external validity) are reported in Annex E.

The endpoints for each study identified as relevant are reported in Annex F. These include also the endpoints identified as key in the uncertainty analysis in the 2015 opinion (EFSA CEF Panel, 2015, Section 4.3.2), except for 'inflammation of the uterus' (pyometra and macrophage infiltration) and serum parameters (histamin and β-hexosamidase) for which no new data were available in the current assessment. For more details see Annex A, Section 2.5.

The key endpoints in the uncertainty analysis in the 2015 EFSA opinion were the following:

- Effects on serum parameters (IgE).
- Lung effects following intraperitoneal sensitisation and inhalatory challenge to ovalbumin (OVA) (airway hyperreactivity, eosinophils in lavage).

- Lung effects following mucosal sensitisation and inhalatory challenge to OVA (inflammation severity, eosinophils in lavage, neutrophils, lymphocytes and T lymphocytes in lavage).
- Lung effects following mucosal sensitisation and inhalatory challenge to OVA and Lipopolysaccharides (LPS) (inflammation severity, eosinophils in lavage, neutrophils, lymphocytes and T lymphocytes in lavage).
- Lung effects following intraperitoneal sensitisation and inhalatory challenge to OVA and alum (eosinophils in lavage, neutrophils and lymphocytes in lavage, airway resistance, airway elastance).

Identification of clusters of relevant endpoints

The immune system is oriented at offering defence to exogenous agents, notably microorganisms, to the host, and preventing deleterious consequences of such pathogens. It operates by a first line of defence, also called innate immune system, which comprises mainly phagocytic and non-specific cytotoxic cells. While killing and degrading the pathogens, antigens are presented by antigen-presenting (dendritic) cells to the antigen-specific arm of the immune system, i.e. lymphocytes that have specific receptors on them that recognise the antigens, and while doing so, expand by proliferation, mature and exert functions such as specific antibody production or specific cytotoxic activity, which terminates the infection. The adaptive immune response includes actions by T cells (i.e. cell-mediated immunity) and B cells (i.e. humoral immunity). Pathogen-specific T cells and B cells, after activation, undergo intensive cell proliferation (i.e. clonal expansion) so there exists a large number of cells that can react to the current threat. Thus, the immune system has the necessary and powerful mechanisms to eradicate threats but must also be tightly regulated to avoid inappropriate reactions.

While defence depends on damage inflicted on the invading pathogens, collateral damage to the tissues of the host may also be inflicted. Whereas inflammation is in principle oriented at destroying the invading pathogen, inflammation may also be adverse to the host as damage to the inflamed tissues may be a consequence. Also, an overreaction of the specific arm of the immune system may occur, i.e. immune responses to agents that are not adverse by themselves but are only adverse because of the consequence of the immune reaction. Such responses are called allergic responses.

Based on the studies available and the nature of the immune system, the relevant immune outcomes identified in the appraised studies were grouped into five clusters:

- Innate immunity
- Cellular immunity
- Humoral immunity
- Inflammation
- Allergic lung inflammation.

Innate immunity

The endpoints considered relevant for the cluster Innate immunity are the cells of the innate immune system, i.e. monocytes/macrophages, natural killer cells, antigen-presenting dendritic cells and their products, such as the antimicrobial molecule lysozyme produced by macrophages. Reduced cell numbers or activity of the cells may result in reduced resistance to pathogens or reduced induction of acquired immunity and should be regarded as adverse. The specific endpoints that were included for the effects on BPA on innate immunity were lysozyme activity, mast cells CysLT, mast cells PGD2, mast cell TNF-α, mast cells IL-13, monocytes/macrophages, MHC class II^+ cells, NK cells, NKT cells and dendritic cells, G-CSF.

Cellular immunity

T cells have a central role in both cell-mediated and humoral immune responses of the adaptive immune system. T cells, so-called because they are primarily produced in the thymus, aid in the immune response by recruiting or activating other immune cells (i.e. T helper cells, $CD4^+$) or by directly killing

infected cells (i.e. cytotoxic T cells, $CD8^+$). They recognise antigens presented by antigen-presenting cells (APCs) via the surface-expressed T-cell receptor (TCR). In the case of antigens processed by APCs by the exogenous pathway, CD4+ T cells bear antigen that has been processed and expressed in the groove of the MHC-II molecules, whereas other antigens found within cells, e.g. target cells, are processed through an endogenous pathway and are delivered by MHC-I to TCR of CD8+ cells. Multiple phenotypes of both CD4+ and CD8+ T cells have been identified with different functions. CD4+ cells are divided into Th1, Th2, Th9, Th17 and T regulatory (Treg) groups, each with a specific profile of cytokine production. CD8+ T cells are comprised of Tc1 and Tc2 subpopulations with cytokine profiles similar to Th1 and Th2 cells. The functions of T cells include promotion of inflammation by cytokine production (Th1 and Th17 cells); helping B cells (Th2 cells); regulation of immunosuppressive responses (Treg) and killing of unwanted target cells (cytotoxic T lymphocytes). The specific endpoints that were included for assessing the effects on BPA on cellular immunity were spleen weight, spleen histology, spleen proliferation, spleen total cell number, T-cell proliferation, Th17 cells, Treg cells, Th1 cells, Th cells (CD4+ cells), Tc cells (CD8+ cells), CD25+ T cells, IFN-γ (not in lung), IL-4 (not in lung), IL-5 (not in lung), IL-13 (not in lung), IL-17 (not in lung), IL-21 (not in lung), IL-23 (not in lung). It should be noted that the spleen contains basically all immune cells, including B cells, T cells, NK cells, macrophages, etc. Therefore, in reality, effects on the spleen could indicate effects on the cellular immunity (T cells), humoral immunity (B cells and antibody production) and innate immunity (NK cells, macrophages).

Humoral immunity

Humoral immunity is provided by antigen-specific antibodies. These are produced by plasma cells, which are differentiated antigen-specific B cells. B cells may recognise antigens, upon which they proliferate, mature and produce antibodies, initially IgM antibodies. Under regulation by helper T lymphocytes isotype switching takes place, and instead of IgM, IgG, IgA and IgE may be produced. IgM, after recognising the antigens on the pathogen to which it is specific, may bind complement and destroy the pathogen. IgA in mucosa may bind the pathogens they recognise, hence bind the pathogens to the mucus and subsequently use the mucus as a vehicle to dispose of the microbes. IgE is one of the isotypes produced and potentially affected by BPA exposure, but typically this antibody is associated with allergic reactions and is therefore included in the cluster allergic lung inflammation. The specific endpoints that were included for the effects on BPA on humoral immunity were IgA+ cells, IgA, IgM production and B-cell proliferation of spleen cells (e.g. using LPS or Pokeweed mitogen as mitogen).

Inflammation

Inflammation is a broad term referring to the presence of active immune cells at a site of infection, which is part of the normal process of pathogen destruction. In addition, tissue damage, by the release of DAMPs (damage-associated molecular patterns), can also trigger inflammation (i.e. sterile inflammation). Inflammation is a common response to a variety of stressors, including xenobiotics. Common features of the inflammatory process include tissue resident cell activation, release of pro-inflammatory mediators and leukocyte recruitment/activation. Hence, xenobiotics can elicit prolonged, severe and/or inappropriate inflammatory responses that play a causal role in the progression of biological events resulting in an adverse effect. The specific endpoints that were included for the effects on BPA on inflammation were IL-1, IL-6, IL-12p70, IL-22, IL-31, TNF-α, VEGF, lung stromal cell-derived factor 1 (SDF1), neutrophils, eosinophils and colonic inflammation score.

Allergic lung inflammation

Allergic lung inflammation may be brought about by serum IgE levels, specific for certain respiratory allergens. When IgE, bound on the membrane of mast cells through specific IgE receptors, is cross-linked by antigens on the allergen, the mast cells release various products such as histamine and serotonin as well as inflammatory mediators. This in turn leads to attraction of inflammatory cells, such as eosinophils, causing damage in the respiratory tract. Inflammation may ultimately lead to reduced capacity of the respiratory function of the lungs. Production of IgE is regulated by an array of cytokines,

released from T lymphocytes as well as from other cell types, such as the mast cells or the inflammatory cells themselves. In addition, the recruitment of inflammatory cells is regulated by pro-inflammatory mediators, produced by different cell types. The specific endpoints that were included for the effects on BPA on allergic lung inflammation were lung IL-4, lung IL-13, lung IL-17, lung IL-33, lung TNF-α, lung (serum anti-OVA) IgE, lung inflammatory score, lung CysLT, lung KC, lung RANTES, lung cellularity in bronchoalveolar lavage (BAL) (total cells, macrophages, neutrophils, eosinophils, lymphocytes).

WoE of the clusters of relevant endpoints

The main information extracted from the studies addressing relevant endpoints in the HOC Immunotoxicity are summarised in Annex G. The outcome of the WoE is described in the text below and presented in a tabulated format in Annex H.

The clusters of the effects of BPA on Immunity considered for the WoE assessment were the following:

- Innate immunity
- Cellular immunity
- Humoral immunity
- Inflammation
- Allergic lung inflammation.

As a general approach for the WoE and subsequent selection of endpoints to be taken forward for risk characterisation, it should be noted that not all parameters in a cluster or subcluster need necessarily to show effects in the same direction, to still prove an effect. This is inherent to the nature of the immune system which is a regulatory network with checks and balances. Whereas all intermediate endpoints may shed light on the likelihood of the cluster to be affected by BPA, many of these are not specific for the eventual adverse outcome and sometimes not even predictive. For this reason, in case a (sub)cluster identifies a likely effect, apical endpoint parameters within this cluster were taken forward for BMD analysis rather than these intermediate ones. Intermediate endpoints comprise predominantly cytokines or other factors mediating communication between different components of the immune system.

Innate immunity

Within this cluster, six studies in mice were identified, of which five studies dealt with exposure during development and one with exposure during adulthood. Furthermore, four studies in rats were identified, two of which dealt with exposure during development, three with exposure during development and adulthood, and one during growth phase/young age. Note that some studies cover different stages of life (i.e. both exposure and endpoint measurement timing).

Developmental exposure (pre-natal and/or post-natal until weaning)

The evidence that investigated the effect of exposure during development on innate immunity included seven studies: five in mice, two in rats. Of the mouse studies there were two allocated to Tier 1: Malaisé et al. (2018) [RefID 11172] and Rogers et al. (2017) [RefID 6257], two to Tier 2: Malaisé et al. (2017) [RefID 4815] and Patel et al. (2015a) [RefID 5696] and one to Tier 3: Bodin et al. (2014) [RefID 623]. The rat studies were both allocated to Tier 1: NTP Clarity Report (2018)/Camacho et al. (2019) [RefID 11370] and Li J et al. (2018a) [RefID 12460], part of the NTP Clarity study. There are no endpoints in this cluster showing a consistent effect following BPA exposure during developmental phases of life. The only parameter that showed a consistent effect was the decrease in the production of lysozyme. This effect was shown in two papers: Malaisé et al. (2017) [RefID 4815] and Malaisé et al. (2018) [RefID 11172] in which C3H/HeN male mice in Malaisé et al. (2017) [RefID 4815] and female mice in Malaisé et al. (2018) [RefID 11172] were exposed to 50 µg/kg bw per day from gestational day 15 until weaning. Measurements were done at PND45 and 170 in Malaisé et al. (2017) [RefID 4815] and PND50 in Malaisé et al. (2018) [RefID 11172]. Both papers were from the same scientific group and may in fact have been provided by one study. In addition, there was only one dose of BPA used.

Therefore, the CEP Panel assigned a likelihood level of ALAN to the innate immunity effects of BPA during the developmental exposure period, hence none of the endpoints was taken forward for BMD analysis. However, they were considered in the uncertainty analysis (see Appendix D).

Developmental and adult exposure (pre-natal and post-natal in pups until adulthood)

Three studies in rats investigated the effect of exposure during development and adulthood on innate immunity. All were allocated in Tier 1: NTP Clarity Report (2018)/Camacho et al. (2019) [RefID 11370], Li J et al. (2018a) [RefID 12460] and Li J et al. (2018b) [RefID 12461]. Both Li papers belong to the NTP Clarity Grantee Studies. No consistent effects were shown. In detail, BPA (0, 2.5, 25, 250, 2500 or 25000 µg/kg bw per day) was administered daily by gavage in 0.3% carboxymethylcellulose vehicle to NCTR (National Centre for Toxicological Research) Sprague–Dawley rats from GD6 through the start of parturition and then directly to pups from the day after birth until termination at 1 or 2 years (continuous-dose group). Overall, no effects were identified. For this reason, the CEP Panel decided that the effect of BPA on innate immunity during developmental and adult stages is Not Likely, so, none of the endpoints was taken forward for BMD analysis.

Growth phase/young age exposure

The studies that investigated the effect of exposure during the growth phase on innate immunity included one study in rats: Ogo et al. (2018) [RefID 11201], which was allocated to Tier 2. This study evaluated the effect of BPA on macrophages following exposure at young age. In this study, rats were exposed to 20 µg/kg bw or 200 µg/kg bw per day during PND36–66. No effects of BPA on macrophages were observed.

As no effects were observed, the CEP Panel assigned a likelihood level of Not Likely to the innate immunity effects of BPA during the growth phase/young age exposure period, so none of the endpoints was taken forward for BMD analysis.

Adult exposure (after puberty)

A single Tier 2 study investigated the effect of exposure during adulthood on innate immunity: Cetkovic-Cyrlje et al. (2017) [RefID 916]. In this study, male C57BL/6 mice were orally exposed to 1 or 10 mg BPA/L (corresponding to 160 and 1600 µg/kg bw per day) starting at 4 weeks of age; diabetes was induced at 9 weeks of age with streptozotocin (STZ). The innate parameters investigated (macrophages and natural killer cells) either showed no effect, or some statistically significant differences but without a clear dose–response relationship. As the study measured different endpoints, the CEP Panel judged this effect as Not Likely.

The CEP Panel assigned a likelihood level of Not Likely to the innate immunity effects of BPA during the adult exposure period, so, none of the endpoints was taken forward for BMD analysis.

Indirect (germline) exposure

No studies were available for this exposure period.

Overall cluster selection of the endpoints/studies for BMD analysis for innate immunity

Overall, the CEP Panel assigned a likelihood level of ALAN, Not Likely, Not Likely and Not Likely to innate immunity effects of BPA in the exposure periods developmental exposure, developmental and adult exposure, growth phase/young age and adult exposure, respectively. The overall likelihood across all exposure periods, i.e. the highest likelihood given in the cluster innate immunity, was ALAN.

The CEP Panel considered that the evidence from the studies available did not show a Likely or Very Likely effect of BPA in any exposure period, therefore, none of the endpoints was taken forward for BMD analysis.

Cellular immunity

Within this cluster, 10 studies in mice were identified, of which six studies dealt with exposure during development, one with exposure during development and adulthood and three during adulthood. Furthermore, eight studies in rats were identified, five of which dealt with exposure during

development, three with exposure during development and adulthood, and one with exposure during the adult phase. Note that some studies cover different stages of life.

Developmental exposure (pre-natal and/or post-natal until weaning)

The studies that investigated the effect of exposure during development on cellular immunity included six studies in mice and five in rats. Of the six studies in mice, three were allocated to Tier 1: Luo et al. (2016) [RefID 4679], Malaisé et al. (2018) [RefID 11172] and O'Brien et al. (2014a) [RefID 5462] ; two were allocated in Tier 2: Malaisé et al. (2017) [RefID 4815] and Patel et al. (2015a) [RefID 5696]; and one was allocated in Tier 3: Bodin et al. (2014) [RefID 623]. Of the five studies in rats, four were allocated in Tier 1: Lejonklou et al. (2017) [RefID 3975], NTP Clarity Report (2018)/Camacho et al. (2019) [RefID 11370], Dunder et al. (2018) [RefID 11866] and Li AJ et al. (2018) [RefID 12460]; and one study was allocated to Tier 3: Tarapore et al. (2017) [RefID 7128].

Following developmental exposure to BPA, a consistent dose-related increase in Th17 cells and associated cytokines (i.e. IL-17, IL-21 and IL-23) was observed at doses as low as 100 nM in drinking water (equivalent to 4.75 µg/kg bw per day) by Luo et al. (2016) [RefID 4679]. In this study, pregnant dams were exposed to BPA (10, 100 or 1000 nM) in drinking water from GD0 to PND21 (equivalent to 0.475, 4.75 and 47.5 µg/kg bw per day). Offspring were analysed at PND21 and PND42.

Three other studies support these findings, showing an effect in the same direction: Malaisé et al. (2017) [RefID 4815] (50 µg/kg bw per day, from GD15 until weaning, males measured at PND45 and 170), Bodin et al. (2014) [RefID 623] (0.1, 1 and 10 mg/L drinking water, equivalent to 30, 300 and 3000 µg/kg bw per day during pregnancy, and 45, 450 and 4500 µg/kg bw per day during lactation, from mating until weaning, females were investigated at 7 and 11 weeks) and Malaisé et al. (2018) [RefID 11172] (pregnant mice were orally exposed to BPA 50 µg/kg bw per day from day 15 of pregnancy until weaning, measures were taken at PND50 only in females). Whereas other parameters, including IFN-γ, IL-13 and other T-cell subpopulations were not consistently affected (Bodin et al., 2014 [RefID 623]; Malaisé et al., 2017 [RefID 4815]; O'Brien et al., 2014a [RefID 5462]). This should not be considered as inconsistency, as under cellular immunity many cells with different functions are included. Th17 cells play a critical role in the induction of the tissue inflammation and tissue destruction, common to many immune-mediated diseases, like psoriasis, rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease and asthma.

The CEP Panel assigned a likelihood level of Likely to the cellular immunity effect of BPA during the developmental exposure period. Since the likelihood level for this cluster is Likely for Th17 cells (Luo et al., 2016 [RefID 4679]), this endpoint was taken forward for BMD analysis (see Chapter 3.2.1) and uncertainty analysis (see Appendix D). The endpoints IL-17, IL-21 and IL-23, also Likely, were not taken forward for BMD analysis because these parameters can be triggered by different stimuli, including physiological stimuli, and are not considered very close to the apical endpoint (allergic lung inflammation).

Developmental and adult exposure (pre-natal and post-natal in pups until adulthood)

The studies that investigated the effect of exposure during development and adulthood on cellular immunity included three studies in rats and one in mice. The studies in rats, Li J et al. (2018b) [RefID 12461], Li AJ et al. (2018) [RefID 12460] and NTP Clarity Report (2018)/Camacho et al. (2019) [RefID 11370], were allocated to Tier 1. The mouse study, Gear and Belcher (2017) [RefID 2230], was allocated to Tier 3.

Following developmental and adult exposure, no consistent effects were observed. Some effects were identified, while most of these alterations were found to be transient and not dose dependent (note: Th17 cells were not evaluated in this exposure period).

As some effects were identified in one study only, while most of these alterations were found to be transient and not dose dependent, the CEP Panel assigned a likelihood level of Not Likely to the cellular immunity effects of BPA during the developmental and adult exposure period, so, none of the endpoints was taken forward for BMD analysis.

Growth phase/young age exposure

No studies were available for this exposure period.

Adult exposure (after puberty)

The studies that investigated the effect of exposure during adulthood on cellular immunity included three studies in mice and one in rats. Of the studies in mice, two were allocated to Tier 1: Dong et al. (2013) [RefID 1676] and DeLuca et al. (2018) [RefID 11805], and one in Tier 2: Cetkovic-Cyrlje et al. (2017) [RefID 916]. The study in rats is allocated to Tier 3: Özaydin et al. (2018a) [RefID 12853].

Overall, no consistent effects were observed following adult exposure, and the CEP Panel assigned a likelihood level of Not Likely to the cellular immunity effects of BPA during adult exposure period, so, none of the endpoints was taken forward for BMD analysis.

Indirect (germline) exposure

No studies were available for this exposure period.

Overall cluster selection of the endpoints/studies for BMD analysis for cellular immunity

Overall, the CEP Panel assigned a likelihood level of Likely to cellular immunity effects of BPA in the exposure period developmental exposure, and Not Likely to developmental and adult and to adult only exposure. The overall likelihood across all exposure periods, i.e. the highest likelihood given in the cluster cellular immunity, was Likely.

The CEP Panel considered that the evidence from the studies available showed a Likely effect of BPA during the developmental exposure period for the endpoint Th17 cells (Luo et al., 2016 [RefID 4679]), therefore, this endpoint was taken forward for BMD analysis (see Chapter 3.2.1).

Humoral Immunity

In the cluster Humoral Immunity only two studies were included. One study, part of the NTP CLARITY study, Li J et al. (2018b) [RefID 12461], investigated the effects of BPA exposure during development in rats. A second study, Malaisé et al. (2018) [RefID 11172], investigated the effects of BPA during development and adulthood in mice.

Developmental exposure (pre-natal and/or post-natal until weaning)

The effect of BPA exposure on humoral immunity following developmental exposure was investigated in one study in mice (Malaisé et al. (2018) [RefID 11172]). For local IgA parameters the study was allocated to Tier 3; for systemic IgA parameters, the study was allocated to Tier 1. Pregnant mice were exposed from GD15 until weaning to BPA 50 µg/kg bw per day. The IgA endpoints were investigated in female mice at PND50 on which a consistent decrease was noted. However, the endpoints were all in one study only and are not independent from each other. In addition, only one dose was applied in this study.

The CEP Panel assigned a likelihood level of ALAN to the humoral immunity effects of BPA during the developmental exposure period, so, none of the endpoints was taken forward for BMD analysis. However, they were considered in the uncertainty analysis (see Appendix D).

Developmental and adult exposure (pre-natal and post-natal in pups until adulthood)

The effect of BPA exposure on humoral immunity following development and adulthood exposure was investigated in one study in rats, Li J et al. (2018b) [RefID 12461], allocated to Tier 1. Following developmental and adult exposure animals were dosed by oral gavage with BPA (2.5, 25, 250, 2500 or 25000 µg/kg bw per day) and were euthanised on PND21, 90, 6 months and 1 year. No consistent effects on LPS-induced proliferation were observed: an increase was observed in females at 2500 µg/kg bw per day at PND90, while a decrease was observed in males at 2.5 µg/kg bw per day, and no effects at 6 months and 1 year. The effects on LPS-induced proliferation were transient with no dose–response. Hence, this effect was judged as Not Likely.

In addition, statistically significantly different values of IgM production were found, but inconsistent between the sexes, and without a clear dose–response. The CEP Panel considered these as chance findings and decided the study did not show a likely effect neither for IgM production nor cell proliferation.

The CEP Panel assigned a likelihood level of Not Likely to the humoral immunity effects of BPA in the developmental and adult exposure period, so, none of the endpoints was taken forward for BMD analysis.

Growth phase/young age

No studies were available for this exposure period.

Adult exposure (after puberty)

No studies were available for this exposure period.

Indirect (germline) exposure

No studies were available for this exposure period.

Overall cluster selection of the endpoints/studies for BMD analysis for humoral immunity

Overall, the CEP Panel assigned a likelihood level of ALAN and Not Likely to humoral immunity effects of BPA in the exposure periods developmental exposure and developmental and adult exposure, respectively. The overall likelihood across all exposure periods, i.e. the highest likelihood given in the cluster humoral immunity was ALAN.

The CEP Panel considered that the evidence from the studies available did not show a Likely or Very Likely effect of BPA in any exposure period, therefore, none of the endpoints was taken forward for BMD analysis.

Inflammation

In this cluster, following developmental exposure to BPA a total of five studies (four in mice and one in rats) were allocated; two studies in rats following developmental and adult exposure, one study following exposure during the growth phase and four studies (two in mice, one in rabbits and one in rats) following adult exposure.

Developmental exposure (pre-natal and/or post-natal until weaning)

The studies that investigated the effect of exposure during development on inflammation included five studies, four in mice and one in rats. Of the four studies in mice, two were allocated to Tier 1: Luo et al. (2014) [RefID 4660] and Luo et al. (2016) [RefID 4679], one paper was allocated to Tier 2: Malaisé et al. (2017) [RefID 4815] and one study was allocated to Tier 3: Bodin et al. (2014) [RefID 623]. The rat study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) was allocated to Tier 1. These five studies covered the parameters 'numbers of neutrophils', 'numbers of eosinophils' as well as 'expression of several interleukins' (IL-1, IL-6, IL-22, TNF-α). Effects were reported at doses as low as 0.34 µg/kg bw per day in Luo et al. (2016) [RefID 4679] and 50 µg/kg bw per day in Malaisé et al. (2017) [RefID 4815], but the effects were not seen in all the studies, were not dose related, and some studies only included one dose (Malaisé et al., 2017 [RefID 4815]; Luo et al., 2014 [RefID 4660]).

As there is insufficient evidence to support an effect, having only a trend in a decrease in neutrophils, the CEP Panel assigned a likelihood level of Not Likely to the inflammation adverse effects of BPA during the developmental exposure period, so, none of the endpoints was taken forward for BMD analysis.

Developmental and adult exposure (pre-natal and post-natal in pups until adulthood)

The studies that investigated the effect of exposure during development and adulthood on inflammation were two studies in rats: NTP Clarity Report (2018)/Camacho et al. (2019) [RefID 11370] and Ben-Jonathan et al. (2018) (NTP Grantee study) [RefID 13786], both allocated to Tier 1. In both studies, BPA (0, 2.5, 25, 250, 2500, and 25000 µg/kg bw per day) was administered daily by gavage in 0.3% carboxymethylcellulose vehicle to NCTR Sprague–Dawley rats from GD6 through the start of parturition and then directly to pups from the day after birth until termination at one or 2 years (continuous-dose group).

No effects on neutrophils were found in the CLARITY Core Study NTP Clarity Report (2018)/Camacho et al. (2019) [RefID 11370]. In the same study, a decrease in blood eosinophils at 250 µg/kg bw per day was found, both in males and females, in the continuous-dose group at interim sacrifice, without a

dose–response; no effects were observed at other time points. In the other study, Ben-Jonathan et al. (2018) (NTP Grantee study) [RefID 13786], no change in IL-6 was observed.

The CEP Panel assigned a likelihood level of Not Likely to the inflammation effects of BPA during the developmental and adult exposure period, so, none of the endpoints was taken forward for BMD analysis.

Growth phase/young age exposure

One study in rats, Ogo et al. (2018) [RefID 11201], that was allocated to Tier 1 investigated the effect of exposure during young age on inflammation. An effect was noted on inflammation at the tissue level in the epididymis in a single rat study, characterised by an increase in IL-6 and neutrophils, following exposure during the growth phase to 20 µg/kg bw or 200 µg/kg bw per day, at PND36–66.

The CEP Panel assigned a likelihood level of Likely to the inflammation effects of BPA in the growth phase/young age exposure period. Since the likelihood level for this cluster is Likely for the endpoint neutrophils in epididymis (Ogo et al., 2018 [RefID 11201]), this was taken forward for BMD analysis (see Chapter 3.2.1). As the effect on endpoint IL-6 in epididymis, also likely, can be triggered by different stimuli including physiological stimuli and it is not considered very close to the apical endpoint (inflammation), it was decided not to use this endpoint for BMD analysis.

Adult exposure (after puberty)

The studies that investigated the effect of exposure during adulthood on inflammation included two studies in mice, one study in rabbits and one in rats. Both studies in mice were allocated to Tier 2: Cetkovic-Cyrlje et al. (2017) [RefID 916], DeLuca et al. (2018) [RefID 11805]. The study in rabbits, Fang et al. (2014) [RefID 1914], was allocated to Tier 1. The study in rats, Özaydin et al. (2018a) [RefID 12853], was allocated to Tier 2. The four studies after adult exposure covered the parameters IL-1α, IL-6, IL-12p70, IL-31, SDF-1α, TNF-α, VEGF, as well as the colon inflammation score. The only dose–response reported is in Özaydin et al. (2018a) [RefID 12853], in which a dose-related increase in IL-6 was observed in male rats exposed to 50 and 500 µg/kg bw per day. In addition, in the adult exposure period, there is inconsistence between different species, models or strains used.

Due to insufficient evidence, studies performed only at one dose and Tier 2 studies showing no effects, the CEP Panel assigned a likelihood level of Not Likely to the inflammation effects of BPA during the adult exposure period, so, none of the endpoints was taken forward for BMD analysis.

Indirect (germline) exposure

No studies were available in this exposure period.

Overall cluster selection of endpoints/studies for BMD analysis for Inflammation

Overall, the CEP Panel assigned a likelihood level of Not Likely, Not Likely, Likely and Not Likely to inflammation effects of BPA in the exposure periods developmental exposure, developmental and adult exposure, growth phase/young age exposure and adult exposure, respectively.

The CEP Panel considered that the evidence from the studies available showed a Likely effect of BPA in the exposure period growth phase/young age for the endpoint neutrophils in epididymis (Ogo et al., 2018 [RefID 11201]), therefore, this endpoint was taken forward for BMD analysis (see Chapter 3.2.1).

The overall likelihood across all exposure periods, i.e. the highest likelihood given in the cluster inflammation, was Likely.

Allergic lung inflammation

The cluster allergic lung inflammation included four studies in mice, of which two dealt with exposure during development, and one with exposure during adulthood.

Developmental exposure (pre-natal and/or post-natal until weaning)

The studies that investigated the effect of exposure during development on allergic lung inflammation included two studies in mice allocated to Tier 2: O'Brien et al. (2014a) [RefID 5462] and O'Brien et al. (2014b) [RefID 5463].

In the study of O'Brien et al. (2014b) [RefID 5463], mice were dosed at 0.05 µg, 50 µg, 50000 µg/kg diet (equivalent to 0.0075 µg, 7.5 µg, 7500 µg/kg bw per day), from 2 weeks before mating until PND21. Animals were tested at 6 months. The study investigated effects on mast cell mediators (CysLT, IL-13, PGD2 and TNF-α). The CEP Panel judged effects on CysLT, IL-13 and TNF-α as ALAN as all values were significantly increased, even if there was not a clear dose–response. For PGD2, there was instead a clear dose-related increase, hence this effect was judged as Likely. As this effect can be triggered by different stimuli including physiological stimuli, and it is not considered very close to the apical endpoint (allergic lung inflammation), it was decided not to use this endpoint for BMD analysis.

In the study O'Brien et al. (2014a) [RefID 5462], mice were dosed at 0.05 µg, 50 µg, 50000 µg/kg diet (equivalent to 0.0075 µg, 7.5 µg, 7500 µg/kg bw per day) 1–2 weeks before mating until PND21. Animals were tested at 3 months (12 weeks). Ovalbumin-specific IgE was dose-relatedly increased in both sexes. This effect was therefore judged as Very Likely and the CEP Panel decided to take this effect forward for BMD analysis (see Chapter 3.2.1).

In the same study, lung inflammatory parameters were investigated, i.e. lung cellularity, IL-13, CysLT, IL-17, IL-4, lung inflammatory score, RANTES and TNF-α. Decreased CysLT and IL-17 were considered by the CEP Panel as Likely effects, observed in both sexes and with the same dose–responses. Since this parameter can be triggered by different stimuli including physiological stimuli and it is not considered very close to the apical endpoint of allergic lung inflammation, it was decided not to use this endpoint for BMD analysis.

Lung cellularity, IL-13, IL-4, TNF-α and lung inflammation score were judged as ALAN, as these parameters were either affected in one sex only or there was no clear dose–response.

The CEP Panel considered the effects on RANTES as Not Likely, as the only changes were observed at the lowest dose, in females only.

The CEP Panel assigned a likelihood level of Likely to the allergic lung inflammation effects of BPA during the developmental exposure period. Since the likelihood level for this allergic lung inflammation is Very Likely for the endpoint ovalbumin-specific IgE (O'Brien et al., 2014a [RefID 5462]) this was taken forward for BMD analysis (see Chapter 3.2.1). The endpoints mast cell PGD2, lung CysLT and lung IL-17, also judged as likely, were not taken forward for BMD analysis because these endpoints can be triggered by different stimuli, including physiological stimuli. In addition, these endpoints are not considered very close to the apical endpoint (allergic lung inflammation). However, they will be considered in the uncertainty analysis (see Appendix D).

Developmental and adult exposure (pre-natal and post-natal in pups until adulthood)

No studies reporting exposure pre-natally and post-natally in pups until adulthood were available.

Growth phase/young age exposure

No studies were available in this exposure period.

Adult exposure (after puberty)

There was one study that investigated the effect of exposure during adulthood on allergic lung inflammation, which was allocated to Tier 1, Tajiki-Nishino et al. (2018) [RefID 13221]. In this study, IL-4, IL-33 and eosinophils in the BAL fluid were measured. A dose-dependent increment in IL-4 and IL-33 was observed in the lung of mice sensitised and challenged to toluene diisocyanate and exposed to BPA at 60 and 200 µg/kg bw per day, which were judged as Likely effects.

The CEP Panel assigned a likelihood level of Likely to the allergic lung inflammation effects of BPA during the adult exposure period. Since the likelihood level for this allergic lung inflammation is Likely for eosinophils in the bronchioalveolar lavage and that this endpoint represents a very close to an adverse apical endpoint, this effect was taken forward for BMD analysis (see Chapter 3.2.1). The cytokine endpoints were also assigned Very Likely or Likely but were not taken forward for BMD analysis because these endpoints can be triggered by different stimuli, including physiological stimuli. In addition, these endpoints are not considered very close to the apical endpoint (allergic lung inflammation). However, they will be considered in the uncertainty analysis (see Appendix D).

Indirect germline exposure

No studies were available in this exposure period.

Overall cluster selection of the endpoints/studies for BMD analysis for allergic lung inflammation

Overall, the CEP Panel assigned a likelihood level of Likely to allergic lung inflammation effects of BPA in the exposure periods developmental exposure and adult exposure. The overall likelihood across all exposure periods, i.e. the highest likelihood given in the cluster allergic lung inflammation, was Likely.

The CEP Panel considered that the evidence from the studies available showed a Very Likely effect of BPA in the developmental exposure period for the endpoint serum OVA-specific IgE (O'Brien et al., 2014a [RefID 5462]) and a Likely effect of BPA in the adult exposure period for the endpoint eosinophils in the BAL (Tajiki-Nishino et al., 2018 [RefID 13221]), therefore, these endpoints were taken forward for BMD analysis (see Chapter 3.2.1).

3.1.3.3. Integration of likelihoods from human and animal studies

Table 9 presents the overall likelihood per cluster for the human and animal stream separately, as well as the integration of the likelihoods from the human and animal studies for Immunotoxicity.

Table 9: Integration of the human and animal studies for Immunotoxicity.

Human stream		**Animal stream**		**Integrated likelihood**
Cluster: Asthma/ allergy		**Cluster: Allergic lung inflammation**		
Exposure during Pregnancy	ALAN	Developmental exposure (pre-natal and/or post-natal until weaning)	Likely	
Exposure during Childhood	ALAN	Adult exposure (after puberty)	Likely	
Overall likelihood:	*ALAN*	*Overall likelihood:*	*Likely*	*Likely*
Cluster: Cellular immunity		**Cluster: Cellular immunity**		
Not applicable		Developmental exposure (pre-natal and/or post-natal until weaning)	Likely	
		Developmental and adult exposure (pre-natal and/or post-natal in pups until adulthood)	Not Likely	
		Adult exposure (after puberty)	Not Likely	
		Overall likelihood:	*Likely*	*Likely*
Cluster: Humoral immunity		**Cluster: Humoral immunity**		
Not applicable		Developmental exposure (pre-natal and/or post-natal until weaning)	ALAN	
		Developmental and adult exposure (pre-natal and/or post-natal in pups until adulthood)	Not Likely	
		Overall likelihood:	*ALAN*	*ALAN*
Cluster: Innate immunity		**Cluster: Innate immunity**		
Not applicable		Developmental exposure (pre-natal and/or post-natal until weaning)	ALAN	
		Developmental and adult exposure (pre-natal and/or post-natal in pups until adulthood)	Not Likely	
		Growth phase/young age exposure	Not Likely	

	Adult exposure (after puberty)	Not Likely	
	Overall likelihood	*ALAN*	*ALAN*
Cluster: Inflammation	**Cluster: Inflammation**		
Not applicable	Developmental exposure (pre-natal and/or post-natal until weaning)	Not Likely	
	Developmental and adult exposure (pre-natal and/or post-natal in pups until adulthood)	Not Likely	
	Growth phase/young age exposure	Likely	
	Adult exposure (after puberty)	Not Likely	
	Overall likelihood	*Likely*	*Likely*

3.1.3.4. *In vitro* and mechanistic studies

Cellular immunity

The mechanisms along which BPA influences the immune system are largely unknown, and this applies to all components of the immune system, including acquired cellular immunity.

Since there was no direct human evidence available for the cluster cellular immunity, the overall likelihood of effects of BPA for this cluster was scored based on the animal evidence. As indicated in the WoE section, the CEP Panel considered that the evidence from the studies available indicated a Likely effect of BPA on cellular immunity during the developmental exposure period (see Chapter 3.1.3.2).

Exposure to low-dose BPA (4.75 μg/kg bw per day) during gestation and lactation led to a sustained, sex-specific and dose-dependent increase in Th17 cells in the offspring mice mediated by specific alteration of their transcription factor and regulatory cytokines IL-17, IL-21 and IL-23 (Luo et al., 2016 [RefID 4679]). The study by Malaisé et al. (2018) [RefID 11172] showed deficient dendritic cell maturation in the lamina propria in the intestine and spleen after exposure to 50 μg/kg bw per day during gestational and lactating phases, which may be the basis for the effects on regulatory T cells in the lamina propria and subsequent effects on the Th17 cells systemically. Also, direct effects of BPA on lymphocytes may play a role, as Cipelli et al. (2014) [RefID 1252] reported effects of *in vitro* exposure at 100 nM BPA on proliferation and the ATP content of human leukaemia T-cell lymphoblasts (Jurkat cells), in which oestrogen receptor 2 (ER2) and oestrogen-related receptor-α (ERRA) appeared to be involved.

Whereas *in vitro* studies included BPA concentrations of 100 nM, it should be mentioned that these concentrations may still be quite high compared with the actual internal exposure in humans and mechanisms identified by *in vitro* studies may therefore not necessarily all be operational.

Humoral Immunity

There was no direct human evidence available for the cluster humoral immunity. Thus, the overall likelihood of effects of BPA for this cluster was scored based on the animal evidence. The CEP Panel considered that the evidence from the animal studies available did not show a Likely effect of BPA on humoral immunity in any exposure period. Only during development, an effect of BPA exposure on IgA production was judged as Likely, but this was found in only one study and not supported by other parameters, hence the CEP Panel considered this effect as ALAN. It should be noted that reagins (i.e. antibodies involved in allergic reactions) were not included in this cluster but are dealt with in the cluster allergic lung inflammation, as IgE is a crucial component of allergic lung inflammation. Obviously, IgE is a component of humoral immunity, under strict regulation of T cells, hence under regulation of the cellular immune system. In the current section, the humoral immune aspects other than IgE and IgG1 are being dealt with.

It should be noted that like IgE, IgA is also regulated by cellular immune components and even if the effect on IgA was not sufficiently convincing to justify a judgement Likely for the cluster, effects on both IgE (dealt with under allergic lung inflammation) and IgA do indicate dysregulation of humoral immunity. An effect of 50 µg/kg bw per day of BPA on IgA may be associated with influences of BPA on the intestinal barrier functions, as suggested by Malaisé et al. (2018) [RefID 11172].

Mechanisms underlying humoral immunity were studied in humans by Chailurkit et al. (2016) [RefID 924], who investigated associations of BPA exposure with autoantibodies (antithyroglobulin, antithyroperoxidase and antithyrotrophin receptor) and found a significant trend for an association of antithyroglobulin and antithyroperoxidase with BPA exposure. These autoantibodies again are under regulation of cellular immunity. In addition, their findings may lend further support to an effect on humoral immunity, brought about by suppression of T-cell regulation and subsequent enhancement of Th-17 mechanisms, leading to enhanced responses as indicated in the section on cellular immunity.

Allergic lung inflammation

After the integration of the human and animal evidence, the overall likelihood of effects of BPA for the cluster allergic lung inflammation was scored Likely (see Chapter 3.1.3.2).

The CEP Panel considered that the evidence from the animal studies available showed a Very Likely effect of BPA in the developmental exposure period for the endpoint serum OVA-specific IgE and a Likely effect of BPA in the adult exposure period for the endpoint eosinophils in the BAL. Even if effects of exposure in the cluster humoral immunity were judged as ALAN, especially after exposure during development, these effects are in line with the judgement on effects on allergic lung inflammation, that are especially evident after exposure during development. This supports the susceptibility of the developing immune system, even if in this current cluster of allergic lung inflammation, also effects were noted after exposure to BPA during adulthood. A study performed after intratracheal instillation of BPA, Koike et al. (2018) [RefID 12355], found an exacerbation of ovalbumin-induced lung inflammation and specific IgE responses by of BPA exposure, by enhancing Th2 responses via disruption of the immune system, further supporting this finding. A possible hypothesis for allergic lung inflammation was put forward by Tajiki-Nishino et al. (2018) [RefID 13221], who suggested that bronchial epithelial cells and TSLP may activate APCs resulting in stimulation of Th cells and subsequent exacerbation of local cytokine level, found after exposure to 0.06 mg/kg bw per day at adulthood and IgE production. Cross-linking of IgE on mast cells triggers these mast cells to release their mediators. O'Brien et al. (2014b) [RefID 5463] showed that perinatal BPA exposure to doses from 7.5 ng/kg bw per day displayed a long-term influence on mast cell-mediated production of pro-inflammatory mediators associated with asthma and global DNA methylation levels, supporting the role for mast cells in pulmonary inflammation associated with allergic airway disease into adulthood.

In addition to regulatory effects on IgE production by mediators from bronchial epithelium, also direct inflammatory effects of mediators from bronchial fibroblasts may play a role in an eventual lung inflammation. Mahemuti et al. (2016) [RefID 4784] exposed human fetal lung fibroblasts (HFLF) to 100 µM BPA *in vitro* and observed that BPA affects the release of growth differentiation factor-15 (GDF15), ET-1, interleukin-6 (IL-6) and interferon γ-induced protein 10 (IP-10), as well as phosphorylation of nuclear factor kappa B (NF-κB) p65. The effects were reported at concentrations higher than the cut-off for inclusion of these studies and may not be relevant for the human situation.

Throughout the studies of effects of BPA on the immune system, there seems to be an age dependency, i.e. a more pronounced effect after exposure during development compared with effects of exposure during later stages in life. This is in accordance with findings by Petzold et al. (2014) [RefID 5811]. These authors observed an asthma-promoting effect after life-long exposure, including during pregnancy and breast feeding, to 5 µg/mL in drinking water during pregnancy (equivalent to 0.9 µg/kg bw per day). After BPA exposure of adult mice during sensitisation to ovalbumin, reduced allergic responses were noted. This reduction could be reverted using the glucocorticoid receptor (GR) antagonist RU486.

Innate immunity

There was no direct human evidence available for the cluster innate immunity. Thus, the overall likelihood of effects of BPA for this cluster was scored based on the animal evidence. The CEP Panel considered that the evidence from the animal studies available showed overall ALAN effects.

A reduction in the functional parameter lysozyme was shown by Malaisé et al. (2017) [RefID 4815] and Malaisé et al. (2018) [RefID 11172]. An increase in plasma G-CSF was reported by Rogers et al. (2017) [RefID 6257] in an experimental model of multiple sclerosis. In that study, the increase in C-CSF was associated with increased number of circulating neutrophils compared with vehicle treatment. Blocking C-CSF by a monoclonal antibody resulted in a decreased incidence and severity of Experimental Allergic Encephalomyelitis. This suggests that the mechanism by which gestational BPA exposure increases risk for autoimmunity could be through priming macrophages that produce G-CSF after activation and subsequent mobilisation of neutrophils by G-CSF. Of note, following adult exposure, was the increased number of antigen-presenting (dendritic) cells observed after intratracheal exposure to BPA by Koike et al. (2018) [RefID 12355], which may be in line with the effects on allergic lung inflammation.

With respect to *in vitro* studies and the mechanistic understanding that go far beyond the *in vivo* studies included in this cluster, the effect of BPA on innate immunity was investigated in the most relevant cell types: macrophages, dendritic cells, neutrophils and mast cells. *In vitro* studies were conducted using both primary cells from humans and rodents or cell lines (e.g. THP-1, U936). Studies indicate that BPA can directly act on innate immune cells modulating cytokine production, with results showing increased pro-inflammatory cytokines and decreased anti-inflammatory cytokines at a concentration of 100 nM (Couleau et al., 2015 [RefID 1316]), at 10 nM (Liu YZ et al., 2014 [RefID 4533]) and at 100 nM (Chen Y et al., 2018 [RefID 11728]); decreased phagocytosis at a concentration of 1 nM (Couleau et al., 2015 [RefID 1316]) and of 100 µM (Berntsen et al., 2018 [RefID 11063]). Increased ROS were observed by Michalowicz et al. (2015) [RefID 5083] but only at concentrations higher than 0.3 µM.

As indicated above, it should be mentioned that the concentrations used for *in vitro* studies may be quite high compared with the actual internal exposure in humans and mechanisms identified by these *in vitro* studies may therefore not necessarily all be operational.

Overall, the mechanistic elucidation of the BPA effect was minimal, however, several studies provide evidence for these effects to be induced by modulation of the extracellular signal-related kinase (ERK)/NF-κB signalling pathway observed by Herz et al. (2017) [RefID 2698] at a concentration of 1 nM, Couleau et al. (2015) [RefID 1316] and Liu YZ et al. (2014) [RefID 4533] at a concentration of 100 nM and O'Brien et al. (2014c) [RefID 5464] at concentrations from 1 nM, potentially mediated via ERs (ERa/b and the membrane receptor GPER or GPR30). Furthermore, Michalowicz et al. (2015) [RefID 5083] and Neri et al. (2015) [RefID 5363] suggest that BPA is capable of damaging innate immune cells through oxidative stress and DNA damage leading to apoptosis and necrosis at concentrations ranging from 6.57 nM to 0.3 µM. Finally, O'Brien et al. (2014c) [RefID 5464] studied the effects of BPA *in vitro*, demonstrating that BPA could enhance mast cell release at concentrations of 100 nM and that this could be mediated partly through the ERK pathway and extracellular Ca^{2+} concentrations, but not dependent on an ER-mediated mechanism. This latest study supports the role for mast cells in pulmonary inflammation associated with allergic airway diseases (O'Brien et al., 2014b [RefID 5463]; Petzold et al., 2014 [RefID 5811]; Koike et al., 2018 [RefID 12355]; Tajiki-Nishino et al., 2018 [RefID 13221]).

Liao et al. (2016) [RefID 4266], using a human birth cohort, investigated the effect of pre-natal exposure to BPA on TLR-induced cytokine responses in neonates. Production of TNF-α, IL-6 and IL-10 was evaluated after stimulating mononuclear cells with TLR ligands (TLR1–4 and TLR7/8). Although the study did not yield a statistically significant result for the epidemiological risk of infection during early infancy, they report an association between cord blood BPA concentration and supressed TLR3-stimulated and TLR4-stimulated TNF-α response and TLR7/8-stimulated IL-6 response. Overall, the studies on innate immunity cells support *in vivo* evidence indicating an immune de-regulation and possibly increased susceptibility to develop inflammatory reactions.

Inflammation

There was no human evidence available for the cluster inflammation. Thus, the overall likelihood of effects of BPA for this cluster was scored based on the animal evidence. The CEP Panel considered that the evidence from the animal studies available showed overall Likely effects.

Some endpoints included in this cluster, e.g. pro-inflammatory cytokines, have been discussed in the previous Chapter, as produced by innate immune cells.

Effects following growth phase/young age were shown in the epididymis by Ogo et al. (2018) [RefID 11201] after exposure from 20 μg/kg bw per day, as a clear indication of increased IL-6 and neutrophils.

Regarding cytokine production, overall the findings support an increase in the production of pro-inflammatory cytokines, but with inconsistent results or results obtained in a single-dose study. At adulthood, an increase in IL-6 was observed in two studies from 50 μg/kg bw per day (Özaydin et al., 2018a [RefID 12853]; Fang et al., 2014 [RefID 1914]), with no effect in another study (Cetkovic-Cvrlje et al., 2017 [RefID 916]). Increased TNF-α was found in two studies (Özaydin et al., 2018a [RefID 12853]; Fang et al., 2014 [RefID 1914]), while a decrease was observed in Cetkovic-Cvrlje et al. (2017) [RefID 916] after exposure to 1.6 mg/kg bw per day. Increases in IL-1a, IL-12p70, IL-31, VEGF were reported in a single-dose study of 50 μg/kg bw per day (DeLuca et al., 2018 [RefID 11805]) and in SDF1a at 0.084 ng/kg bw per day in Koike et al. (2018) [RefID 12355]. The increase in pro-inflammatory cytokines is consistent with *in vitro* results, as also described in the previous section.

Two mechanistic studies in humans reported inflammation markers in Asian populations. Song et al. (2017) [RefID 6815] supplemented an *in vitro* study with evidence from epidemiological studies and investigated the association between urinary BPA levels and well known inflammation-related markers including WBC, CRP, IL-10, ALT, AST and γ-GTP. Significant positive associations between BPA level and WBC, ALT and γ-GTP levels were found. Yang et al. (2016) [RefID 8375] also supplemented their *in vivo* and *in vitro* investigations with evidence from epidemiological studies on ALT, γ-GT and high-sensitivity C-reactive protein (hs-CRP), leptin and TNF-α. BPA was associated with inflammation markers (leptin, TNF-α) in lean subjects but not in overweight/obese subjects and stratified analyses suggested a possible attenuation by sex and BMI.

In addition, there are studies indicating modulation of signalling pathways at concentrations equal to or below 100 nM (e.g. ERK, JNK, NF-κB) by Couleau et al. (2015) [RefID 1316] at 100 nM, Liu YZ et al. (2014) [RefID 4533] at 10 nM, Song et al. (2017) [RefID 6815] at 100 nM and Zhu et al. (2015) [RefID 9100] at 10 nM; modulation of cytokine gene expression and secretion by Camarca et al. (2016) [RefID 805] at 0.1 nM, Couleau et al. (2015) [RefID 1316] at 100 nM, Liu YZ et al. (2014) [RefID 4533] at 10 nM, Zhu et al. (2015) [RefID 9100], Chen Y et al. (2018) [RefID 11728] at 100 nM, Li Q et al. (2018) [RefID 12475] at 8.76 nM, Tajiki-Nishino et al. (2018) [RefID 13221] at 1 nM; modulation of histone methylation by Li Q et al. (2018) [RefID 12475] at 8.76 nM and modulation of ER signalling pathways, where effects were reversed by ERa/b antagonists by Couleau et al. (2015) [RefID 1316] at 100 nM, Liu YZ et al. (2014) [RefID 4533] at 10 nM, Chen Y et al. (2018) [RefID 11728] at 100 nM. In contrast to the latter findings, Chakhtoura et al. (2017) [RefID 927] found no effects on cytokine expression and no influence on dendritic cell maturation at concentrations as low as 0.05 nM, while the positive control treatment at the same concentration with β-E2 did.

Again, whereas *in vitro* studies included BPA concentrations of 100 nM, it should be mentioned that these concentrations may still be quite high compared with the actual internal exposure in humans and mechanisms identified may therefore not necessarily all be operational. Nevertheless, taking all results together, many of the studies reviewed highlighted a possible role of BPA in inflammatory processes. Modulation of ERK1/2 phosphorylation, NF-κB activation, modulation of the ERs, GR and androgen (AR), as well as cytokine/chemokine secretion, are relatively common hypothesised mechanisms for the effects observed.

Concluding remarks

Different components of the immune system seem to be affected. Effects may be on non-specific cells belonging to the immune system or influencing the immune system, such as APCs and epithelial cells, that through presentation of antigens to T lymphocytes or release of mediators, influence the regulatory homeostasis of the immune system, suppressing T regulatory cells and stimulating Th17 cells, leading to enhanced production of IgE. IgE, after being cross-linked at the surface of mast cells, may lead to the release of inflammatory mediators, that in concert with inflammatory mediators from other cell types, may lead to inflammatory reactions, including those in the respiratory tract. Such increases in inflammation have also been observed in the epididymis after exposure to BPA, which may in part follow similar mechanisms. It is currently not clear how BPA interacts with the various cells comprising

the immune system or cells such as epithelial cells or fibroblasts, of which the mediators influence the immune system, but a role for GR, ER2 and the ERRA and subsequent activation of transcription factors may be plausible. The response to BPA may differ according the experimental condition. This refers to the exposure regimen, i.e. the exposure at different life stages as well as the exposure in relation to the allergenic challenge. It is likely that such conditions will also have an impact on effects in humans.

3.1.3.5. Conclusion on hazard identification for Immunotoxicity of BPA

In the 2015 EFSA opinion on BPA (EFSA CEF Panel, 2015) it was stated that based on human studies, there were indications that BPA may be linked to immunological outcomes, although a causal link could not be established. In addition, studies in animals lent support to the possibility of immune effects. Effects of concern were especially increase in IgE and allergic lung inflammation, but like the human studies, also animal studies suffered from shortcomings, for which reason the CEF Panel did not take these effects forward for the risk characterisation carried out in 2015. In a later statement in 2016, after evaluating two additional studies of effects of exposure to BPA on the immune system, in which potential allergic conditions were investigated, the CEF Panel confirmed its position that the studies available at that time suggested effects on the immune system, but that the studies were not sufficiently robust to take them forward for risk characterisation (EFSA CEF Panel, 2016).

The available information that has been evaluated in the current evaluation is in line with these earlier observations and indicates now more firmly that there is a hazard with respect to adverse outcomes of exposure on the immune system, notably an effect on cellular immunity and parameters indicating allergic lung inflammation. Even if mechanisms along which the immune system is affected by BPA are not clear, it is clear from the studies shedding some light on these mechanisms, that effects may be on non-specific cells, such as APCs and epithelial cells, that through presentation of antigens to T lymphocytes or release of mediators influence the regulatory homeostasis of the immune system. This may lead to suppression of T regulatory cells and subsequent stimulation of Th17 cells, leading to enhanced production of IgE. IgE, after being cross-linked at the surface of mast cells, may lead to the release of inflammatory mediators, that in concert with inflammatory mediators from other cell types may lead to inflammatory reactions (see Chapter 3.1.3.4 on mechanisms of immunotoxicity of BPA). Whereas the parameters investigated mainly comprised intermediate endpoints, such as interleukins, mast cell mediators and specific antibodies related to allergy, and while indications for inflammation (as visualised by histology and cellular infiltrations and shown by broncho-alveolar lavage) were noted, the eventual disease endpoint of altered lung functionality has been investigated in only a few studies. These seemed to indicate an effect, although were not judged to be of high quality.

In addition to effects in the respiratory tract, an inflammatory effect was also seen in the epididymis after exposure to BPA, which may be brought about by partly similar mechanisms. Based on the available information, the effects in the cluster innate immunity were judged as ALAN, but an increased number of antigen-presenting (dendritic) cells was observed after intratracheal exposure to BPA, which underscores the effect that BPA can have on the homeostasis of the immune system. It is currently not clear how BPA interacts with the various cells comprising the immune system or cells such as epithelial cells or fibroblasts, of which the mediators influence the immune system, but a role for GR, ER2 and ERRA and subsequent activation of transcription factors may be plausible.

Overall, the CEP Panel considers that a hazard exists for adverse effects of BPA on the immune system, that may result, depending on the dose, most likely in inflammatory reactions such as in the respiratory tract. Whereas the developing immune system is generally considered more vulnerable to toxic damage than the fully mature immune system, effects were noted both after exposure during developmental stages as well as at adulthood, hence the hazard exists throughout the different life stages.

Using a WoE approach, the CEP Panel assigned a likelihood level of Likely to BPA-induced effects on the Th17 cells, on the neutrophils in epididymis, on eosinophils in the BAL, and of Very Likely to BPA-induced effects on serum OVA-specific IgE. Therefore, these endpoints were brought forward for BMD analysis (see Chapter 3.2.1).

3.1.4. Metabolic effects

3.1.4.1. Epidemiological studies

For the HOC Metabolic, a total of 27 studies was appraised by the CEP Panel. The details of the appraisals (internal validity) are reported in Annex B.

Identification of the clusters to be considered for WoE

On the basis on the approach described in Chapter 2.3.2 'Definition of Health Outcome Categories and Clusters', the following Clusters (C) and Exposure periods (Exp) were brought forward to WoE analysis:

- C: Obesity
  - Exp: Adulthood
  - Exp: Pregnancy
  - Exp: Childhood
- C: Cardiometabolic effects
  - Exp: Pregnancy
- C: Thyroid effects
  - Exp: Pregnancy
- C: Type 2 diabetes mellitus
  - Exp: Adulthood
- C: Gestational Diabetes Mellitus
  - Exp: Adulthood.

WoE of the relevant clusters

The main information extracted from the studies included in relevant clusters in the HOC Metabolic are summarised in Annex C. The outcome of the WoE is described in the text below and presented in a tabulated format in Annex D.

Cluster Obesity

A large number of obesity-related endpoints was studied in the 13 epidemiological longitudinal studies assessing different exposure periods and all using spot urine samples. The observed heterogeneity for population characteristics and endpoint definitions was considerable. The associations under study reached statistical significance for waist circumference (n = 3) (Valvi et al., 2013 [RefID 7384]; Hoepner et al., 2016 [RefID 2732]; Hao et al., 2017 [RefID 9400]), central obesity (n = 1) (Hao et al., 2017 [RefID 9400]) and annual weight gain (n = 1) (Song Y et al., 2014 [RefID 6839]). In the text below, the assessed studies are described in brief. Their detailed description and risk of bias assessment are provided in Annexes C and D.

Exposure during pregnancy

Eleven publications corresponding to nine cohort studies assessed the association between BPA exposure measured during pregnancy and obesity-related endpoints with a cumulative sample size of 3,536 participants (Valvi et al., 2013 [RefID 7384]; Ashley-Martin et al., 2014 [RefID 280]; Braun et al., 2014b [RefID 709]; Buckley et al., 2016 [RefID 748]; Hoepner et al., 2016 [RefID 2732]; Vafeiadi et al., 2016 [RefID 7368]; Bae et al., 2017 [RefID 350][15]; Perng et al., 2017 [RefID 5792]; Watkins et al., 2017a [RefID 7875]; Yang TC et al., 2017 [RefID 8408]; Junge et al., 2018 [RefID 12262]). The populations under study were of small (<500) sample size except for one study that included 1,237 participants (Ashley-Martin et al., 2014 [RefID 280]); three studies studied European populations (Valvi

[15] This study considered as a cross-sectional study in the EFSA supporting publication 'Implementation of the evidence-based risk assessment for the re-evaluation of Bisphenol A: preparatory work on cross-sectional studies' (AERU, University of Hertfordshire, 2020) was recategorized by the EFSA WG on BPA re-evaluation as a cohort study and therefore considered in the WoE assessment.

et al., 2013 [RefID 7384]; Vafeiadi et al., 2016 [RefID 7368]; Junge et al., 2018 [RefID 12262]), while four studies studied populations in North America (Ashley-Martin et al., 2014 [RefID 280]; Braun et al., 2014b [RefID 709]; Buckley et al., 2016 [RefID 748]; Hoepner et al., 2016 [RefID 2732]) and one study was conducted in Mexico (Watkins et al., 2017a [RefID 7875]; Yang TC et al., 2017 [RefID 8408]; Yang et al., 2018 [RefID 13545]). BPA exposure was measured via spot urine sampling in all eight studies and was comparable across studies. The assessed endpoints that were related to obesity were BW, obesity, BMI (n = 6), rapid growth, weight change, waist circumference (n = 2), skinfold thickness, fat mass (n = 2), leptin and adiponectin. The follow-up of the included studies ranged from 1 to 14 years. For FMI, % body fat and waist circumference, statistically significant associations were observed in one, one and two studies, respectively. None of the six studies (8 publications) that assessed BMI reported statistically significant findings; moreover, all six studies used different follow-up points rendering a formal synthesis uninformative. Of note, results on BMI and overweight risk coming from the INMA study that were presented by Valvi et al. (2013) [RefID 7384] were corroborated by Valvi et al. (2013) [RefID 7384] using a 7-year follow-up. Thus, the currently available longitudinal epidemiological evidence is characterised by a small number of small studies, suboptimal exposure assessment and considerable heterogeneity in the assessed endpoints. Moreover, there are no studies to replicate the observed 'positive' associations; for waist circumference, an endpoint that emerged from the studies assessing exposure during adulthood, the two available studies that reported statistically significant results used fundamentally different follow-up points (14 months and 7 years) and their respective results cannot be consolidated.

On the basis of the above, is the CEP Panel concluded that a positive association between exposure to BPA during pregnancy and obesity is Not Likely.

Exposure during childhood

Three small studies (Lee et al., 2013 [RefID 3911]; Braun et al., 2014b [RefID 709]; Vafeiadi et al., 2016 [RefID 7368]) in Greece, USA and South Korea investigated the association between BPA exposure in pregnancy and childhood and obesity-related endpoints. No statistically significant associations were observed.

On the basis of the above, it is concluded that the evidence for a positive association between BPA exposure during childhood and obesity is Not Likely.

Exposure during adulthood

Three cohort studies (Song Y et al., 2014 [RefID 6839]; Rönn et al., 2015 [RefID 6277]; Hao et al., 2017 [RefID 9400]) assessed the association between BPA exposure measured in adulthood and obesity-related endpoints with a cumulative sample size of 2,759 participants. The populations under study were of comparable sample size but varied in their characteristics; the one study of a European population (Rönn et al., 2015 [RefID 6277]) included 70-year-old community dwellers in Sweden. BPA exposure was measured via a single spot urine sample in two studies, via serum sampling in one study and exposure levels varied between studies; in the study including individuals from Europe the total BPA median interquartile range was 3.9 (2.08–6.57) ng/mL for men and 2.1 (1.97–6.51) ng/mL for women. The assessed endpoints were central obesity, annual weight gain, waist circumference, fat trunk/leg ratio, subcutaneous adipose tissue (SAT), visceral adipose tissue (VAT), visceral/subcutaneous adipose tissue ratio (VAT/SAT), fat (leg), fat (trunk) and body fat. The follow-up of the included studies ranged from 2 to 10 years and no single endpoint was assessed in more than one study. For the incidence of central obesity, annual weight gain and waist circumference, statistically significant associations were observed. For the body fat and fat distribution indices, no statistically significant effects were observed. Thus, the currently available longitudinal epidemiological evidence is characterised by a small number of studies, suboptimal exposure assessment and considerable heterogeneity in the assessed populations, exposure levels and endpoints. Moreover, there are no studies to replicate the observed positive associations between exposure to BPA and obesity.

On the basis of the above, the CEP Panel concluded that a positive association between exposure to BPA during adulthood and obesity is ALAN.

Overall conclusions

On the basis of the above, the CEP Panel concluded that the evidence for an association between BPA exposure and obesity is ALAN.

Cluster Cardiometabolic effects

Exposure during pregnancy

Three cohort studies (Vafeiadi et al., 2016 [RefID 7368]; Bae et al., 2017 [RefID 350]; Perng et al., 2017 [RefID 5792]) assessed cardiovascular risk factors related to the metabolic syndrome (total cholesterol, LDL, HDL, triglycerides, diastolic blood pressure, systolic blood pressure and pulse pressure). The follow-up of these studies ranged from 4 to 14 years.

One study (Bae et al., 2017 [RefID 350]) reported respectively a statistically significant increase of diastolic blood pressure and a statistically significant decrease of systolic blood pressure, while no statistically significant results were found as regards to pulse pressure. The other two studies (Vafeiadi et al., 2016 [RefID 7368]; Perng et al., 2017 [RefID 5792]) did not find any statistically significant results related to the measured endpoints (total cholesterol, LDL, HDL, triglycerides, systolic blood pressure and diastolic blood pressure).

The currently available longitudinal epidemiological evidence is characterised by a small number of small studies and suboptimal exposure assessment. One study reports two conflicting statistically significant results on blood pressure, while the other studies do not report any statistically significant results.

Overall conclusions

On the basis of the above, the CEP Panel concluded that the evidence for a positive association between BPA exposure and cardiometabolic effects is Not Likely.

Cluster Thyroid effects

Three cohort studies assessed the association between BPA exposure measured in spot urine samples and thyroid hormones' profile using different exposure periods. In the text below, the assessed studies are described in brief. Their detailed description and risk of bias assessment related to these studies are provided in Annexes C and D. Of these, one study evaluated BPA exposure during adulthood (Aung et al., 2017 [RefID 303]) and it was not considered further in the WoE as it represents a single entry for this cluster. In summary, the limited currently available epidemiological evidence does not put forward a thyroid-related endpoint as a critical one for risk assessment.

Exposure during pregnancy

Two birth cohort studies in USA (Chevrier et al., 2013 [RefID 1154]; Romano et al., 2015 [RefID 6270]) assessed whether maternal BPA concentrations in spot urine samples during pregnancy were associated with neonatal (cord blood) TSH (n = 364) or neonatal thyroid hormones (n = 249). No statistically significant associations were observed.

On the basis of the above, it is concluded that the evidence for a positive association between BPA exposure during pregnancy and thyroid effects is Not Likely.

Overall conclusions

On the basis of the above, the CEP Panel concluded that the evidence for an association between BPA exposure and abnormal thyroid function is Not Likely.

Cluster Type 2 diabetes mellitus (T2DM)

Six studies (four cohort, two case–control) (Lee et al., 2013 [RefID 3911]; Sun et al., 2014 [RefID 6986]; Hu et al., 2015 [RefID 2818]; Bi et al., 2016 [RefID 571]; Watkins et al., 2016 [RefID 7874]; Shu et al., 2018 [RefID 13119]) assessed the association between BPA exposure measured in spot urine samples and endpoints related to T2DM using different exposure periods. Their detailed

description and risk of bias assessment related to these studies are provided in Annexes C and D. Of these, two studies evaluated BPA exposure during pregnancy (Watkins et al., 2016 [RefID 7874]) and during childhood (Lee et al., 2013 [RefID 3911]), respectively, and they were not considered further in the WoE as they represent single entries for this cluster.

Exposure during adulthood

Four studies evaluated BPA exposure during adulthood and were considered in the WoE (Sun et al., 2014 [RefID 6986]; Hu et al., 2015 [RefID 2818]; Bi et al., 2016 [RefID 571]; Shu et al., 2018 [RefID 13119]). Their detailed description and risk of bias assessment related to these studies are provided in Annexes C and D. In summary, the limited currently available epidemiological evidence does not put forward a T2DM-related endpoint as a critical one for risk assessment.

Two cohorts (Hu et al., 2015 [RefID 2818]) and two case–control studies (Sun et al., 2014 [RefID 6986]; Shu et al., 2018 [RefID 13119]) assessed the association between BPA exposure measured in adulthood and T2DM-related endpoints with a cumulative sample size of 4504 participants. The populations under study were of various sample sizes and varied in their characteristics. The assessed endpoints were T2DM and eGFR in T2DM patients. The largest studies were a nested case–control study within the Nurses' Health Study and the Nurses' Health Study II (NHSII) (n = 1942) in a population of predominantly European ancestry (Sun et al., 2014 [RefID 6986]) and a cohort study in a China (n = 2209, n cases = 242) (Bi et al., 2016 [RefID 571]). BPA levels were statistically significantly associated with incident-T2DM only in NHSII and after adjustment for BMI [per quartile OR (95%CI), 1.34 (0.70, 2.27), 1.91 (1.11, 3.29), 2.08 (1.17, 3.69)]. Conversely, Bi et al. (2016) [RefID 571] reported no significant association of risk of incident T2D with BPA. Based on the above, the currently available longitudinal epidemiological evidence is characterised by a small number of studies, suboptimal exposure assessment and considerable heterogeneity in the assessed populations, exposure levels and endpoints. Moreover, there are no studies to replicate the observed partially 'positive' associations.

On the basis of the above, the CEP Panel concluded that a positive association between BPA exposure during adulthood and T2D is ALAN.

Overall conclusions

On the basis of the above, the CEP Panel concluded that a positive association between BPA exposure and type 2 diabetes is ALAN.

Cluster Gestational diabetes mellitus

Exposure during adulthood

Three cohort studies (Bellavia et al., 2018 [RefID 11590] n = 350; Fisher et al., 2018 [RefID 11946] n = 232 and Shapiro et al., 2015 [RefID 6605] n = 1885) with their follow-up restricted within pregnancy assessed the association between BPA exposure measured in spot urine samples (n = 2) or serum (n = 1) and endpoints related to GDM. Their detailed description and risk of bias assessment related to these studies are provided in Annexes C and D. All three studies include participants from Europe or North America and they shared similarities for their population characteristics and endpoint definitions. No association under study reached statistical significance.

On the basis of the above, the CEP Panel concluded that a positive association between BPA exposure during adulthood and GDM is Not Likely.

Overall conclusions

On the basis of the above, the CEP Panel concluded that a positive association between BPA exposure and gestational diabetes mellitus is Not Likely.

Cross-sectional studies

Obesity

A total of 20 studies (Nicolucci et al., 2013 [RefID 5391]; Choi et al., 2014 [RefID 1185]; Eng et al., 2014 [RefID 1817]; Ko et al., 2014 [RefID 10443]; Wells et al., 2014 [RefID 7921]; Agay-Shay et al., 2015 [RefID 54]; Akin et al., 2015 [RefID 93]; Andra and Makris, 2015 [RefID 197]; Geens et al., 2015 [RefID 2238]; Milic et al., 2015 [RefID 5098]; Pornkunwilai et al., 2015 [RefID 5889]; Savastano et al., 2015 [RefID 6495]; Xue et al., 2015 [RefID 8263]; Corbasson et al., 2016 [RefID 1292]; Metwally et al., 2016 [RefID 9673]; Do et al., 2017 [RefID 1640]; Hong et al., 2017 [RefID 2762]; Li J et al., 2017 [RefID 4058]; Liu et al., 2017 [RefID 11165]; Charisiadis et al., 2018 [RefID 11691]) examined cross-sectionally the relationship between BPA exposure and obesity, BMI and body composition measurements. Of those, nine studies were conducted in children or adolescents (Nicolucci et al., 2013 [RefID 5391]; Choi et al., 2014 [RefID 1185]; Eng et al., 2014 [RefID 1817]; Wells et al., 2014 [RefID 7921]; Agay-Shay et al., 2015 [RefID 54]; Akin et al., 2015 [RefID 93]; Pornkunwilai et al., 2015 [RefID 5889]; Xue et al., 2015 [RefID 8263]; Li J et al., 2017 [RefID 4058]). The populations under study were diverse in terms of sample size, age, sex, comorbidities, exposure levels and geographical origin. The endpoints assessed also varied considerably and included obesity, overweight, weight, BMI, insulin, insulin resistance, body fat, fat mass, lean mass, hip and waist circumference, waist to height ratio, adiponectin, adipokine hormones, leptin. Multiple analyses were performed per study. The cross-sectional studies in adults were generally small and some yielded statistically significant results. Although, the associations were not entirely consistent in terms of directionality, findings from some individual studies could be interpreted as being indicative of an association with obesity. The results from these cross-sectional analyses are in accordance with the results from the few prospective cohorts on obesity. Of course, with only a single measure of BPA cross-sectionally it seems biologically implausible that short time exposure quantified at that point in time could affect obesity risk. An alternative explanation for these cross-sectional findings is that those with obesity may be differently exposed to BPA through difference in lifestyle or behaviour. Alternatively, these associations may also reflect biological differences in rate of excretion of BPA. Studies relying on repeated measures of BPA would to some extent be able to address these limitations.

Cardiometabolic effects

Thirteen cross-sectional studies assessed the association between BPA exposure and blood pressure and vascular health (Sabanayagam et al., 2013 [RefID 6353]; Khalil et al., 2014 [RefID 3467]; Ko et al., 2014 [RefID 10443]; Shiue, 2014 [RefID 6705]; Aekplakorn et al., 2015b [RefID 47]; Lin et al., 2015 [RefID 4307]; Wang TG et al., 2015 [RefID 7755]; Metwally et al., 2016 [RefID 9673]; Turgut et al., 2016 [RefID 7330]; Kataria et al., 2017 [RefID 3407]; Menale et al., 2017 [RefID 5026]; Mouneimne et al., 2017 [RefID 5237]; Amin et al., 2018 [RefID 11498]). The available cross-sectional evidence on blood pressure is not characterised by consistency, while the small studies related to biomarkers of cardiovascular health used heterogeneous endpoints. Eleven cross-sectional studies (Eng et al., 2014 [RefID 1817]; Ko et al., 2014 [RefID 10443]; Lin et al., 2015 [RefID 4307]; Savastano et al., 2015 [RefID 6495]; Turgut et al., 2016 [RefID 7330]; Milosevic et al., 2017 [RefID 5101]; Mouneimne et al., 2017 [RefID 5237]; Amin et al., 2018 [RefID 11498]; Carlsson et al., 2018 [RefID 11070]; Lee I et al., 2018 [RefID 12428]; Mohsen et al., 2018 [RefID 12746]) investigated the relationship between BPA exposure and lipid profile characterised by various population characteristics and sample sizes. Of these, only a few studies showed statistically significant associations between BPA exposure and total cholesterol, LDL, triglycerides and dyslipidemia.

Thyroid effects

A total of 10 studies (Sriphrapradang et al., 2013 [RefID 6870]; Wang TG et al., 2013 [RefID 7753]; Wang N et al., 2015 [RefID 7702]; Aker et al., 2016 [RefID 92]; Chailurkit et al., 2016 [RefID 924]; Minatoya et al., 2017b [RefID 5106]; Park et al., 2017 [RefID 5656]; Zhou ZZ et al., 2017 [RefID 9089]; Przybyla et al., 2018 [RefID 12920]; Sanlidag et al., 2018 [RefID 13042]) examined cross-sectionally the relationship between BPA exposure and endpoints related to thyroid function. Of those, three studies were conducted in children or adolescents (Wang N et al., 2015 [RefID 7702]; Minatoya et al., 2017b [RefID 5106]; Sanlidag et al., 2018 [RefID 13042]) and one study was conducted in pregnant women (Aker et al., 2016 [RefID 92]). The populations under study were diverse in terms of sample size, age, sex, comorbidities, exposure levels and geographical origin. The endpoints assessed also varied considerably and included TSH, Triiodothyronine (T_3), Thyroxine (T_4), thyroid autoimmunity, antithyroglobulin, antithyroperioxidase, antithyrotrophin receptor, thyroid volume, thyroid nodules, hyperthyroidism, euthyroidism, nodular goitre, papillary thyroid carcinoma and thyroid secretory

capacity. Multiple analyses were performed per study. Overall, the associations reported in these studies are Not Likely to be causal.

Type 2 diabetes mellitus (T2DM)

A total of 18 studies (Sabanayagam et al., 2013 [RefID 6353]; Ahmadkhaniha et al., 2014 [RefID 67]; Beydoun et al., 2014 [RefID 551]; Aekplakorn et al., 2015a [RefID 46]; Savastano et al., 2015 [RefID 6495]; Bi et al., 2016 [RefID 571]; Piecha et al., 2016 [RefID 5831]; Tai and Chen, 2016 [RefID 7070]; Turgut et al., 2016 [RefID 7330]; Chailurkit et al., 2017 [RefID 11073]; Hong et al.,2017 [RefID 2762]; Kataria et al., 2017 [RefID 3407]; Menale et al., 2017 [RefID 5026]; Mouneimne et al., 2017 [RefID 5237]; Carlsson et al., 2018 [RefID 11070]; Dallio et al., 2018 [RefID 11782]; Li AJ et al., 2018 [RefID 12448]; Verstraete et al., 2018 [RefID 13308]) examined cross-sectionally the relationship between BPA exposure and endpoints related to type 2 diabetes mellitus (T2DM). Of those, three studies were conducted in children or adolescents (Kataria et al., 2017 [RefID 3407]; Menale et al., 2017 [RefID 5026]; Carlsson et al., 2018 [RefID 11070]). The populations under study were diverse in terms of sample size, age, sex, comorbidities, exposure levels and geographical origin. The endpoints assessed also varied considerably and included diabetes, impaired fasting glucose, haemoglobin A1c, hyperinsulinemia, insulin levels, glucose homeostasis, 2-hour post-loading plasma glucose, insulin resistance, Homeostatic Model Assessment (HOMA), and resistin. Multiple analyses were performed per study. Overall, the associations reported in these studies are Not Likely to be causal.

Gestational diabetes mellitus (GDM)

Two studies (Robledo et al., 2013 [RefID 6218]; Wang X et al., 2017 [RefID 7773]) examined cross-sectionally the relationship between BPA exposure and endpoints related to gestational diabetes. One of the studies showed a statistically significantly association between higher urinary BPA concentrations and reduced risk of GDM (Wang X et al., 2017 [RefID 7773]), while the other one yielded statistically non-significant findings (Robledo et al., 2013 [RefID 6218]). Overall, the associations reported in these studies are Not Likely to be causal.

3.1.4.2. Animal studies

For the HoC Metabolic effects a total of 82 studies were appraised by the CEP Panel. The details of the appraisals (internal and external validity) are reported in Annex E.

The endpoints for each study identified as relevant in this opinion are reported in Annex F. The data of these studies used for the WoE are shown in Annex G.

Glucose regulation was already a key endpoint in the uncertainty analysis in the 2015 EFSA opinion (EFSA CEF Panel, 2015, Section 4.3.2). For more details see Annex A, Chapter 2.5.

Identification of clusters of relevant endpoints

The clusters of relevant endpoints assessed in the studies on effects of BPA on metabolism were the following:

- Obesity
- Fat deposition in the liver
- Glucose regulation
- Blood lipids
- Uric acid
- Type 1 diabetes mellitus (T1DM)
- Other metabolic hormones
- Thyroid hormones.

Obesity

Environmental chemicals may both increase obesity (have obesogenic effect) and decrease obesity (have a toxic effect), which both are considered adverse. Obesity is associated with multiple metabolic

alterations that are risk factors for diabetes mellitus with its consequences due to macrovascular and microvascular changes, such as cardiovascular diseases, and is related to non-alcoholic fatty liver disease (NAFLD) with the risk to develop non-alcoholic steatohepatitis (NASH) (Napoli and Pozzilli, 2014). Whereas non-alcoholic liver disease is reversible, NASH may progress to cirrhosis and liver cancer (Willebrords et al., 2015). It is believed that insulin resistance in the adipose tissue, liver and skeletal muscle is crucial in the pathogenesis of these metabolic abnormalities. Adipocytes are key regulators of whole-body energy homeostasis and altered adipose tissue glucose metabolism is also an important cause of insulin resistance and metabolic dysfunction. Adipose tissue contributes to the development of obesity-related glucose abnormalities through excessive release of free fatty acids (FFA), adipokines, cytokines and macrophage infiltration, causing inflammation. Increased body weight and body fat mass are also linked to several types of cancer, i.e. colorectal, liver and postmenopausal breast cancer (WCRF/AICR, 2018).

All the endpoints in the clusters are interrelated and may potentially affect humans adversely. Although there are several slightly different definitions on the metabolic syndrome, obesity, insulin resistance, atherogenic dyslipidemia and hypertension comprise the metabolic syndrome, and are risk factors for cardiovascular disease and T2DM (Huang, 2009). Several of these endpoints are included as metabolic endpoints in this WoE.

The specific endpoints that were included for the effects of BPA in the cluster obesity from the metabolic studies were body weight, BMI, body fat mass, visceral adipocyte size and non-visceral adipocyte size.

Body weight was regarded as a transversal endpoint[16]. Data on body weight were therefore collected from the HOC Metabolic effects, as well as from General toxicity and Reproductive toxicity. Body weight was recorded at one or several time points and as body weight gain over time.

Body mass index (BMI) is defined in humans as the body mass in kg divided by the square of the body height, expressed as kg/m^2 (Batsis et al., 2016). Persons with BMI > 25 are categorised as overweight, whereas persons with BMI > 30 are categorised as obese. In the studies on mice, BMI was defined as $[BW/(BL)^2] \times 100$, where BW is body weight (in g) and BL is body length (in cm). The BL was measured from nose to anus.

Body fat mass was used as a term to describe fat masses measured in the whole or large parts of the body by equipment such as Echo Magnetic Resonance Imaging System (ECoMRI), dual energy X-ray absorptiometry (DEXA) or computed tomography scans (CT) in the studies assessed in this opinion, as opposed to measurements of individual adipose tissues (pat pads).

Adipose tissue is a central metabolic organ, however, unlike other organs it is compartmentalised into individual depots distributed throughout the body. In humans, intra-abdominal fat, often referred to as visceral adipose tissue, which surrounds the inner organs, is associated with increased risk of insulin resistance and dyslipidemia and is regarded as an independent risk factor for type 2 diabetes mellitus, hypertension and all-cause mortality (Chusyd et al., 2016). In contrast, the upper-body subcutaneous adipose tissue may be beneficial to health due to its ability to act as long-term fat (energy) storage site, protecting against ectopic fat depositions in organs such as liver and pancreas and the associated lipotoxicity. Regarding individual fat deposits in the body, there is some controversy regarding the terminology used for the various fat pads, and the correlation between the various fat pads, in both their composition and function, in humans versus those in rodents (Chusyd et al., 2016). In the papers assessed in this opinion, the expression **visceral white adipose tissue (WAT) weight** was used for weight of various adipose tissues in the abdominal area of rodents, specified in the papers as retroperitoneal, gonadal/perigonadal/perigonadal WAT (pWAT), epididymal/epididymal WAT (eWAT), ovarian/periovarian, parametrial, renal/perirenal and mesenterial adipose tissues or fat pads. Weight of WAT outside of the abdominal area, called **non-visceral WAT weight**, was specified in the papers as inguinal, subcutaneous and subcutaneous mammary – caudal. However, complicating matters even more, there are also data indicating that individual adipose tissue depots are independent organs, developing from different precursor populations and serving different metabolic functions. For instance,

[16] Transversal endpoints refer to endpoints relevant in more than one HOC and evaluated collectively.

there is growing support for the idea that even within visceral fat deposits subpopulations of adipocytes could have beneficial metabolic effects (Schoettl et al., 2018).

Brown adipose tissue (BAT) protects against hypothermia in small mammals and newborn human infants through thermogenesis but was thought to be absent in adult humans (Wang GX et al., 2015). However, recent studies using positron emission tomography (PET) demonstrated that metabolically active BAT is present in some adults. Activation of BAT leads to increased energy expenditure, reduced adiposity and lower plasma glucose and lipid levels, thus, contributing to better homeostasis. Brown fat is emerging as a promising target for therapeutic intervention in obesity and metabolic disease (Betz and Enerbäck, 2018). Activation of BAT in humans is associated with marked improvement in metabolic parameters such as levels of FFA and insulin sensitivity. In older persons, the amount of BAT was inversely correlated with BMI (Cypess et al., 2009). Because changes of the weight of brown fat tissue are of unclear relevance, data on BAT were not included in the assessment.

In the studies assessed in this opinion, data on non-visceral BAT weight, comprised of interscapular or unspecified BAT weight, were available.

It was decided that in this assessment, only endpoints considered biologically relevant and statistically significant or showed a clear trend in the data in at least one Tier 1 or Tier 2 study were included in the WoE (see Annex A). Although statistically significant in some studies, the data on visceral or non-visceral WAT weight or non-visceral BAT weight were considered as Tier 3 because blinding was not mentioned in any such papers, which is important to avoid bias for the manual excised and weighed adipose tissues or fat pads. Hence, because there were no Tier 1 or Tier 2 studies showing significant effects on these endpoints, the available data on these endpoints were not included in the WoE.

There are principally two ways of fat accumulation in the adipocytes in the body, either that the adipocytes increase in number, without increasing in size (hyperplastic morphology) or they increase in size, because of a larger fat content per cell (hypertrophic morphology) (Tandon et al., 2018). Increased adipocyte size is associated with adverse health effects, such as insulin resistance, diabetes and cardiovascular disease, both in visceral and subcutaneous, i.e. non-visceral, fat (Tandon et al., 2018). The data on adipocyte size were also divided into two endpoints. Visceral adipocyte size was specified in the papers as size of visceral, retroperitoneal, perigonadal/gonadal WAT, epididymal, periovarian, perirenal/perirenal WAT and mesenteric adipocytes. Non-visceral adipocyte size was specified as size of adipocytes obtained from subcutaneous, inguinal and interscapular WAT. Results were also included in the endpoint non-visceral adipocyte size if it was not stated in the publication from which fat pad the adipocytes were obtained.

Fat deposition in the liver

The endpoints considered for fat deposition in the liver were liver cholesterol and liver triglycerides. One role of the liver in fat metabolism includes oxidising triglycerides to produce energy (Trefts et al., 2017). The liver breaks down fatty acids and uses the break down product acetoacetate and β-hydroxybutyrate as energy supply. The liver also exports acetoacetate and β-hydroxybutyrate into the blood which transports them into tissues outside the liver where it is converted into acetyl-CoA, which then enters the citric acid cycle and is oxidised in the mitochondria for energy. The liver also synthesises lipoprotein, and it is the major site for converting excess carbohydrates and proteins into fatty acids and triglyceride, which are exported and stored in adipose tissue. The liver also synthesises large quantities of cholesterol and phospholipids. Some of this is packaged with lipoproteins and made available to the rest of the body. The remainder is excreted in bile as cholesterol or after conversion to bile acids.

The endpoint liver fat % is relevant as fat accumulation in the liver may have adverse health effects. It is normal for the liver to contain some fat. However, if >5% of the liver's weight is fat, then it is called a fatty liver (steatosis). The condition is often called NAFLD, which is an umbrella term for a range of liver conditions affecting people who drink little to no alcohol, but have too much fat stored in their liver cells (Kneeman et al., 2012). It is often associated with obesity, type 2 diabetes mellitus, dyslipidaemia and insulin resistance. The more severe form of NAFLD is called NASH, characterised by inflammation and enlargement of the liver. This may progress to advanced scarring (cirrhosis) and liver failure. Because there were no Tier 1 or Tier 2 studies showing significant effects or a trend for liver fat %, the available data on this endpoint were not included in the WoE.

Glucose regulation

Insulin is a hormone that regulates lipid and carbohydrate metabolism and energy homeostasis (Wilcox, 2005). After food intake, insulin is secreted by the β-cells of the pancreas, and it regulates the blood glucose to stay in the normal range by facilitating the uptake of glucose by glucose transporters in the liver and in other cells in the body. Insulin resistance is a condition where the cells, in particular in muscles, fat and liver have a reduced response to insulin, which leads to a reduced uptake of glucose from the blood into the cells and hence, hyperglycaemia. To compensate, the β-cells in pancreas produce more insulin, causing high blood levels of insulin (hyperinsulinemia). Since insulin is important to regulate the blood glucose levels, the relationship between glucose and insulin is tight and the effects on both are evaluated together. Impaired glucose regulation is a risk factor linked to adverse health effects, and insulin resistance is one of the components in metabolic syndrome (Huang, 2009). Obesity is a risk factor for developing insulin resistance.

Glucose regulation was assessed by several endpoints in the metabolic papers. **Glucose levels** were measured in full blood, serum or plasma in animals that were fasting, fed or this information was not given in the papers. **Insulin levels** were measured in serum or plasma in fasting or fed animals or this information was not given. In addition to measurements of existing levels of glucose and insulin, functional tests were also performed. In the **glucose tolerance test (GTT)**, glucose was administered via oral, intraperitoneal (i.p.) or intravenous (i.v.) route or the route was not stated in the papers, and changes in glucose levels over time were measured. In **insulin tolerance test (ITT)**, insulin was injected via the i.p. route and changes in glucose levels over time were measured. The data in GTT and ITT are expressed as AUC of the glucose levels measured over time. In GTT, impaired glucose regulation is seen as higher and prolonged levels of glucose in the blood after a glucose challenge, whereas in ITT, it is seen as higher levels of glucose decreasing slower over time after the insulin challenge.

Pancreas weight is also considered a metabolic endpoint, since insulin and glucagon are produced in the β-cells and α-cells of the pancreas, respectively (Wilcox, 2005). The endpoint **β-cell morphometry** was given in the papers as 'insulin-stained β-cells as ratio of β-cells/total pancreas', β-cell mass, β-cell fraction (%), insulin-reactive cells in pancreas (%) or H-score (taking degree of staining into account). The endpoint **α-cell morphometry** was given as 'glucagon-stained α-cells as ratio of α-cells/total pancreas' or α-cell mass.

Blood lipids

The endpoints indicating a possible effect on metabolism of lipids were cholesterol (in some studies also reported as 'total cholesterol'), HDL cholesterol, LDL cholesterol, triglycerides and FFA.

Impaired lipid metabolism (dyslipidemia) is one component of metabolic syndrome (Huang, 2009). Depending on the various definitions of metabolic syndrome, dyslipidemia is defined with increased values of triglycerides and decreased HDL cholesterol, outside of certain ranges. The adversity of elevated cholesterol is due to the fact that it is a risk factor for cardiovascular disease in humans, involved in the development of atherosclerosis (Avci et al., 2018). The adversity of elevated cholesterol has to be judged on the relationship between its subfraction, HDL cholesterol, which has a protective function on cardiovascular disease in humans, and LDL cholesterol, which is related to an increased risk for cardiovascular events (Avci et al., 2018). Thus, HDL cholesterol and LDL cholesterol should be assessed together. In the animal studies, mainly rodents have been investigated, in which HDL levels are higher than LDL levels, which is opposite to the situation in humans (Kaabia et al., 2018). Also, non-human primates had higher HDL than LDL levels. Hence, the predictive value of results in these species might be in question. FFA cause both insulin resistance and inflammation in the major insulin target tissues, skeletal muscle, liver and endothelial cells, and thus, are an important link between obesity, insulin resistance, inflammation and the development of T2DM, hypertension, dyslipidaemia, disorders of coagulation and atherosclerotic vascular disease (Boden, 2008).

Uric acid

The effects of BPA on **uric acid in serum, urine or liver** were assessed as elevated uric acid is associated with obesity and data in experimental animals indicate that purine catabolism in adipose tissue could be enhanced in obesity (Tsushima et al., 2013). Uric acid is the degradation product of the

metabolism of purines. Purines are constituents of the diet which contains nucleic acids. They are also endogenously formed, party by *de novo* purine biosynthesis.

Increased level of **uric acid in blood** (hyperuricaemia) can result from increase of the rate of purine biosynthesis *de novo* in the liver, by increased dietary uptake and by a decrease in renal clearance of uric acid or a combination of the processes. Another cause for increased levels of uric acid may be increase in glucagon levels due to glucagon-induced catabolism of proteins.

In humans, uric acid is the final end product of purine metabolism and is excreted by the kidneys as **uric acid in the urine** or via intestine. In some species, including rats and mice, most of the uric acid is further metabolised by the enzyme uricase to allantoin. Because in humans uricase is mutated and non-functional, and the ionic form urate is extensively reabsorbed in the renal tubuli, the urate acid levels in plasma are ten-fold higher than those of other mammals (Mandal and Mount, 2015).

Hyperuricaemia is a frequent finding in patients with hypertension, partly explained by the effect of drugs (i.e. diuretics) given against hypertension. A positive association exists between serum urate and body weight. In an obese mouse model (*ob/ob*), adipose tissue could produce and secrete uric acid through the activity of xanthine oxidoreductase (XOR), catalysing purines to uric acid, and the production was enhanced in obesity (Tsushima et al., 2013). There are also some findings indicating an association between hyperlipidaemia and hyperuricaemia without a biological explanation. Hyperuricaemia is noted in patients with degenerative vascular disease and after acute myocardial infarction, however, the interrelationship is caused probably both by effects of the drugs given in those conditions and by effects of the underlying disease on urate excretion (Emmerson, 1978; Fang and Alderman, 2000; Strasak et al., 2008). Hyperuricemia is also strongly associated with insulin resistance syndrome, an established risk factor for T2DM, and individuals with higher serum uric acid, including younger adults, had a higher future risk of T2DM (Bhole et al., 2010). Uric acid is mainly produced in the liver, and both higher baseline and increased serum uric acid have been identified as risk factors for developing fatty liver (Jensen et al., 2018).

Type 1 Diabetes Mellitus (T1DM)

In T1DM, an autoimmune response gradually destroys the β-cells in the pancreas which leads to a severely reduced insulin production with the consequence of continuously elevated blood glucose (Katsarou et al., 2017). T1DM develops most often in young people but can also appear in adults, and if not well medically treated may lead to microvascular and macrovascular complications. Among the papers on metabolic effects, effects of BPA were only studied as the incidence of T1DM in specific mice models, such as diabetes-prone non-obese diabetic (NOD) mice or mice with streptozotocin-induced T1DM.

Other metabolic hormones

Hormones related to obesity, glucose regulation and lipid metabolism were also among the metabolic endpoints. Adiponectin and leptin levels were measured in serum or plasma, glucagon was measured in serum and resistin in plasma.

Leptin and **adiponectin** are cytokines produced by adipocytes (Ghantous et al., 2015). Generally, obesity is associated with high levels of the circulating leptin and low levels of adiponectin. Leptin's physiological role is to regulate hunger by inducing a feeling of satiety. Studies show that lack of leptin causes severe obesity because of the resulting lack of appetite control and is strongly linked with insulin resistance and with T2DM (Facey et al., 2017).

Leptin is produced also by other cells such as cardiomyocytes and vascular smooth muscle cells. It may mechanistically be involved in causing the cardiovascular risks linked to obesity (Ghantous et al., 2015). However, as it has also been shown that it may have beneficial effects on reperfusion of ischaemic damage. (Zhang WF et al., 2019) Thus, it mediates adverse and beneficial effects in humans. In the context of this assessment, leptin may also be seen as an indicator of the number of adipocytes.

Adiponectin is produced by brown and white adipose tissue and is inversely related to metabolic and cardiovascular diseases (Ghantous et al., 2015). Adiponectin levels in plasma are inversely correlated with adiposity and directly correlated with insulin sensitivity. It contributes to the normal functioning of the cardiovascular system and can be regarded as a cardioprotective hormone (Ghantous et al., 2015).

In the context of this assessment, adiponectin may also be seen as an indicator of the number of adipocytes.

Glucagon is produced by the α-cells in the pancreas and has opposite effects of insulin. It activates adenylate cyclase in the liver, which is the first step in transforming glycogen to glucose in the liver cells (Finan et al., 2020). Glucose is released into the blood and increases the blood glucose level. However, glucagon is also involved in the gluconeogenesis from proteins. It was hypothesised that dysregulated α-cell function and increased glucagon in the blood were essential contributors to hyperglycaemia. However, newer studies indicated that glucagon has a more complex biology than previously thought (Finan et al., 2020).

Resistin is mainly secreted by adipocytes in rodents and in peripheral blood mononuclear cells (PBMC) and macrophages in humans (Parreno et al., 2018). It plays an important role in many mechanisms in rodent studies, including insulin resistance, lipid metabolism and inflammation. In a systematic review and meta-analysis, it was shown that in persons with T2DM and obesity, the resistin levels were positively associated with insulin resistance in persons with increased levels of resistin in their blood, but not in those with normal blood resistin levels (Su et al., 2019). Serum resistin was involved in the pathogenesis of arteriosclerosis (Parreno et al., 2018). In addition, serum resistin levels were increased in patients with hypertension, coronary heart disease and cerebrovascular disease and are related to the development and worsening of heart failure.

Thyroid hormones

T_3 and T_4, as well as TT_3, TT_4), FT_3, FT_4 and reverse T_3/total T_4 (rT_3/TT_4) ratio, were measured in serum. TSH was also measured in the publications but was not included among the relevant endpoints in this assessment, because no Tier 1 or 2 studies showed statistically significant effects or a clear trend.

A reciprocal interaction between the hypothalamus–pituitary–thyroid axis and the adipose tissue is required for proper homeostasis of energy balance (Ceccarini et al., 2015). In both lean and obese subjects, thyroid hormones T_3 and T_4 (and TSH) levels are strongly influenced by the individual nutritional status. Furthermore, obesity can be associated with variations of circulating thyroid hormones T_3 and T_4 (and TSH). Whether obesity is a risk factor for thyroid diseases (e.g. autoimmunity or cancer) is still under debate. It is well established that hypothyroidism may induce obesity, but the relationship may be bidirectional. Obesity was significantly associated with an increased risk of hypothyroidism in a recent systematic review and meta-analysis (Song et al., 2019).

The association between thyroid hormones and cardiovascular conditions has been well studied, specifically, the effects of hypothyroidism on cardiomyopathy and hyperthyroidism with arrhythmias, and the literature demonstrated a clear correlation between hypothyroidism, even subclinical, and cardiac dysfunction (Khan et al., 2020). However, there were mixed findings when studying patients with hyperthyroidism and heart failure.

Thyroid hormones were regarded as transversal endpoints[17].

WoE of the clusters of relevant endpoints

The main information extracted from the studies addressing relevant endpoints in the HOC Metabolic effects are summarised in Annex G. The outcome of the weight of the evidence is described in the text below and presented in a tabulated format in Annex H.

In the WoE, the metabolic endpoints were divided into several exposure categories: developmental (pre-natal and/or post-natal until weaning), developmental and adult (pre-natal and/or post-natal in pups until adulthood), growth phase/young age and adult exposure (after puberty), indirect (germline) exposure.

A majority of the metabolic studies had an experimental design with maternal exposure, i.e. the offspring that were studied for the metabolic endpoints were exposed to BPA during their developmental

[17] Transversal endpoints refer to endpoints relevant in more than one HOC and evaluated collectively.

phase, as embryos when the dams were exposed during pregnancy and/or through mother's milk, when the dams were exposed during the lactation period. In some studies, the BPA exposure of the offspring also continued after the weaning, or they were not treated, but observed for a period after weaning before termination. In some studies, the metabolic effects were compared between animals on a standard chow diet and a high-fat diet (HFD) or high-fat/high cholesterol diet.

The **clusters** of metabolic endpoints assessed in the studies on effects of BPA on metabolism were the following:

- Obesity
- Fat deposition in the liver
- Glucose regulation
- Blood lipids
- Uric acid
- Type 1 diabetes mellitus (T1DM)
- Metabolic hormones.

Body weight and thyroid hormones were considered as transversal endpoints, and the data on them were collected from the HOCs Metabolic effects, General toxicity and Reproductive and developmental Toxicity, and from Metabolic effects and Reproductive and developmental Toxicity, respectively.

Obesity

The specific endpoints that were assessed for the effects of BPA in the cluster obesity were body weight, BMI, body fat mass, visceral adipocyte size and non-visceral adipocyte size.

Body weight was treated as a transversal endpoint in this opinion. Data on this transversal endpoint were collected from the HOCs Metabolic effects, General toxicity and Reproductive toxicity. This resulted in very high numbers of studies on body weight for most of the exposure periods. Therefore, the WoE of this endpoint was done by comparing the number of studies demonstrating no effects, increasing effects or decreasing effects on body weight, because it was not possible to compare all the various study parameters and outcomes in detail simultaneously for such high numbers of studies. In addition, the tier allocation and the range of doses administered in each category of effects were taken into consideration.

In this assessment, a total of 114 studies (i.e. unique RefIDs) reported data included in the cluster obesity. Many of the studies reported more than one result, i.e. effects on different endpoints, doses, time points, sex and sometimes also on different diets or on two strains, and in some studies also data from more than one exposure period. Thus, the number of individual results reported is much higher than the number of studies.

Within the cluster obesity, 85 studies were on mice, of which 32 studies had exposure in the developmental until weaning period, 13 had exposure during the developmental until adulthood period, 13 had exposure during the growth phase, 24 were exposed as adults and three had germline exposure. Of the 96 studies on rats, 36 studies had exposure during the developmental until weaning period, 13 had exposure during the developmental until adulthood period, eight had exposure during the growth phase, 35 were exposed as adults and four had germline exposure. In addition, nine studies were on other animal species. One study was on Mongolian gerbils, with exposure during the developmental until weaning period, three studies were on rabbits exposed as adults, two studies were on sheep, both with exposure during the developmental until weaning period, and three studies were on monkeys; one study on common marmosets, exposed as adults, and two studies on rhesus macaques, one had exposure during the developmental until weaning period and one as adults.

<u>Developmental exposure (pre-natal and/or post-natal until weaning)</u>

For the endpoint body weight, there were 59 studies in which the exposure of the animals was under the developmental until weaning period; 27 studies on mice, 30 studies on rats and one study each on Mongolian gerbils and monkeys (rhesus macaques). In Park et al. (2018) [RefID 12869], it was not clear if the mice were exposed to 10000 µg/kg bw per day by i.p. injection or by gavage for the age

nine to 21 weeks. Thus, the dose and/or administration route of these two studies could not be taken into consideration in the WoE. In Rubin et al. (2017) [RefID 6319] and Kass et al. (2015) [RefID 3402], BPA was given via subcutaneous osmotic minipumps, and in Ke et al. (2016) [RefID 3447], BPA was given by subcutaneous injections, thus, the doses were converted to oral doses in these studies. In Junge et al. (2018) [RefID 12262], Meng Y et al. (2018) [RefID 12707] and Desai et al. (2018) [RefID 11817], BPA doses in µg/kg bw per day were calculated from intake of drinking water.

There were 23 studies that did not show any effects on body weight at all. Of these, there were 10 studies on mice, of which there were four, three and three studies, respectively, in Tier 1 (Taylor et al., 2018 [RefID 13239]; MacKay et al., 2017 [RefID 4767]; Tucker et al., 2018 [RefID 13275]; Meng Y et al., 2018 [RefID 12707]), Tier 2 (Ke et al., 2016 [RefID 3447]; Meng Z et al., 2018 [RefID 12708]; Shi et al., 2018 [RefID 13099]) and Tier 3 (Bodin et al., 2014 [RefID 623]; Eckstrum et al., 2018 [RefID 11874]; Hijazi et al., 2018 [RefID 2707]). There were 11 studies on rats (of which there were six, four and one studies, respectively, in Tier 1 (Cao et al., 2015 [RefID 831]; Ding et al., 2014 [RefID 1620]; Lejonklou et al., 2017 [RefID 3975]; Lejonklou et al., 2016 [RefID 3974]; Ferguson SA et al., 2014 [RefID 1998]; Lind et al., 2017 [RefID 4350]), Tier 2 (Leung et al., 2017 [RefID 3990]; Spörndly-Nees et al., 2018 [RefID 13164]; Kass et al., 2015 [RefID 3402] and Santamaria et al., 2016 [RefID 6448]) and Tier 3 (Tarapore et al., 2017 [RefID 7128]). In addition, there was one study on Mongolian gerbils (de Lima et al., 2015 [RefID 1470]), which was Tier 2, and one study on monkeys (rhesus macaques) (Calhoun et al., 2014 [RefID 798]), which was Tier 2. Kass et al. (2015) [RefID 3402] contained two separate experiments, with pre-natal or perinatal exposure. The doses that were found not to have an effect on body weight were in the range 0.05–50000 µg/kg bw per day.

There were 21 studies that showed increased body weight after BPA exposure. Of these, there were nine studies on mice, of which there were one, four and four studies respectively, in Tier 1 (van Esterik et al., 2014 [RefID 7393]), Tier 2 (Malaisé et al., 2017 [RefID 4815]; Junge et al., 2018 [RefID 12262]; Susiarjo et al., 2015 [RefID 7022] and Wang W et al., 2014 [RefID 7759]) and Tier 3 (Rubin et al., 2017 [RefID 6319]; Ziv-Gal et al., 2015 [RefID 9143]; Dobrzynska et al., 2015 [RefID 1644] and Patel et al., 2013 [RefID 5697]). There were 12 studies on rats, of which eight, four and zero studies in Tier 1 (Hass et al., 2016 [RefID 2610]; Jiang et al., 2014 [RefID 3190]; Song SZ et al., 2014 [RefID 6829]; Desai et al., 2018 [RefID 11817]; Dunder et al., 2018 [RefID 11866]; Xia et al., 2014 [RefID 8103]; Quan et al., 2017 [RefID 6025] and Uchtmann et al., 2019 [RefID 13784]), Tier 2 (Ma et al., 2013 [RefID 4748]; Mao et al., 2017 [RefID 4865]; Zhang et al., 2013 [RefID 8798] and Anteur et al., 2016 [RefID 13773]) and Tier 3, respectively. The doses that were found to increase the body weight were in the range 0.45–10000 µg/kg bw per day.

There were 15 studies that showed decreased body weight after BPA exposure. Of these, there were eight studies on mice, of which there were one, four and three studies in Tier 1 (van Esterik et al., 2014 [RefID 7393]), Tier 2 (Malaisé et al., 2017 [RefID 4815]; Suglia et al., 2016 [RefID 6943]; Susiarjo et al., 2015 [RefID 7022] and Krementsov et al., 2013 [RefID 3692]) and Tier 3 (Kalb et al., 2016 [RefID 3312]; Dobrzynska et al., 2015 [RefID 1644] and Patel et al., 2013 [RefID 5697]), respectively. There were seven studies on rats, of which three, four and zero studies were in Tier 1 (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]; Bernardo et al., 2015 [RefID 533] and Brandt et al., 2014 [RefID 700]), Tier 2 (Greenberg, 2018 (NTP Grantee study) [RefID 13785]; Chang et al., 2016 [RefID 965]; Santos-Silva et al., 2018 [RefID 13047] and Anteur et al., 2016 [RefID 13773]) and Tier 3, respectively. The doses that were found to decrease the body weight were in the range 4–25000 µg/kg bw per day.

The decreasing effect observed in Dobrzynska et al. (2015) [RefID 1644] was with 20000 µg/kg bw per day, and in Greenberg (2018) (NTP Grantee study) [RefID 13785] with 25000 µg/kg bw per day, i.e. above the cut-off value of 10000 µg/kg bw per day.

There was approximately the same number of studies that did not find any effects of BPA on body weight (23), (10 Tier 1, nine Tier 2, four Tier 3), as found an increased effect (i.e. an obesogenic effect) (21), (nine Tier 1, eight Tier 2, four Tier 3) and also quite a high number of studies that observed decreasing body weight (i.e. potentially a toxic effect) (15) (four Tier 1, eight Tier 2, three Tier 3). The dose ranges were also more or less in the same orders between these three categories of effects. Thus, the CEP Panel judged this endpoint as ALAN.

For the endpoint BMI, there were one study in mice in which the exposure of the animals was under the developmental until weaning period. In the Tier 3 study (Patel et al., 2013 [RefID 5697]), F0 dams were exposed from GD11.5 to pups' weaning (PND21) with an assumed dose of 0.5, 5.0 or 200 µg/kg bw per day. At weaning, there was no effects of any doses on BMI for F1 female offspring, whereas BMI was decreased for F1 males with 200 µg/kg bw per day only. At months 1–4, F1 females had decreased BMI with 200 µg/kg bw per day at 2 months of age only, and increased BMI with 5.0 µg at 4 months only, whereas F1 males had decreased BMI 200 µg/kg bw at 1 month only and increased BMI with 5.0 µg/kg bw at 4 months only. At termination, there were no effects on BMI with any doses either for females or males. The CEP Panel judged this evidence as Inadequate.

For the endpoint body fat mass, there were six studies in which the exposure of the animals was during the developmental until weaning period. Of these, three studies were on mice, two studies were on rats and one study on sheep.

Two mouse studies were Tier 2 (Susiarjo et al., 2015 [RefID 7022] and Junge et al., 2018 [RefID 12262]) and one study was Tier 3 (Rubin et al., 2017 [RefID 6319]). In Susiarjo et al. (2015) [RefID 7022], F0 dams were exposed to 10 or 10000 µg/kg bw per day from 2 weeks before mating and through pregnancy and lactation. At PND98–PND117, whole body fat % was increased in male offspring with both doses, whereas in female offspring, there were no effects of either dose. In Junge et al. (2018) [RefID 12262], BPA doses in µg/kg bw per day were calculated from intake of drinking water. F0 dams were exposed to 0.45 µg/kg bw per day from 1 week before mating to giving birth. In F1 male and female offspring combined, whole body fat mass as % of bw was increased (+53%). In the mouse study Rubin et al. (2017) [RefID 6319], BPA was given via subcutaneous osmotic minipumps, thus, the doses were converted to oral doses, and the mice were exposed to BPA in two different periods. First, F0 dams were exposed to 60, 600, 5600 or 55600 µg/kg bw per day (converted oral doses) from GD8 to LD16. The overall effect of treatment (all doses) on whole body fat (g) was increased in F1 males and females, but not statistically significantly different from controls for any individual doses in either sex. In males, the strongest effect was with 5,600 µg/kg bw per day (increased 19%, 20% and 17.7% at PND50, 90 and 130, respectively). The overall effect of treatment (all doses) on whole body fat (%) was increased in F1 males and females, but not significantly different from controls for any individual doses in either sex. In males, the strongest effect was with 5600 µg/kg bw per day (increased 15%, 17% and 13% vs. controls at PND50, 90 and 130, respectively). When the mice were exposed to the same doses from GD8 to LD16 and PND21–PND35 (Rubin et al., 2017 [RefID 6319]), the overall treatment effect (all doses) on whole body fat mass (g) or with any individual doses were not significant in F1 males. In F1 females, the overall treatment effect on body fat mass (g) was increased, and the dose 600 µg/kg bw was increased vs. controls and increased vs. 60 and 56000 µg/kg bw at PND141. The overall treatment effect on whole body fat (%) was increased in F1 males and females, but there were no significant differences between the doses in F1 males. For whole body fat (%) in F1 females, 600 µg/kg bw was significantly increased vs. 60 µg/kg bw, and nearly significantly increased vs. control and 56000 µg/kg bw with P adjusted for hyperactive females ($p = 0.007$).

In the Tier 1 rat study Desai et al. (2018) [RefID 11817], BPA doses in µg/kg bw per day were calculated from intake of drinking water. F0 dams were exposed to 250 µg/kg bw per day from 2 weeks before mating and through pregnancy and lactation. In male offspring, whole body fat (%) was increased at both weeks 3 and 24, whereas in female offspring, no effects were observed at either time point. In the Tier 2 rat study Santos-Silva et al. (2018) [RefID 13047], BPA was given by subcutaneous injections, thus, the doses were converted to oral doses. The offspring were exposed on PND3–PND15 through milk from F0 dams exposed to 1785 and 178500 µg/kg bw per day. At PND180, no effects on whole body fat (%) were found with either dose in either sex.

In the Tier 2 study Veiga-Lopez et al. (2016) [RefID 7424], in their 'study 2', F0 sheep were exposed to 40500 µg/kg bw per day GD30–GD90 (term at ~147 days). In female offspring, no effects were found on subcutaneous, visceral or both (total) fat mass volume by whole-body scans at age 19 months on either normal diet or an overfed diet. Male offspring were not studied.

There were two studies (both Tier 2) that showed no effects on body fat mass in two species and four studies (one Tier 1, two Tier 2, one Tier 3) that showed increasing body fat mass in two species. Apparently, the lower dose range (0.45–10000 µg/kg bw) had more effects than the higher dose range

(500–178500 µg/kg bw), but these dose ranges overlapped. The CEP Panel judged this endpoint as ALAN.

For the endpoint **visceral adipocyte size**, there were four studies in which the exposure of the animals was under the developmental until weaning period. One study was on mice, two studies were on rats and one study was on sheep.

In the Tier 1 mouse study by van Esterik et al. (2014) [RefID 7393], the F1 female mice were exposed to HFD weeks 17–23, whereas the F1 males were given normal diet the whole time. Both sexes were exposed to 3, 10, 30, 100, 300, 1000 or 3000 µg/kg bw per day during gestation and lactation. In females, decreased adipocyte size was observed in perirenal WAT (−17%) (not known with which dose(s)), whereas no effects were seen in males with any dose. The effects in females were reported as MDR by the authors (data only shown with symbols).

Both rat studies were Tier 1. In Lejonklou et al. (2017) [RefID 3975], no effects on gonadal white adipocyte size as number/area were observed in female or male rats exposed to either 0.5 or 50 µg/kg bw from GD3.5 to PND22. In the other rat study from Desai et al. (2018) [RefID 11817], BPA doses in µg/kg bw per day were calculated from intake of drinking water. The males were exposed to 250 µg/kg bw via their dams from 2 weeks before mating and through pregnancy and lactation and increased retroperitoneal adipocyte size as area was observed at week 3.

In the Tier 2 sheep study Veiga-Lopez et al. (2016) [RefID 7424], the female sheep were given normal diet or were overfed from 14 weeks until termination at ~21.5 months of age. They were exposed to 40500 µg/kg bw per day GD30–GD90 (term at ~147 days), and increased area and diameter of visceral adipocytes at termination at 21 months of age were found both when given normal diet or overfed.

No effects on visceral adipocyte size were observed in one study (Tier 1, in rats), increased effects were seen in two studies (one Tier 1 in rats, one Tier 2 in sheep) and decreased effects were seen in one study (Tier 1 in mice). Based on this limited evidence, the CEP Panel judged this endpoint as ALAN.

For the endpoint **non-visceral adipocyte size**, there were two rat studies in which the exposure of the animals was under the developmental until weaning period.

In the Tier 1 study Lejonklou et al. (2017) [RefID 3975], the rats were exposed to 0.5 or 50 µg/kg bw from GD3.5 to PND22 and terminated at 5 weeks of age. In females, increased inguinal white adipocytes (iWAT) size was observed with 0.5 µg, but not with 50 µg, whereas in males, there were no effects with either dose, although the highest dose was significantly higher than the lowest dose. No effects were observed on interscapular WAT with either dose as number/area in either sex. In the other Tier 1 study by Dunder et al. (2018) [RefID 11866], the rats were exposed to 0.5 or 50 µg/kg bw from GD3.5–PND22 and studied at 2 weeks and 52 weeks of age. No effects on iWAT size as number/area were observed with either dose at any time point with any sex.

An increasing effect on non-visceral adipocyte size was observed in only one of the two available Tier 1 rat studies, both studies used both sexes. The effect was observed in one of two adipose tissues in females only and only at the lowest of two doses tested. Based on this limited evidence, the CEP Panel judged this endpoint as Not Likely.

The likelihood level ALAN was assigned for effects of BPA on body weight, Inadequate evidence for BMI, ALAN for effects on body fat mass, ALAN for visceral adipose size and Not Likely for non-visceral adipocyte size. Thus, in this exposure period, most, three of four, endpoints with available studies or adequate evidence were scored ALAN. The CEP Panel assigned a likelihood level of ALAN to the obesity effects of BPA in the exposure period developmental until weaning, thus, none of the endpoints included in this cluster was taken forward for BMD analysis. However, the ALAN endpoints were considered in the uncertainty analysis (see Appendix D).

<u>Developmental and adult exposure (pre-natal and/or post-natal in pups until adulthood)</u>

For the endpoint body weight, there were 21 studies in which the exposure of the animals was under the developmental until adulthood period, 10 studies on mice and 11 studies on rats.

There were seven studies that did not show any effects on body weight at all. Of these, there was one study on mice (Kasneci et al., 2017 [RefID 3399]), which was Tier 2. There were six studies on rats, of which there were three, two and one study, respectively, in Tier 1 (Ben-Jonathan, 2018 (NTP Grantee

study) [RefID 13786]; Gonzalez-Cadavid, 2018 (NTP Grantee study) [RefID 13787] and Leung et al., 2020 [RefID 13789]), Tier 2 (Greenberg, 2018 (NTP Grantee study) [RefID 13785] and Auxietre et al., 2014 [RefID 305]) and Tier 3 (Wang J et al., 2014 [RefID 7640]). The doses that were found not to have an effect on body weight were in the range 0.5–25000 µg/kg bw per day.

There were nine studies that showed increasing body weight after BPA exposure. Of these, there were six studies on mice, of which there were one, two and three studies, respectively, in Tier 1 (Chatsantiprapa et al., 2016 [RefID 9822]), Tier 2 (Ke et al., 2016 [RefID 3447] and Patel et al., 2014 [RefID 5695]) and Tier 3 (Biasiotto et al., 2016 [RefID 575]; Dobrzynska et al., 2018 [RefID 11837] and Patel et al., 2013 [RefID 5697]). There were three studies on rats, of which two, zero and one studies on Tier 1 (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370] and Boudalia et al., 2014 [RefID 670]), Tier 2 and Tier 3 (Jeong et al., 2017 [RefID 3133]), respectively. The doses that were found to increase the body weight were in the range 0.5–25000 µg/kg bw per day.

The increasing effect observed in NTP Clarity Report (2018)/Camacho et al. (2019) [RefID 11370] was with 25000 µg/kg bw per day in the sensitivity analysis, i.e. above the cut-off value of 10000 µg/kg bw per day.

There were five studies that showed decreased body weight after BPA exposure. Of these, there were three studies on mice, of which there was one in Tier 1 (Patel et al., 2015b [RefID 5698]), one in Tier 2 (Patel et al., 2014 [RefID 5695]) and one in Tier 3 (Dobrzynska et al., 2018 [RefID 11837]). In Patel et al. (2015b) [RefID 5698], the reduced body weight was seen in F1 males on a HFD, but not on a normal diet. There were two studies on rats, which both were in Tier 1 (Boudalia et al., 2014 [RefID 670] and Dere et al., 2018 [RefID 11815]). The doses that were found to decrease the body weight were in the range 0.6 + 1.1 (two periods) to 250000 µg/kg bw per day.

The decreasing effect observed in Dobrzynska et al. (2018) [RefID 11837] was with 20000 µg/kg bw per day, i.e. above the cut-off value of 10000 µg/kg bw per day.

There was approximately the same number of studies that did not find any effects of BPA on body weight (seven), (three Tier 1, three Tier 2, one Tier 3), as found an increasing body weight (i.e. an obesogenic effect) (nine) (three Tier 1, two Tier 2, four Tier 3), and somewhat lower number of studies that observed a decreased effect (i.e. potentially a toxic effect) (five), (three Tier 1, one Tier 2, one Tier 3). The dose ranges were more or less in the same orders between these three categories of effects. Thus, the CEP Panel judged this endpoint as ALAN.

For the endpoint **BMI**, there was two studies on mice in which the exposure of the animals was under the developmental until adulthood period. In the Tier 2 study Patel et al. (2014) [RefID 5695], the mice were exposed to 5 µg/kg bw per day on GD11.5–PND21, and 0.6 and 0.7 µg/kg bw per day for F1 males and females (doses converted from drinking water), respectively, on PND21–4 months, and were given either normal diet or a HFD from PND21–4 months. On a normal diet, no effects were observed either in females or males at weaning, or at week 4, 8 or 12. On an HFD, there were no effects in females or males at weeks four and eight, and in females, no effects at week 12. In males, BMI was decreased at week 12. In the Tier 3 study Patel et al. (2013) [RefID 5697], F0 dams were exposed from GD11.5 to pups' weaning (PND21) and F1 female and male offspring were exposed directly further from PND21 to 4 months of age to assumed doses of 0.5 or 5.0 µg/kg bw per day. At weaning, there were no effects on BMI for F1 females and males of either dose. At months 1–4, BMI was increased both for F1 females and males with 5.0 µg/kg bw per day at 4 months only. At termination, there were no effects on BMI either for F1 females or males.

Based on this limited evidence, with one Tier 2 study finding decreased effects on BMI on a HFD and no effects on a normal diet, and one Tier 3 study finding increased effects on normal diet, the CEP Panel judged this endpoint as ALAN.

For the endpoint **body fat mass**, there was only one Tier 3 study on mice (Biasiotto et al., 2016 [RefID 575]) in which the exposure of the animals was under the developmental until adulthood period. In this study, increased body fat mass was found only with the dose 5 µg/kg bw in F1 males, and no effects were found with 0.5, 50 or 500 µg/kg bw after exposure on PND90–PND140. Females were not studied. The CEP Panel considered this evidence as Inadequate.

For the endpoint **visceral adipocyte size**, there was one study in which the exposure of the animals was under the developmental until adulthood period. This was a study on rats (Ben-Jonathan, 2018 (NTP Grantee study) [RefID 13786]). In this Tier 1 study, the rats were exposed to 2.5, 25, 250, 2500 or 25000 µg/kg bw per day from GD6 until PND90 or PND180. At both PND90 and PND180, no effects were observed in either F1 females (in periovarian adipose tissue) or in F1 males (in epididymal adipose tissue) with any doses. Based on this limited evidence, the CEP Panel judges this endpoint as Not Likely.

For the endpoint **non-visceral adipocyte size**, there was one study in which the exposure of the animals was under the developmental until adulthood period. This was a Tier 1 study on rats [Ben-Jonathan, 2018 (NTP Grantee study) [RefID 13786]]. The rats were exposed to 2.5, 25, 250, 2500 or 25000 µg/kg bw per day from GD6 until PND90 or PND180. At PND90, no effects in either F1 females or males on inguinal adipocyte size were observed with any doses. At PND180 (6 months), the only effect was decreased size of inguinal adipocytes with the highest dose 25000 µg/kg bw in females. Based on this limited evidence, the CEP Panel judged this endpoint as Not Likely for an obesogenous effect of BPA.

The likelihood level ALAN was assigned for effects of BPA on body weight, ALAN for effects on BMI, Inadequate evidence for effects on body fat mass and Not Likely for both visceral and non-visceral adipocyte size. Thus, two of four endpoints with adequate evidence were scored ALAN and two were scored Not Likely. There was approximately the same number of studies that did not find any effects of BPA on body weight (seven), as found an increasing body weight (10), and somewhat lower number of studies that observed a decreased effect (five), thus, the weight of the evidence was judged clearly as ALAN for the endpoint body weight. The same was the case for BMI, were the limited evidence pointed in opposite directions. For visceral and non-visceral adipocyte size, the evidence was less convincing since the likelihood of both endpoints were based on only one study. The CEP Panel assigned a likelihood level of ALAN to the obesity effects of BPA in the exposure period developmental until adulthood, thus, none of the endpoints included in this cluster was taken forward for BMD analysis. However, the ALAN endpoints were considered in the uncertainty analysis (see Appendix D).

Growth phase/young age exposure

For the endpoint body weight, there were 16 studies in which the exposure of the animals was under the growth phase, eight studies on mice and eight studies on rats.

There were 11 studies that did not show any effects on body weight at all. Of these, there were four studies on mice, of which there was one study in Tier 1 (Cetkovic-Cvrlje et al., 2017 [RefID 916]), two studies in Tier 2 (Dong et al., 2013 [RefID 1676] and Ke et al., 2016 [RefID 3447]) and one study in Tier 3 (Dobrzynska et al., 2014 [RefID 1645]). In Dong et al. (2013) [RefID 1676], BPA doses in µg/kg bw per day were calculated from intake of drinking water. There were seven studies on rats, of which two studies were Tier 1 (Gurmeet et al., 2014 [RefID 2502] and Ullah et al., 2018a [RefID 13281]) and five studies were Tier 2 (Yang YJ et al., 2014 [RefID 10269]; Zhang J et al., 2017 [RefID 8770]; Müller et al., 2018 [RefID 12781]; Brouard et al., 2016 [RefID 734] and Zaid et al., 2014 [RefID 10261]). In Brouard et al. (2016) [RefID 734], BPA was given by subcutaneous injections, and the doses were converted to oral doses. The doses that were found not to have an effect on body weight were in the range 0.5–100000 µg/kg bw per day.

There were five studies that showed increased body weight after BPA exposure. Of these, there were four studies on mice, of which there were one, two and one studies, respectively, in Tier 1 (Yang et al., 2016 [RefID 8375]), Tier 2 (Lin Y et al., 2017 [RefID 4338] and Wyatt et al., 2016 [RefID 8080]) and Tier 3 (Rubin et al., 2017 [RefID 6319]). In Rubin et al. (2017) [RefID 6319], BPA was given via subcutaneous osmotic minipumps, and the doses were converted to oral doses. There was one study on rats (Ullah et al., 2018b [RefID 13282]), which was Tier 3. In this study, the doses used were not clear (in most places the doses 5000, 25000 and 50000 µg/kg bw were mentioned, but also 5000, 50000 and 500000 µg/kg bw were mentioned once), and the BPA doses in µg/kg bw per day were calculated from intake of drinking water. The doses that were found to increase the body weight were in the range 2.5–5000 µg/kg bw per day.

There were approximately twice as many studies that observed no effects of BPA on body weight (11), (three Tier 1, seven Tier 2, one Tier 3), as found an increasing effect on body weight (five), (one Tier

1, two Tier 2, two Tier 3). The dose ranges overlapped in both effect categories. Thus, the CEP Panel judged this endpoint as ALAN.

For the endpoint **BMI**, there were no studies in which the exposure of the animals was under the growth phase.

For the endpoint **body fat mass**, there were two mouse studies in which the exposure of the animals was under the growth phase.

In the Tier 1 study by Yang et al. (2016) [RefID 8375], mice were given a normal diet or a high-fat diet (HFD) and exposed to 5, 50, 500 or 5000 µg/kg bw per day for 30 days from 5 weeks of age. In males and females, whole-body fat mass (% bw) was increased with all doses on normal diet. However, the same study found no effects in either sex with any dose on a HFD. In the Tier 3 mouse study by Rubin et al. (2017) [RefID 6319], BPA was given via subcutaneous osmotic minipumps and the doses were converted to oral doses. The F0 dams were exposed to 60, 600, 5600 or 55600 µg/kg bw per day from GD8 to LD16 and PND21–PND35. The overall treatment effect on whole-body fat mass (g) or with any individual doses was not significant in F1 males. In F1 females, the overall treatment effect on body fat mass (g) was increased, and the dose 600 µg/kg bw was increased vs. controls and increased vs. 60 and 55600 µg/kg bw at PND141. The overall treatment effect on whole body fat (%) was increased in F1 males and females, but there were no significant differences between the doses in F1 males. For whole body fat (%) in F1 females, 600 µg/kg bw was significantly increased vs. 60 µg/kg bw, and nearly significantly increased vs. control and 55600 µg/kg bw with P adjusted for hyperactive females ($P = 0.007$).

An increasing effect on body fat mass was demonstrated in parts only of two mouse studies. The increasing effect was seen at a large dose range on normal diet, but not on HFD, in both sexes in a Tier 1 mouse study. An increasing effect on body fat mass was only seen with a lower dose on only females in the other Tier 3 mouse study. Based on this limited evidence, the CEP Panel judged this endpoint as ALAN.

For the endpoint **visceral adipocyte size**, there were two studies on mice in which the exposure of the animals was under the growth phase.

In this Tier 2 study by Patel et al. (2014) [RefID 5695], the mice were given HFD from PND21–4 months and exposed to 5 µg/kg bw per day of BPA on GD11.5–PND21, and 0.6 and 0.7 µg/kg bw per day for F1 males and females (doses converted from drinking water), from PND21 to 4 months. There were no effects on mesenteric adipocyte area (% of vehicle) in either females or males. In the other Tier 2 study by Wyatt et al. (2016) [RefID 8080], two strains of male mice, C57BL/6J and DBA/2J, were exposed to 28 µg/kg bw per day from 4 weeks to 11 weeks of age. Perigonadal adipocyte size as area was increased in both strains.

No effects on **visceral adipocyte size** was demonstrated on either sex given a HFD in one Tier 2 mouse study, whereas increasing effects were seen on two strains of male mice in another Tier 2 study. Based on this limited evidence, the CEP Panel judged this endpoint as ALAN.

For the endpoint **non-visceral adipocyte size**, there was one study in which the exposure of the animals was under the growth phase. This was a Tier 3 study on mice (Yang et al., 2016 [RefID 8375]), in which male mice were exposed to 5, 50, 500 or 5000 µg/kg bw per day for 30 days from 5 weeks of age and given a normal diet. The diameter of iWAT was increased with all doses. Females and HFD were not studied. The CEP Panel considered this evidence as Inadequate.

There were no studies on BMI in this exposure period. The likelihood level ALAN was assigned for effects of BPA on body weight, ALAN for effects on body fat mass and visceral adipocyte size and Inadequate evidence for non-visceral adipocyte size. In this exposure period, all three of the endpoints with available studies or adequate data were scored ALAN. The CEP Panel assigned a likelihood level of ALAN to the obesity effects of BPA in the exposure period growth phase, thus, none of the endpoints included in this cluster was taken forward for BMD analysis. However, the ALAN endpoints were considered in the uncertainty analysis (see Appendix D).

Adult exposure (after puberty)

For the endpoint body weight, there were 56 studies in which the exposure of the animals was as adults; 22 studies on mice, 31 studies on rats, one study on rabbits and two studies on monkeys (one study each on rhesus macaques and common marmosets, respectively). In Park et al. (2018) [RefID 12869], it was not clear from the study if the mice were exposed to 10000 µg/kg bw per day by i.p. injection or by gavage, and, thus, the dose and administration route could not be taken into consideration in the WoE. In Rubin et al. (2017) [RefID 6319] and Kass et al. (2015) [RefID 3402], BPA was given via subcutaneous osmotic minipumps and the doses were converted to oral doses. In Santos-Silva et al. (2018) [RefID 13047] and Chouhan et al. (2015) [RefID 1216], BPA was given by subcutaneous or intraperitoneal injections, respectively, and the doses were converted to oral doses. In Meng Y et al. (2018) [RefID 12707], Desai et al. (2018) [RefID 11817], Dong et al. (2013) [RefID 1676] and Ullah et al. (2018b) [RefID 13282], BPA doses in µg/kg bw per day were calculated from intake of drinking water.

There were 43 studies that did not show any effects on body weight at all. Of these, there were 18 studies on mice, of which there were seven, six and five studies, respectively, in Tier 1 (van Esterik et al., 2014 [RefID 7393]; Cetkovic-Cvrlje et al., 2017 [RefID 916]; MacKay et al., 2017 [RefID 4767]; Chatsantiprapa et al., 2016 [RefID 9822]; Wang HF et al., 2016 [RefID 7618]; Xu XH et al., 2015 [RefID 8232] and Meng Y et al., 2018 [RefID 12707]), Tier 2 (Dong et al., 2013 [RefID 1676]; Marmugi et al., 2014 [RefID 4884]; Lv Q et al., 2017 [RefID 4697]; Kim MJ et al., 2014 [RefID 3534]; Ma et al., 2018 [RefID 12637]; Hu et al., 2018 [RefID 11119]) and Tier 3 (Liang et al., 2018 [RefID 12508]; Rubin et al., 2017 [RefID 6319]; Yuan et al., 2018 [RefID 13593]; Chouhan et al., 2015 [RefID 1216] and Dobrzynska et al., 2014 [RefID 1645]). There were 23 studies on rats, of which there were 15, seven and one studies, respectively, in Tier 1 (Vahdati Hassani et al., 2017 [RefID 2614]; Cao et al., 2015 [RefID 831]; Ding et al., 2014 [RefID 1620]; Altamirano et al., 2015 [RefID 155]; NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]; Desai et al., 2018 [RefID 11817]; Song SZ et al., 2014 [RefID 6829]; Jiang et al., 2014 [RefID 3190]; Xia et al., 2014 [RefID 8103]; Poormoosavi et al., 2018 [RefID 12913]; Lejonklou et al., 2017 [RefID 3975]; Bernardo et al., 2015 [RefID 533]; Boudalia et al., 2014 [RefID 670]; Olukole et al., 2018 [RefID 12841] and Brandt et al., 2014 [RefID 700]), Tier 2 (Ding et al., 2016 [RefID 1621]; Santos-Silva et al., 2018 [RefID 13047]; Zhang J et al., 2017 [RefID 8770]; Kass et al., 2015 [RefID 3402]; Jiang et al., 2016 [RefID 3179]; Huang DY et al., 2018 [RefID 12167] and Santamaria et al., 2016 [RefID 6448]) and Tier 3 (Wu et al., 2016 [RefID 8036]). The study Kass et al. (2015) [RefID 3402] contained two experiments. In addition, there was one study on rabbits (Fang et al., 2014 [RefID 1914]), which was Tier 2, and one study on monkeys (rhesus macaques), which was Tier 2. The doses that were found not to have an effect on body weight were in the range 0.03–178500 µg/kg bw per day.

There were seven studies that showed increasing body weight after BPA exposure. Of these, there were three studies on mice (Lin Y et al., 2017 [RefID 4338]; Wyatt et al., 2016 [RefID 8080] and Park et al., 2018 [RefID 12869]), all three studies were in Tier 2. There were four studies on rats, of which one study was in Tier 1 (Thilagavathi et al., 2017 [RefID 9247]), two studies were in Tier 2 (Mahmoudi et al., 2018 [RefID 12656] and Amraoui et al., 2018 [RefID 11503]) and one study was in Tier 3 (Ullah et al., 2018b [RefID 13282]). The doses that were found to increase the body weight were in the range 2.5–10000 µg/kg bw per day.

There were six studies that showed decreasing body weight after BPA exposure. Of these, there was one study on mice (Sivashanmugan et al., 2017 [RefID 6773]), which was in Tier 2. There were four studies on rats, of which one and three studies were in Tier 1 (Thilagavathi et al., 2017 [RefID 9247]) and Tier 2 (Abdel-Rahman et al., 2018 [RefID 11426]; Kazemi et al., 2017 [RefID 3441] and Ola-Davies and Olukole, 2018 [RefID 12837]), respectively. In addition, one study on monkeys (common marmosets) (Vijaykumar et al., 2017 [RefID 7477]) was in Tier 3. The doses that were found to decrease the body weight were in the range 2.5–400000 µg/kg bw per day.

The decreasing effects observed in Sivashanmugan et al. (2017) [RefID 6773] were with 100000 and 400000 µg/kg bw per day, i.e. above the cut-off value of 10000 µg/kg bw per day.

The large majority of the studies showed no effects on body weight (43 studies), (22 Tier 1, 15 Tier 2, six Tier 3), whereas approximately the same number of studies showed increased effect (i.e. an obesogenic effect) (seven studies), (one Tier 1, five Tier 2, one Tier 3) and a decreased effect (i.e.

potentially a toxic effect) (six studies) (one Tier 1, four Tier 2, one Tier 3). The dose ranges overlapped for these three categories of effects. Thus, the CEP Panel judged this endpoint as Not Likely.

For the endpoint **BMI**, there was one study in which the exposure of the animals was as adults. In this Tier 2 rat study Mahmoudi et al. (2018) [RefID 12656], it was not explained how BMI was measured, except that it was given as g/cm^2. In this study Mahmoudi et al. (2018) [RefID 12656], male rats were exposed to 10000 µg/kg bw from 7 weeks to ~15.5 weeks of age. BMI was increased (23 ± 0.02%). Females were not studied. The CEP Panel considered this evidence as Inadequate.

For the endpoint **body fat mass**, there were two studies in which the exposure of the animals was as adults, one on mice and one on rats.

In the Tier 2 study by Kim MJ et al. (2014) [RefID 3534], the apolipoprotein E knockout (ApoE$^{-/-}$) mouse model for atherosclerosis was used. Male ApoE$^{-/-}$ mice were exposed to 50 µg/kg bw per day of BPA from 8 weeks of age for 12 weeks on a high-fat/high cholesterol diet. No effects were observed on whole body fat %. Females were not studied.

In the Tier 1 study by Desai et al. (2018) [RefID 11817], BPA doses in µg/kg bw per day were calculated from intake of drinking water. The F0 rat dams were exposed from 2 weeks before mating and through pregnancy and lactation with 250 µg/kg bw per day. No effect on whole body fat weight was observed in these F0 dams.

No effects of BPA of body fat mass were observed in the two available studies (one Tier 1 study on rats and one Tier 2 study on mice). Based on this limited evidence, the CEP Panel judged this endpoint as Not Likely.

For the endpoint **visceral adipocyte size**, there were three studies in which the exposure of the animals was as adults. One study was on rats, one study on mice and one study on rabbits.

In the Tier 2 study by Wyatt et al. (2016) [RefID 8080], two strains of male mice, C57BL/6J and DBA/2J, were exposed to 28 µg/kg bw per day from 4 weeks to 11 weeks of age. Perigonadal adipocyte size as area was increased in both strains. Females were not studied.

In the Tier 1 study by Desai et al. (2018) [RefID 11817], BPA doses in µg/kg bw per day were calculated from intake of drinking water. The F0 rat dams were exposed to 250 µg/kg bw per day from 2 weeks before mating and through pregnancy and lactation. No effect on retroperitoneal adipocyte size as area was found.

In the Tier 2 study by Fang et al. (2014) [RefID 1914], male rabbits were exposed to 400 µg/kg bw per day 12 weeks from 14 weeks of age. Visceral adipocyte diameter was non-significantly increased (+11%). The visceral adipocyte size distribution (%) was shifted towards larger cells and there were significant differences in some of the cell size categories. Females were not studied.

No effects on visceral adipocyte size were observed in one Tier 1 study on female rats, whereas increasing effects were seen in two Tier 2 studies; on two strains of male mice and on male rabbits. Based on this limited evidence, the CEP Panel considered this endpoint ALAN.

For the endpoint **non-visceral adipocyte size**, there were two studies in which the exposure of the animals was as adults. One study was on rats and one study on rabbits.

In the Tier 2 study by Mahmoudi et al. (2018) [RefID 12656], male rats were exposed to 10000 µg/kg bw per day from seven to ~15.5 weeks of age. Increased adipocyte size (not stated which adipocytes) as increased adipocyte surface area and as decreased number/area were observed. Females were not studied.

In the Tier 2 study by Fang et al. (2014) [RefID 1914], male rabbits were exposed to 400 µg/kg bw per day 12 weeks from 14 weeks of age. Increased diameter of subcutaneous adipocytes was observed. Subcutaneous adipocyte size distribution (%) curves were shifted towards larger cells, with mostly significant cell size differences. Females were not studied.

Increasing effects on non-visceral adipocyte size were demonstrated in two Tier 2 studies with single doses on male rats and rabbits. Based on this limited evidence, the CEP Panel judged this endpoint as ALAN.

The likelihood level Not Likely was assigned for effects of BPA on body weight, Inadequate evidence for BMI, Not Likely for effects on body fat mass and ALAN for both visceral adipocyte size and non-visceral adipocyte size. In this exposure period, the large majority of the studies (43) on body weight showed no effects, whereas approximately the same number of studies (seven) showed increasing effect and decreasing effect (six), thus, the weight of the evidence was considered clearly Not Likely for the endpoint body weight. The same was the case for body fat mass, where the only two studies available found no effects. For visceral adipocyte size, the effects were not consistent and for non-visceral adipocyte size the evidence was less convincing. The CEP Panel assigned a likelihood level of Not Likely to the obesity effects of BPA during adult exposure, thus, none of the endpoints included in this cluster was taken forward for BMD analysis.

Indirect (germline) exposure

For the endpoint **body weight**, there were six studies in which the exposure of the animals was via the germline, two studies on mice and four studies on rats.

There were five studies that did not show any effects on body weight at all. Of these, there were two studies on mice (Susiarjo et al., 2015 [RefID 7022] and Ziv-Gal et al., 2015 [RefID 9143]), in Tier 2 and 3, respectively. There were three studies on rats (Li GQ et al., 2014 [RefID 4039]; Mao et al., 2015 [RefID 4864] and Auxietre et al., 2014 [RefID 305]), which all were Tier 2. The doses that were found not to have an effect on body weight were in the range 0.5–10000 µg/kg bw per day.

There was one Tier 1 rat study that showed increased body weight of F2 offspring after BPA exposure (Altamirano et al., 2015 [RefID 155]). The dose 2.6 µg/kg bw per day had increasing effect when given during the lactation period, whereas 0.03 µg/kg bw per day had no effect.

One Tier 1 rat study showed an increasing effect on body weight in F2 offspring, whereas five studies found no effects (four Tier 2 studies; one on mice and three on rats, and one Tier 3 study on mice). Thus, the CEP Panel judged this endpoint as Not Likely.

For the endpoint **BMI**, there were no studies in which the exposure of the animals was via the germline.

For the endpoint **body fat mass**, there was one study in which the exposure of the animals was via the germline. In this Tier 2 mouse study Susiarjo et al. (2015) [RefID 7022], F0 dams were exposed to 10 or 10000 µg/kg bw per day from 2 weeks before mating and through pregnancy and lactation. At PND98–117, whole body fat % was increased in F2 males only with highest dose, 10000 µg/kg bw. F2 females were not studied.

Increasing effects on body fat mass was only seen in F2 males with one dose in one Tier 2 mouse study. Based on this limited evidence, the CEP Panel considered this endpoint ALAN.

For the endpoints **visceral adipocyte size and non-visceral adipocyte size**, there were no studies in which the exposure of the animals was via the germline.

There were no studies on BMI, visceral adipocyte size and non-visceral adipocyte size in this exposure period. The likelihood level Not Likely was assigned for effects of BPA on body weight and ALAN for effects on body fat mass. In this exposure period, the evidence for the endpoint body weight was considered more convincing that the evidence for body fat mass, since for body weight an increasing effect was found for a single dose in only one study and five studies found no effects, whereas for body fat mass an increasing effect was found only in males in one study. The CEP Panel assigned a likelihood level of Not Likely to the obesity effects of BPA in the exposure period germline exposure, thus, none of the endpoints included in this cluster was taken forward for BMD analysis.

Overall cluster selection of the endpoints/studies for BMD analysis for obesity

Overall, the CEP Panel assigned a likelihood level to the obesity effects of BPA of ALAN in the exposure periods developmental until weaning, developmental until adulthood and growth phase and Not Likely after after exposure as adults and exposure via the germline. The overall likelihood across all exposure periods, i.e. the highest likelihood given in the cluster obesity, was ALAN.

The CEP Panel considered that the evidence from the studies available did not show a Likely or Very Likely effect of BPA on the cluster obesity in any exposure period, therefore, none of the endpoints included in this cluster was taken forward for BMD analysis.

Fat deposition in the liver

The specific endpoints that were included for the effects of BPA in the cluster fat deposition in the liver were liver cholesterol and liver triglycerides.

In this assessment, there are in total 12 unique studies that reported data included in the cluster fat deposition in the liver. Among these studies, there were 12 results on liver cholesterol and 17 results on liver triglycerides, in total 29 results.

For all endpoints within the cluster fat deposition in the liver, 18 results were on mice, of which five had exposure during the developmental until weaning period, two had exposure during the developmental until adulthood period, five had exposure during the growth phase, six were exposed as adults and none had exposure via the germline. Of the 11 results on rats, three had exposure during the developmental until weaning period, none had exposure during the developmental until adulthood period, none had exposure during the growth phase, eight were exposed as adults and none had exposure via the germline.

Developmental exposure (pre-natal and/or post-natal until weaning)

For the endpoint **liver cholesterol**, there were three studies in which the exposure of the animals was under the developmental until weaning period. Two studies were on mice and one study was on rats.

Both mouse studies were Tier 2. In one of the mouse studies (Ke et al., 2016 [RefID 3447]), males were exposed to 0.5 µg/kg bw per day from birth to 8 weeks of age. Increased liver cholesterol was observed at 8 weeks of age. Females were not studied. In the other mouse study Meng Z et al. (2018) [RefID 12708], females were exposed to 100 µg/kg bw per day from GD7 to PND21 and terminated at 5 weeks of age. No effects on liver cholesterol were observed. Males were not studied.

In the Tier 2 rat study Santos-Silva et al. (2018) [RefID 13047], BPA was given by subcutaneous injections to the dams and the doses were converted to oral doses. The offspring were exposed on PND3–PND15 through milk from F0 dams exposed to 1785 and 178500 µg/kg bw per day. No effects of either dose were seen in either sex at PND15 or PND21. At PND180, decreased liver cholesterol was observed in females only with 1785 µg/kg bw, whereas in males, no effects were observed with either dose.

The three Tier 2 studies had either no effects (on female mice, males not tested, single dose), increasing effects (on male mice, females not tested, single dose) or decreasing effects (effect only in one of two doses on female rats, no effects of any doses on males; two other timepoints, no effects in either sex of either doses) on liver cholesterol. Based on this limited evidence, the CEP Panel judged the effects on this endpoint as Not Likely.

For the endpoint **liver triglycerides**, there were five studies in which the exposure of the animals was under the developmental until weaning period. Three studies were on mice and two studies were on rats.

Two Tier 2 mouse studies using single dose found no effects with 0.5 µg/kg bw per day (Ke et al., 2016 [RefID 3447]) and with 100 µg/kg bw per day (Meng Z et al., 2018 [RefID 12708]). The third mouse study by Rubin et al. (2017) [RefID 6319] was Tier 3 and on females only. BPA was given via subcutaneous osmotic minipumps and the doses were converted to oral doses. The mice were exposed in two different periods, either on GD8 to LD16, or GD8 to LD16 and PND21–PND35. They did not find any effects after exposure from GD8 to LD16, but after exposure to 600, 5600 or 55600 µg/kg bw from GD8 to LD16 and PND21–PND35, increased liver triglycerides were found with 5600 and 55600 µg/kg bw, but not with 600 µg/kg bw.

Of the two rat studies, one was Tier 1 (Jiang et al., 2014 [RefID 3190]) and found increased liver triglycerides in male rats at 15 and 26 weeks, but not at 3 weeks, after exposure to 40 µg/kg bw per day from GD0 to weaning PND21. Females were not studied. The other rat study Santos-Silva et al. (2018) [RefID 13047] was Tier 2. BPA was given by subcutaneous injections and the doses were converted to oral doses. No effects were found of either dose at PND15 or PND21 in male or female offspring after exposure on PND3–PND15 through milk from F0 dams exposed to 1785 and 178500 µg/kg bw.

Approximately the same number of studies had no effect on liver triglycerides (three studies), all Tier 2 (two on mice, both single dose, either males or females; one on rats, both sexes, two doses, two time points), and had an increasing effect (two studies), one Tier 1 (male rats, single dose, at two of three timepoints), one Tier 3 (mouse, females only, effects of two of three doses after the longest exposure period). The dose ranges overlapped in the three effect categories. The CEP Panel judged the likelihood of this endpoint as ALAN.

The likelihood level Not Likely was assigned for effects of BPA on liver cholesterol and ALAN for effects on liver triglycerides. In this exposure period, the evidence for liver triglycerides was considered more convincing than the evidence for liver cholesterol. The CEP Panel assigned a likelihood level of ALAN to the fat deposition in the liver effects of BPA in the exposure period developmental until weaning, thus, none of the endpoints included in this cluster was taken forward for BMD analysis. However, the ALAN endpoints were considered in the uncertainty analysis (see Appendix D).

Developmental and adult exposure (pre-natal and/or post-natal in pups until adulthood)

For the endpoint **liver cholesterol**, there was one study in which the exposure of the animals was under the developmental until adulthood period. This was a Tier 2 study on mice (Ke et al., 2016 [RefID 3447]), in which male mice were exposed to 0.5 µg/kg bw per day from birth to 10 months of age (40 weeks). Liver cholesterol was increased at 10 months. Females were not studied. The CEP Panel considered this evidence as Inadequate.

For the endpoint **liver triglycerides**, there was one study in which the exposure of the animals was under the developmental until adulthood period. This Tier 2 study was on male mice (Ke et al., 2016 [RefID 3447]). After exposure to 0.5 µg/kg bw per day of BPA from birth to 10 months of age (40 weeks), increased liver triglycerides were found at 10 months. Females were not studied. The CEP Panel considered this evidence as Inadequate.

The CEP Panel considered that there was Inadequate evidence on the effects of BPA on fat deposition in the liver in the exposure period developmental until adulthood, thus, none of the endpoints included in this cluster was taken forward for BMD analysis.

Growth phase/young age exposure

For the endpoint **liver cholesterol**, there were two studies on mice in which the exposure of the animals was under the growth phase.

In the Tier 2 study by Ke et al. (2016) [RefID 3447], male mice were exposed to 0.5 µg/kg bw per day from birth to 8 weeks of age. Liver cholesterol was increased at 8 weeks. Females were not studied. In the other Tier 2 study Lin Y et al. (2017) [RefID 4338], male mice were exposed to 50 µg/kg bw per day from 3 weeks of age for 90 days until ~16 weeks. No effect on liver cholesterol was observed. Females were not studied.

The two Tier 2 studies found either no effects or increasing effects on liver cholesterol, both studying only a single dose, only in males. Based on this limited evidence, the CEP Panel judged this endpoint as ALAN.

For the endpoint liver triglycerides, there were three studies in which the exposure of the animals was under the growth phase, all on mice.

In one Tier 2 study (Ke et al., 2016 [RefID 3447]), in males exposed to 0.5 µg/kg bw per day from birth to 8 weeks of age, no effects on liver triglycerides were observed at 8 weeks. Females were not studied. In another Tier 2 study on males (Lin Y et al., 2017 [RefID 4338]), increased liver triglycerides were observed after exposure to 50 µg/kg bw per day from 3 weeks of age for 90 days until ~16 weeks. Females were not studied. The third study was Tier 3 (Rubin et al., 2017 [RefID 6319]), in which BPA was given via subcutaneous osmotic minipumps and the doses converted to oral doses. After exposure to 600, 5600 or 55600 µg/kg bw from GD8 to LD16 and PND21–PND35, increased liver triglycerides were found with 5600 and 55600 µg/kg bw, but not with 600 µg/kg bw, in females. Males were not studied. The dose 55600 µg/kg bw per day showing increasing effect in Rubin et al. (2017) [RefID 6319] was above the cut-off value of 10000 µg/kg bw per day.

One study found no effects on liver triglycerides (Tier 2, male mice, single dose) and two studies demonstrated increasing effects (one Tier 2, male mice, single dose, and one Tier 3, female mice,

effects with two of three doses, however, one of the doses was above the oral cut-off). Based on this limited evidence, the CEP Panel judged this endpoint as ALAN.

The likelihood level ALAN was assigned for the effects of BPA on liver cholesterol and ALAN for effects on liver triglycerides. The CEP Panel assigned a likelihood level of ALAN to the fat deposition in the liver effects of BPA in the exposure period growth phase, thus, none of the endpoints included in this cluster was taken forward for BMD analysis. However, the ALAN endpoints were considered in the uncertainty analysis (see Appendix D).

Adult exposure (after puberty)

For the endpoint **liver cholesterol**, there were six studies in which the exposure of the animals was as adults. Two studies were on mice and four studies were on rats.

One Tier 2 mouse study (Lin Y et al., 2017 [RefID 4338]), in which males were exposed to 50 µg/kg bw per day from 3 weeks of age for 90 days until ~16 weeks, found no effects. Females were not studied. The other Tier 2 mouse study Marmugi et al. (2014) [RefID 4884], in which males were exposed to 5, 50, 500 or 5000 µg/kg bw per day from 6 weeks of age for 8 months found increased liver cholesterol only with 5000 µg/kg bw and no effects with the other doses. Females were not studied.

Among the studies on rats, two studies used only a single dose (both 50 µg/kg bw) and were Tier 1 (Ding et al., 2014 [RefID 1620]) or Tier 2 (Ding et al., 2016 [RefID 1621]) and showed no effects on males either on a normal diet or a HFD. The third rat study using a single dose was Tier 2 (Mahmoudi et al., 2018 [RefID 12656]) and found increased liver cholesterol after exposure of males to 10000 µg/kg bw per day from seven to ~15.5 weeks of age. Females were not studied. The fourth rat study Santos-Silva et al. (2018) [RefID 13047] was Tier 2. BPA was given by subcutaneous injections and the doses were converted to oral doses. In F0 dams exposed to 1785 and 178500 µg/kg bw per day, no effects of either dose were found on PND21 of their offspring.

Among the Tier 2 studies, it seemed that longer exposure (8 months or 8½ weeks) to higher doses of BPA (5000 or 10000 µg/kg bw) increased liver cholesterol in male mice and rats, respectively. However, one other mouse study (Tier 2, males only, single dose) and three other rat studies (one Tier 1 and one Tier 2, both single dose, males only, on two diets, and one Tier 2, females only, with two doses) failed to show any effects after exposure for similar time periods. Thus, most studies showed no effects and the CEP Panel judged this endpoint as Not Likely.

For the endpoint **liver triglycerides**, there were eight studies in which the exposure of the animals was as adults. Four studies were on mice and four studies were on rats.

All four studies on mice were Tier 2 and only performed in males. In one of these studies Marmugi et al. (2014) [RefID 4884], no effects with any dose were observed on males after exposure to 5, 50, 500 or 5000 µg/kg bw per day from 6 weeks of age for 8 months. Another study Lv Q et al. (2017) [RefID 4697], found increased liver triglycerides after exposure of males to all doses (5, 50 or 500 µg/kg bw per day) from 6 weeks of age for 8 weeks. A third study on males (Lin Y et al., 2017 [RefID 4338]), observed increased liver triglycerides after exposure to 50 µg/kg bw per day from 3 weeks of age for 90 days until ~16 weeks. The fourth study performed on males (Yang SM et al., 2017 [RefID 8398]) observed increased triglyceride content/protein in the liver after exposure to 5, 50 or 500 µg/kg bw per day from 6 weeks of age for 8 weeks. The increase was +58, +69 and +83% for the three doses, respectively, and showed dose–response.

Among the rat studies, one Tier 1 study (Ding et al., 2014 [RefID 1620]) and one Tier 2 study (Ding et al., 2016 [RefID 1621]) found no effects after a single dose (50 µg/kg bw) on either a normal diet or a HFD. In another Tier 2 rat study (Santos-Silva et al., 2018 [RefID 13047]), BPA was given by subcutaneous injections and the doses were converted to oral doses. No effects were found on F0 dams exposed on their offspring's PND3–PND15 to 1785 and 178500 µg/kg bw on PND21 of their offspring. The fourth rat study, Tier 2 (Mahmoudi et al., 2018 [RefID 12656]) found increased liver triglycerides after exposure of males to 10000 µg/kg bw per day from seven to ~15.5 weeks of age.

The same number of studies showed no effects on **liver triglycerides** (four studies), one Tier 1 study and one Tier 2 study on rats (both males only, single dose, on two diets), two Tier 2 studies (one in male mice and one in female rats, three or two doses), or increasing effects (four studies), all Tier 2

(three on male mice, one on male rats, two single dose, two with two or three doses). The dose ranges overlapped in the three effect categories. The CEP Panel judged this endpoint as ALAN.

The likelihood level Not Likely was assigned for the effects of BPA on liver cholesterol and ALAN for effects on liver triglycerides. In this exposure period, the evidence for liver triglycerides was considered more convincing than the evidence for liver cholesterol (more studies, more of them with two or three doses vs. one dose). The CEP Panel assigned a likelihood level of ALAN to the fat deposition in the liver effects of BPA in the exposure period adult exposure, thus, none of the endpoints included in this cluster was taken forward for BMD analysis. However, the ALAN endpoints were considered in the uncertainty analysis (see Appendix D).

Indirect (germline) exposure

For the endpoints, **liver cholesterol and liver triglyceride**, there were no studies in which the exposure of the animals was via the germline.

Overall cluster selection of the endpoints/studies for BMD analysis for fat deposition in the liver

Overall, the CEP Panel assigned a level to the fat deposition in the liver effects of BPA of ALAN in the exposure periods developmental until weaning, growth phase and adulthood, and there was Inadequate Evidence to conclude on the likelihood of effects in the exposure period developmental until adulthood. There were no studies with germline exposure. The overall likelihood across all exposure periods, i.e. the highest likelihood given in the cluster fat deposition in the liver, was ALAN.

The CEP Panel considered that the evidence from the studies available did not show a Likely or Very Likely effect of BPA on the cluster fat deposition in the liver in any exposure period, therefore, none of the endpoints was taken forward for BMD analysis.

Glucose regulation

The specific endpoints that were included for the effects of BPA in the cluster glucose regulation were glucose level, insulin level, intraperitoneal (i.p.) GTT (ipGTT), intravenous (i.v.) GTT (ivGTT), oral GTT (oGTT) or GTT where the route of administration was not stated in the studies, i.p. insulin tolerance test (ipITT), pancreas weight, β-cell morphometry and α-cell morphometry.

Glucose level was measured in full blood, serum or plasma in animals that were fasting or fed, or this information was not stated in the publications. Insulin level was measured in serum or plasma in fasting or fed animals or this information was not stated. In addition, functional tests were performed. In GTT, glucose was administered via various routes (i.p., i.v. or oral) or the route was not stated in the publications, and changes in glucose levels over time were measured. In insulin tolerance test (ITT), insulin was injected via the i.p. route, and changes in glucose levels over time were measured. In some studies, also insulin levels were measured in the ipGTT tests. Since insulin is important to regulate the blood glucose levels, the relationship between glucose and insulin is tight and effects on both should be evaluated together. Pancreas weight is also a metabolic endpoint, since insulin and glucagon are produced in the β-cells and α-cells in the pancreas, respectively. The endpoint β-cell morphometry was given in the studies as insulin-stained β-cells as ratio of β-cells/total pancreas, β-cell mass, β-cell fraction (%) or as insulin-reactive cells in pancreas (%). The endpoint α-cell morphometry was given as glucagon-stained α-cells as ratio of α-cells/total pancreas or as α-cell mass.

In this assessment, there were in total 38 unique studies that reported data included in the cluster glucose regulation. Among these studies, there were 40 results on glucose level, 29 on insulin level, 16 on ipGTT, one on ivGTT, two on oGTT, one on GTT where the administration route was not stated, seven on ipITT, five on pancreas weight, eight on β-cell morphometry and two on α-cell morphometry, in total 111 results.

For all endpoints within the cluster glucose regulation, 54 results were on mice, of which 16 studies had exposure during the developmental until weaning period, five had exposure during the developmental until adulthood period, 13 had exposure during the growth phase, 16 were exposed as adults and four had exposure via the germline. Of the 54 studies on rats, 25 studies had exposure during the developmental until weaning period, seven had exposure during the developmental until adulthood period, two had exposure during the growth phase, 11 were exposed as adults and nine had exposure via the germline. In addition, one publication with two experiments from sheep had exposure

during the developmental until weaning period, one study on rabbits and one on monkeys (common marmosets) had both exposure as adults.

Developmental exposure (pre-natal and/or post-natal until weaning)

For the endpoint **glucose level**, there were 12 studies in which the exposure of the animals was under the developmental until weaning period. Three studies were on mice, nine studies were on rats.

Of the mouse studies, two were in Tier 2 and showed no effects of BPA. In one Tier 2 study (Susiarjo et al., 2015 [RefID 7022]), the F0 dams were exposed to 10 µg/kg bw or 10000 µg/kg bw per day from 2 weeks before mating and through pregnancy and lactation. In the male offspring, no effects were observed with any dose. Female offspring were not studied. In the other Tier 2 study (Ke et al., 2016 [RefID 3447]), only male mice were exposed to 0.5 µg/kg bw per day from birth to 8 weeks of age. No effect was observed on the glucose level at 8 weeks.

One mouse study was Tier 3 and showed increasing effect. In this study by Rubin et al. (2017) [RefID 6319], BPA was given via subcutaneous osmotic minipumps and the doses were converted to oral doses. The mice were exposed in two different periods, either on GD8 to LD16, or GD8 to LD16 and PND21–PND35. On female offspring of F0 dams exposed to 600 or 5600 µg/kg bw per day from GD8 to LD16, there were no effects at 28 or 34 weeks. When the dams were exposed to the same doses from GD8 to LD16 and PND21–PND35, the glucose level was increased with 600 µg/kg bw at 34 weeks, but not at 28 weeks in the female offspring. No effects were seen with 5600 µg/kg bw at either time point. Male offspring were not studied.

Five of the rat studies found no effects on the glucose level. Two of the Tier 1 rat studies (Altamirano et al., 2015 [RefID 155] and NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) found no effects of BPA on glucose levels, of which one used two doses (Altamirano et al., 2015 [RefID 155]) and one used six doses (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]). In the third Tier 1 rat study Ding et al. (2014) [RefID 1620], F0 sires were exposed to 50 µg/kg bw for 35 weeks on a normal or a HFD. In male or female offspring, no effects were observed at 10 weeks of age with either diet. Two Tier 2 rat studies found no effects (Ma et al., 2013 [RefID 4748] and Greenberg, 2018 (NTP Grantee study) [RefID 13785]), using a single dose or five doses, respectively.

Three rat studies found an increasing effect on the glucose level. One Tier 1 study (Song SZ et al., 2014 [RefID 6829]) exposed only male rats to 80 or 758 µg/kg bw from GD6 through lactation and terminated them at PND100. With the 758 µg/kg bw dose only, the glucose level was increased at the puberty stage (PND50), whereas it was increased with both doses at the adult stage (PND100). Females were not studied. In the Tier 2 study Mao et al. (2017) [RefID 4865], the rats were exposed to 40 µg/kg bw GD0–PND21. In male offspring, the glucose level was increased at 3 weeks, but not at 9 weeks, whereas in female offspring, no effects were observed at 3 weeks or 9 weeks. In the Tier 2 study Zhang et al. (2013) [RefID 8798], only female rats were exposed on GD6–PND21 to 33 or 310 µg/dam during gestation, 80 or 758 µg/kg bw per day at the end of gestation. In F1 females, the glucose level was increased with 80 µg, but not with 758 µg at 7 weeks of age. Males were not studied.

In the Tier 2 study by Santos-Silva et al. (2018) [RefID 13047], BPA was given by subcutaneous injections and the doses were converted to oral doses. The F0 rat dams were exposed on PND3–PND15 to 1785 and 178500 µg/kg bw per day. At PND15 and PND21, no effects of either dose were found on either sex exposed through milk from the dams. At PND180, decreased glucose levels were found with 178500 µg/kg bw in females, whereas no effects were found on males with either dose.

The dose 178500 µg/kg bw inducing a decreasing effect in Santos-Silva et al. (2018) [RefID 13047] was above the cut-off value of 10000 µg/kg bw per day.

Most the studies (seven) showed no effects on the glucose level (three Tier 1 and four Tier 2), only four studies found an increasing effect demonstrated in one sex only (one Tier 1, two Tier 2 and one Tier 3) and one Tier 2 study found a decreasing effect. The CEP Panel judged this endpoint as Not Likely.

For the endpoint insulin level, 11 studies in which the exposure of the animals was under the developmental until weaning period. Four studies were on mice and seven studies were on rats.

Among the mouse studies, there was one Tier 1 study (van Esterik et al., 2014 [RefID 7393]), two Tier 2 studies (Susiarjo et al., 2015 [RefID 7022] and Ke et al., 2016 [RefID 3447]) and one Tier 3 study (Rubin et al., 2017 [RefID 6319]). In van Esterik et al. (2014) [RefID 7393], the F1 female mice were given a HFD weeks 17–23, whereas the F1 males were given a normal diet the whole time. Two of these studies found increasing effects of BPA on insulin levels (Susiarjo et al., 2015 [RefID 7022] and Rubin et al., 2017 [RefID 6319]). In Susiarjo et al. (2015) [RefID 7022], the dams were exposed to 10 or 10000 µg/kg bw per day from 2 weeks before mating and through pregnancy and lactation. In F1 males, increased insulin level was found with 10 µg/kg bw, but not with 10000 µg/kg bw (i.e. it was nearly significant, p = 0.08). F1 females were not studied. In Rubin et al. (2017) [RefID 6319], BPA was given via subcutaneous osmotic minipumps and the doses converted to oral doses. The mice were exposed in two different periods, either on GD8 to LD16, or GD8 to LD16 and PND21–PND35. When the dams were exposed to 600 or 5600 µg/kg bw for day from GD8 to LD16, no effects on insulin level were observed at 28 or 34 weeks in F1 females. When the same exposure was on GD8 to LD16 and PND21–PND35, the insulin level was increased in F1 females with 600 µg/kg bw both at 28 and 34 weeks, but not with 5600 µg/kg bw at either time point. F1 males were not studied.

Among the rat studies, three were Tier 1 (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]; Song SZ et al., 2014 [RefID 6829] and Ding et al., 2014 [RefID 1620]), of which one used a single dose (Ding et al., 2014 [RefID 1620]), one used two doses (Song SZ et al., 2014 [RefID 6829]) and one used six doses (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]). In Ding et al. (2014) [RefID 1620], the rats were given a normal diet or a HFD. Only one of the Tier 1 studies found any effects of BPA on insulin levels. In Song SZ et al. (2014) [RefID 6829], male rats were exposed to 80 or 758 µg/kg bw from GD6 through lactation and terminated at PND100. The insulin level was increased only with the dose 758 µg/kg bw at the puberty stage (PND50) and with both doses at the adult stage (PND100). There were four Tier 2 studies on rats, of which two studies found increasing effects on the insulin level. In Ma et al. (2013) [RefID 4748], F0 dams were exposed to 50 µg/kg bw through gestation and lactation, and increased insulin level was found both at three and 21 weeks in F1 males. F1 females were not studied. In Zhang et al. (2013) [RefID 8798], F0 dams were exposed to 33 or 310 µg/dam during gestation, 80 or 758 µg/kg bw per day at the end of gestation, on GD6–PND21. In female offspring, the insulin level was increased with both doses. Male offspring were not studied.

In the Tier 2 study by Santos-Silva et al. (2018) [RefID 13047], BPA was given by subcutaneous injections and the doses were converted to oral doses. The F0 dams were exposed on PND3–PND15 to 1785 and 178500 µg/kg bw per day. At PND15 and PND21, on F1 females exposed through milk, no effects were found of either dose, in F1 males, the insulin level decreased with both doses at PND15, but no effects were seen on PND21. At PND180, the insulin levels were decreased with both doses in F1 females, whereas no effects were seen on F1 males with either dose.

The dose 178500 µg/kg bw inducing decreasing effect in Santos-Silva et al. (2018) [RefID 13047] was above the cut-off value of 10000 µg/kg bw per day.

The same number of studies had no effects on the insulin level (five studies; three Tier 1, two Tier 2), or increasing effects (five studies; one Tier 1, three Tier 2, one Tier 3), and one study had a decreasing effect (Tier 2). The dose ranges overlapped in the three effect categories. Thus, the CEP Panel judged this endpoint as ALAN.

For the endpoint ipGTT, there were six studies in which the exposure of the animals was under the developmental until weaning period. Four studies were on mice, and two studies were on rats.

The one Tier 1 single-dose study on mice (van Esterik et al., 2014 [RefID 7393]) found no effects of BPA on the AUC for glucose in ipGTT. The same was the result in the Tier 2 single-dose study on mice (Wang D et al., 2018 [RefID 13341]). In this study, BPA was given by subcutaneous injections and the doses were converted to oral doses. The same result of no effects was also found in the Tier 3 study on mice using two doses (Rubin et al., 2017 [RefID 6319]). In this study, BPA was given via subcutaneous osmotic minipumps and the doses were converted to oral doses and the exposure period was GD8 to LD16 and PND21–PND35. The only mouse study finding an effect, was in the Tier 2 study Susiarjo et al. (2015) [RefID 7022], in which the F0 dams were exposed to 10 or 10000 µg/kg bw per day from 2 weeks before mating and through pregnancy and lactation. In F1 male offspring, the 10000

5047 µg/kg bw dose increased glucose AUC at PND98–PND117, whereas the increased AUC by 10 µg/kg bw
5048 nearly reached significance (p = 0.06). In F1 female offspring, no effects were found with either dose.

5049 Both rat studies found increasing effects of BPA in the ipGTT. In the Tier 2 study by Chang et al. (2016)
5050 [RefID 965], the F0 dams were exposed to 10 µg/kg bw through gestation and lactation. The AUC for
5051 glucose was not increased for F1 males and females combined at 4 weeks and 8 weeks, whereas it was
5052 increased at 20 weeks. For both male and female offspring separately, there were no effects at 4 weeks
5053 and 8 weeks, but glucose AUC was increased at 20 weeks in both sexes. In the Tier 2 study by Mao et
5054 al. (2017) [RefID 4865], the rats were exposed to 40 µg/kg bw on GD0–PND21. Both male and female
5055 offspring had increased glucose AUC at 9 weeks, but none had increased AUC at 3 weeks.

5056 The same number of studies had no effects in ipGTT (three studies; one in each Tier) or showed
5057 increasing effects (three studies; all Tier 2). The dose ranges overlapped in the three effect categories.
5058 Thus, the CEP Panel judged this endpoint as ALAN.

5059 For the endpoint ivGTT, there was only one study in which the exposure of the animals was under the
5060 developmental until weaning period. Two experiments, called study 1 and study 2, were reported in
5061 sheep in this Tier 2 study Veiga-Lopez et al. (2016) [RefID 7424]. In study 1, female F0 sheep were
5062 exposed to 4050, 40500 or 405000 µg/kg bw per day at GD30–GD90 (term at ~147 days). Glucose
5063 and insulin were measured after 24 hours fasting at the pre-pubertal stage (age ~6 weeks) and after
5064 48 hours fasting at the post-pubertal stage (age ~13 months) in female F1 offspring on a normal diet.
5065 In study 2, female F0 sheep were exposed to 40500 µg/kg bw per day at GD30–GD90 (term at ~147
5066 days). Glucose and insulin were measured after 48 hours fasting at ~15 months of age in female F1
5067 offspring on either a normal diet or on an overfed diet. In study 1, on normal diet, only the dose 4050
5068 µg increased glucose at 6 weeks of age (pre-pubertal stage). At 13 months (post-pubertal stage), there
5069 were no effects on glucose of any doses. In the same study, at 6 weeks of age (pre-pubertal stage)
5070 there were no effects on insulin of any dose in female sheep. At 13 months (post-pubertal stage), the
5071 dose 40500 µg/kg bw increased significantly ($p < 0.05$) mean cumulative insulin response at 20 minutes
5072 and nearly significantly at 180 minutes ($p = 0.054$), increased significantly mean cumulative
5073 insulin/glucose ratio response at both 20 and 180 minutes, and increased acute insulin response (AIR)
5074 at five minutes. The other doses had no effects on insulin levels in this study. In study 2, at 15 months
5075 (post-pubertal stage), there were no effects on glucose on either diet (normal or overfed). In the same
5076 study, no differences were observed in insulin responses (acute response, AUC, cumulative response
5077 over 180 minutes, insulin sensitivity index or insulin/glucose ratio) on either diet. Male F1 offspring
5078 were not studied in either study 1 or 2.

5079 One Tier 2 study with two experiments in female sheep showed mixed results of no effects or increased
5080 effects in **ivGTT** in various parts of the experiments. Based on this limited evidence, the CEP Panel
5081 judged this endpoint as ALAN.

5082 For the endpoint **oGTT**, there was one study in which the exposure of the animals was under the
5083 developmental until weaning period. This Tier 2 study was on mice (Malaisé et al., 2017 [RefID 4815]).
5084 The F0 dams were exposed to 50 µg/kg bw on GD15 to PND21. In male offspring, glucose AUC was
5085 increased at PND35. Female offspring were not studied. The CEP Panel considered this evidence as
5086 Inadequate.

5087 For the endpoint **GTT without know administration route**, there were no studies in which the
5088 exposure of the animals was under the developmental until weaning period.

5089 For the endpoint **ipITT**, there were five studies in which the exposure of the animals was under the
5090 developmental until weaning period. Three studies were on mice and two studies were on rats.

5091 Of the three mouse studies, two were Tier 2 (Malaisé et al., 2017 [RefID 4815] and Wang D et al.,
5092 2018 [RefID 13341]) and one was Tier 3 (Rubin et al., 2017 [RefID 6319]). The single-dose study with
5093 2222 µg/kg bw (Wang D et al., 2018 [RefID 13341]), in which BPA was given by subcutaneous
5094 injections and the doses converted to oral doses, found no effects on glucose AUC in males. In Malaisé
5095 et al. (2017) [RefID 4815], F0 dams were exposed to 50 µg/kg on bw GD15 to PND21. In F1 males,
5096 glucose AUC was increased at PND125. F1 females were not studied. In Rubin et al. (2017) [RefID
5097 6319], BPA was given via subcutaneous osmotic minipumps and the doses were converted to oral
5098 doses. The mice were exposed in two different periods, either on GD8 to LD16, or GD8 to LD16 and
5099 PND21–PND35. When F0 dams were exposed to 600 or 5600 µg/kg bw per day on GD8–LD16, no

effects were found on the glucose level in female offspring with either dose at 40 weeks. Male offspring were not studied. When F0 dams were exposed to the same doses on GD8–LD16 and PND21–PND35, female offspring showed an overall increased effect of BPA on the glucose level, a nearly significant increase (P = 0.07) with 600 µg/kg bw for overall comparison and increased glucose levels with 600 and 5600 µg/kg bw at 45 minutes, at 40 weeks. Male offspring were not studied.

The two rat studies were both Tier 2 using a single dose. In Mao et al. (2017) [RefID 4865], F0 dams were exposed to 40 µg/kg bw on GD0–PND21. In F1 males, at 9 weeks, glucose levels (as % of 0 value) were increased at 15, 45 and 60 minutes, and at 3 weeks at 10 and 60 minutes. In F1 females, glucose levels were increased at 9 weeks at 30, 45 and 60 minutes, but not at 3 weeks for any measured time points. In Chang et al. (2016) [RefID 965], the F0 dams were exposed to 10 µg/kg bw through gestation and lactation. In F1 males and females combined, at 4 weeks and 8 weeks, there were no effects on glucose AUC, whereas it was increased at 20 weeks. In F1 males separately, at 4 weeks, there were no effects, whereas at 8 weeks and 20 weeks, glucose AUC was increased. In F1 females separately, there were no effects on glucose AUC at either 4, 8 or 20 weeks.

Three Tier 2 and one Tier 3 studies in two species (mouse, rat) found increasing effects and only one Tier 2 study did not find an effect in ipITT. However, all studies except the Tier 3 study were with only one dose and mostly studied or demonstrating effects in only one sex. The CEP Panel judged this endpoint as ALAN.

For the endpoint pancreas weight, there were two studies, both on rats, in which the exposure of the animals was under the developmental until weaning period.

In the Tier 2 study by Greenberg (2018) (NTP Grantee study) [RefID 13785], the rats were exposed to 2.5, 25, 250, 2500 or 25000 µg/kg bw from GD6 until PND21 (stop-dose study) and kept unexposed to 1 year. No effects of any doses were observed in either sex. In the other Tier 2 study Chang et al. (2016) [RefID 965], the rats were exposed to 10 µg/kg bw through gestation and lactation. For males and females combined, no effects on pancreas to body weight ratio were observed on GD15.5.

No effects were demonstrated on pancreas weight in the two available Tier 2 studies, using both sexes of rats with one or five doses. Based on this limited evidence, the CEP Panel judged this endpoint as Not Likely.

For the endpoint β-cell morphometry, there were three studies in which the exposure of the animals was under the developmental until weaning period. One study was on mice and two studies were on rats.

In the Tier 1 study on male mice by Bansal et al. (2017) [RefID 9499], F0 dams were exposed to 10 or 10000 µg/kg bw per day from 2 weeks before mating until PND21. In male offspring, β-cell mass/body weight was decreased with 10 µg/kg bw, but not with 10000 µg/kg bw. Females were not studied.

In the Tier 2 rat study by Chang et al. (2016) [RefID 965], they were exposed to 10 µg/kg bw through gestation and lactation and examined at birth. For female and male offspring combined, absolute β-cell mass at birth was decreased. In the other Tier 2 study (Greenberg, 2018 (NTP Grantee study) [RefID 13785]), the rats were exposed to 2.5, 25, 250, 2500 or 25000 µg/kg bw from GD6 until PND21 (stop-dose study) and kept unexposed to 1 year. Insulin-stained β-cells as ratio of β-cells/total pancreas was decreased in females with 25000 µg, whereas no effects were observed with the other doses. In males, no effects were observed of any doses. For β-cell mass, there were no effects of any doses in either sex.

The dose 25000 µg/kg bw inducing decreasing effect in (Greenberg, 2018 (NTP Grantee study) [RefID 13785]) was above the cut-off value of 10000 µg/kg bw per day.

Decreasing effects were observed in three studies (one Tier 1 mouse study and two Tier 2 rat studies). However, the decreasing effects were only studied or observed on combined sexes at birth and on one sex of adults, did not show consistent results between sexes, showed effects with very varying doses (10 µg/kg bw, and 25000 µg/kg bw, above the oral cut-off) and not consistent results for related parameters. Based on this limited and inconsistent evidence, the CEP Panel judged this endpoint as ALAN.

For the endpoint α-cell morphometry, there was one study in which the exposure of the animals was under the developmental until weaning period. This Tier 2 study was on rats (Greenberg, 2018 (NTP Grantee study) [RefID 13785]). They were exposed to 2.5, 25, 250, 2500 or 25000 µg/kg bw from GD6 until PND21 (stop-dose study) and kept unexposed to 1 year. Glucagon-stained α-cells as ratio of α-cells/total pancreas was increased in males with 2.5 and 25 µg/kg bw, but no effects were observed with the other doses. In the females, no effects were observed with any doses. For α-cell mass, no effects of any doses were observed in either sex.

Increasing effects on α-cell morphometry were seen in one Tier 2 rat study in only one sex and on one of two related parameters. Based on this limited evidence, the CEP Panel judged this endpoint as ALAN.

There were no studies in which the administration route for GTT was not mentioned in this exposure period. The likelihood level Not Likely was assigned for effects of BPA on glucose level, ALAN for effects on insulin level, ALAN for ipGTT, ALAN for ivGTT, Inadequate evidence for oGTT, ALAN for ipITT, Not Likely for pancreas weight and ALAN for both β-cell morphometry and α-cell morphometry. In this exposure period, the majority, six of eight, endpoints for which there were available studies or adequate evidence were scored ALAN.

The CEP Panel assigned a likelihood level of ALAN to the glucose regulation effects of BPA in the exposure period developmental until weaning, thus, none of the endpoints included in this cluster was taken forward for BMD analysis. However, the ALAN endpoints were considered in the uncertainty analysis (see Appendix D).

Developmental and adult exposure (pre-natal and/or post-natal in pups until adulthood)

For the endpoint **glucose level**, there were four studies in which the exposure of the animals was under the developmental until adulthood period. Two studies were on mice and two studies were on rats.

In the Tier 2 study by Ke et al. (2016) [RefID 3447], male mice were exposed to 0.5 µg/kg bw per day from birth to 10 months of age (40 weeks). The glucose level was increased at 10 months. Females were not studied. The other Tier 2 study on mice of both sexes using a single dose and a normal diet or a HFD (Patel et al., 2014 [RefID 5695]), found no effects of BPA on the glucose level in either sex on either diet.

One Tier 1 study on rats using six doses of BPA (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) and one Tier 2 study on rats using five doses (Greenberg, 2018 (NTP Grantee study) [RefID 13785]), did not find any effects on glucose level with any doses in either sex.

No effects on glucose level were observed in one Tier 1 and two Tier 2 studies, all on both sexes and two of them with a large dose range, whereas an increasing effect was seen only in one Tier 2 study using a single dose on one sex. Based on this limited evidence, the CEP Panel judged this endpoint as Not Likely.

For the endpoint insulin level, there were three studies in which the exposure of the animals was under the developmental until adulthood period. One study was on mice and two studies were on rats.

The only study that found an effect was the Tier 2 mouse study Ke et al. (2016) [RefID 3447], in which male mice were exposed to 0.5 µg/kg bw per day from birth to 10 months of age (40 weeks). The insulin level was increased at 10 months. Females were not studied.

One rat study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) was Tier 1, and one rat study (Greenberg, 2018 (NTP Grantee study) [RefID 13785]) was Tier 2, using six and five doses, respectively, and none of these studies found any effects of BPA on the insulin level.

No effects on insulin level were observed in one Tier 1 and one Tier 2 study, both studies on both sexes and with a large dose range, whereas increasing effects were seen only in one Tier 2 study using a single dose on one sex. Based on this limited evidence, the CEP Panel judged this endpoint as Not Likely.

For the endpoint ipGTT, there was only one study in which the exposure of the animals was under the developmental until adulthood period. This Tier 2 study Patel et al. (2014) [RefID 5695] was performed on mice, which were given either a normal diet or a HFD. The mice were exposed to 5 µg/kg bw per

day on GD11.5–PND21, and 0.6 and 0.7 µg/kg bw per day for F1 males and females (doses converted from drinking water), from PND21–4 months. Neither male nor female F1 offspring showed an effect on glucose AUC when the mice were given a normal diet. In the same experiment, when the mice were given a HFD, there was no effect in male offspring, and in female offspring, glucose AUC was increased only at the time point 120 minutes. The CEP Panel considered this evidence as Inadequate.

For the endpoints ivGTT, oGTT, glucose GTT without know administration route, ipITT and pancreas weight there were no studies in which the exposure of the animals was under the developmental until adulthood period.

In the Tier 3 study on mice (Biasiotto et al., 2016 [RefID 575]), males were exposed to 0.5, 5, 50 or 500 µg/kg bw per day from week 2 of pregnancy to birth and then from weaning for 140 days. Increased pancreas weight as fold increase was observed with 500 µg/kg bw, but no effects were found with the other doses. Females were not studied.

In the Tier 2 study by Greenberg (2018) (NTP Grantee study) [RefID 13785], the rats were exposed to 2.5, 25, 250, 2500 or 25000 µg/kg bw from GD6 until PND21 and further until 12 months continuously. No effects of any doses were observed in either sex.

No effects on **pancreas weight** were observed in a Tier 2 study on either sex with a large dose range, whereas an increasing effect was seen only in one Tier 3 study, studying only one sex. Based on this limited evidence, the CEP Panel judged this endpoint as Not Likely.

For the endpoint β-cell morphometry, there was one study in which the exposure of the animals was under the developmental until adulthood period. This Tier 2 study Greenberg (2018) (NTP Grantee study) [RefID 13785] was on rats. The rats were exposed to 2.5, 25, 250, 2500 or 25000 µg/kg bw from GD6 until PND21 and further until 12 months continuously. For insulin-stained β-cells as ratio of β-cells/total pancreas, no effects of any doses were seen in either sex. For β-cell mass, no effects were seen of any dose in either sex.

No effects were demonstrated on two parameters for β-cell morphometry in the only available Tier 2 study on either sex with a large dose range. Based on this limited evidence, the CEP Panel judged this endpoint as Not Likely.

For the endpoint α-cell morphometry, there was one study in which the exposure of the animals was under the developmental until adulthood period. This Tier 2 study was on rats (Greenberg, 2018 (NTP Grantee study) [RefID 13785]). The rats were exposed to 2.5, 25, 250, 2500 or 25000 µg/kg bw from GD6 until PND21 and further until 12 months continuously. For glucagon-stained α-cells as ratio of α-cells/total pancreas, no effects of any doses were observed in either sex. However, α-cell mass was increased in females with 2.5, 250 and 25000 µg/kg bw, with no effects of the other doses. In males, there were no effects of any doses.

The dose 25,000 µg/kg bw inducing increasing effect in Greenberg (2018) (NTP Grantee study) [RefID 13785] was above the cut-off value of 10000 µg/kg bw per day.

Increasing effects were demonstrated on only one of two parameters for α-cell morphometry on only one of the sexes in only one Tier 2 study. Based on this limited evidence, the CEP Panel judged this endpoint as ALAN.

There were no studies on ivGTT, oGTT, in which the administration route for GTT was not mentioned and ipITT in this exposure period. The likelihood level Not Likely was assigned for effects of BPA on glucose level, Not Likely for effects on insulin level, Inadequate evidence for ipGTT, Not Likely for pancreas weight, Not Likely for β-cell morphometry and ALAN for α-cell morphometry. In this exposure period, the majority, four of five, of the endpoints for which there were available studies or adequate evidence were scored Not Likely. The CEP Panel assigned a likelihood level of Not Likely to the glucose regulation effects of BPA in the exposure period developmental until adulthood, thus, none of the endpoints included in this cluster was taken forward for BMD analysis. However, the ALAN endpoints were considered in the uncertainty analysis (see Appendix D).

Growth phase/young age exposure

For the endpoint glucose level, there were five studies, all on mice, in which the exposure of the animals was under the growth phase.

Of the five studies on mice, three studies were Tier 2 (Ke et al., 2016 [RefID 3447]; Lin Y et al. (2017) [RefID 4338] and Wyatt et al., 2016 [RefID 8080]), of which only the last study had three doses, whereas the other two used a single dose. None of these three the studies found any effects of BPA on the glucose level. One Tier 1 study with two doses (Cetkovic-Cvrlje et al., 2017 [RefID 916]) did not find any effects either. The only study that found any effects was a Tier 3 study (Rubin et al., 2017 [RefID 6319]), in which BPA was given via subcutaneous osmotic minipumps and the doses were converted to oral doses. The F0 dams were exposed to 600 or 5600 µg/kg bw per day on GD8–LD16 and PND21–PND35. The female offspring showed increased glucose level with 600 µg/kg bw at 34 weeks, but not at 28 weeks. No effects were seen with 5600 µg/kg bw at any time point. The male offspring were not studied.

Four mouse studies (one Tier 1 and three Tier 2) found no effects on glucose level and only one Tier 3 mouse study found increasing effects when tested only on one sex. Thus, the CEP Panel judged this endpoint as Not Likely.

For the endpoint **insulin level**, there were five the studies in which the exposure of the animals was under the growth phase. Four the studies were on mice and one study was on rats.

Of the four mouse the studies, three found effects on the insulin level, of these two were Tier 2 (Lin Y et al., 2017 [RefID 4338] and Wyatt et al., 2016 [RefID 8080]) and one was Tier 3 (Rubin et al., 2017 [RefID 6319]). In Lin Y et al. (2017) [RefID 4338], male mice were exposed to 0.5 µg/kg bw per day from 3 weeks of age for 90 days until ~16 weeks, and increased insulin level was found. Females were not studied. In Wyatt et al. (2016) [RefID 8080], two strains of mice (C57BL/6J and DBA/2J) were exposed to 2.8, 28 or 280 µg/kg bw per day from 4 weeks to 11 weeks of age. In males, no effects on the insulin level were found of any dose in either strain. In females, the insulin level was increased in C57BL/6J mice only with the doses 2.8 and 280 µg/kg bw, and in DBA/2J mice with the doses 2.8, 28 and 280 µg/kg bw. In Lin Y et al. (2017) [RefID 4338], the insulin level was increased with 0.5 µg/kg bw per day in males. Females were not studied. In Rubin et al. (2017) [RefID 6319], BPA was given via subcutaneous osmotic minipumps and the doses were converted to oral doses. F0 dams were exposed to 600 or 5600 µg/kg bw per day on GD8–LD16 and PND21–PND35. In female offspring, the insulin level was increased with 600 µg/kg bw both at 28 and 34 weeks, whereas there were no effects with 5600 µg. Male offspring were not studied. In the Tier 2 study by Ke et al. (2016) [RefID 3447], no effects were found in male mice exposed to 0.5 µg/kg bw per day from birth to 8 weeks of age. Females were not studied.

In the only rat study by Yang YJ et al. (2014) [RefID 10269], which was Tier 2, rats (sex not specified) were exposed to 1 or 100 µg/kg bw per day from 5 weeks to 10 weeks of age and terminated 1 week later. The insulin level was increased with both doses.

Four of five studies observed increasing effects on insulin level in two species (three Tier 2 studies; two studies on mice, including one on two strains, and one study on rats, and one Tier 3 mouse study). Only one Tier 2 study found no effects when tested only on male mice with a single dose. The CEP Panel considered this endpoint Likely.

For the endpoint ipGTT, there were two studies, both on mice, in which the exposure of the animals was under the growth phase.

In the Tier 1 study by Yang et al. (2016) [RefID 8375] with four doses (5, 50, 500 and 5000 µg/kg bw per day), no effects were found on males either on a normal diet or a HFD. In the other Tier 3 mouse study (Rubin et al., 2017 [RefID 6319]), BPA was given via subcutaneous osmotic minipumps and the doses were converted to oral doses. F0 dams were exposed to 600 or 5,600 µg/kg bw per day on GD8–LD16 and PND21–PND35. No effects were found on female offspring in ipGTT. Male offspring were not studied.

Two mouse studies, one Tier 1 and one Tier 3, observed no effects on ipGTT. Based on this limited evidence, the CEP Panel judged this endpoint as Not Likely.

For the endpoint ivGTT, there were no studies in which the exposure of the animals was during the growth phase.

For the endpoint oGTT, there was one study in which the exposure of the animals was under the growth phase. In this Tier 2 study by Yang YJ et al. (2014) [RefID 10269], the rats (sex not specified) were

exposed to 1 or 100 µg/kg bw per day from 5 weeks to 10 weeks of age and terminated 1 week later. The glucose level was increased with 100 µg/kg bw at 60, 90 and 120 minutes, whereas no effects were observed with 1 µg/kg bw.

Increasing effects on oGTT were observed on unspecified sex(s) in only one Tier 2 rat study. Based on this limited evidence, the CEP Panel considered this endpoint ALAN.

For the endpoint GTT without known administration route, there was one study in which the exposure of the animals was under the growth phase. In this Tier 2 study by Wyatt et al. (2016) [RefID 8080], two strains of mice (C57BL/6J and DBA/2J) were exposed to 2.8, 28 or 280 µg/kg bw per day from 4 weeks to 11 weeks of age. No effects on glucose AUC were observed in males or females with any dose in either strain.

No effects on GTT without known administration route were observed on either sex in two strains of mice in one Tier 2 study. Based on this limited evidence, the CEP Panel judged this endpoint as Not Likely.

For the endpoint ipITT, there was one study in which the exposure of the animals was under the growth phase. In this Tier 3 study Rubin et al. (2017) [RefID 6319], BPA was given via subcutaneous osmotic minipumps and the doses were converted to oral doses. F0 dams were exposed to 600 or 5600 µg/kg bw per day on GD8–LD16 and PND21–PND35.The F1 females showed an overall increased effect of treatment on the glucose level, a nearly significant increase (P = 0.07) with 600 µg/kg bw for overall comparison and increased glucose levels with 600 and 5600 µg/kg bw at 45 minutes, at 40 weeks. F1 males were not studied. The CEP Panel considered this evidence as Inadequate.

For the endpoints pancreas weight, β-cell morphometry and α-cell morphometry there were no studies in which the exposure of the animals was during the growth phase.

There were no studies on ivGTT, pancreas weight, β-cell morphometry and α-cell morphometry in this exposure period. The likelihood level Not Likely was assigned for effects of BPA on glucose level, Likely for effects on insulin level, Not Likely for ipGTT, ALAN for oGTT, Not Likely for GTT with unknown administration route and Inadequate evidence for ipITT. In this exposure period, the majority, three of five, of the endpoints for which there were available studies or adequate evidence were scored Not Likely. The CEP Panel assigned a likelihood level of Not Likely to the glucose regulation effects of BPA in the exposure period growth phase, thus, none of the endpoints included in this cluster was taken forward for BMD analysis. The Likely and ALAN endpoints were considered in uncertainty analysis (Appendix D)

<u>Adult exposure (after puberty)</u>

For the endpoint glucose level, there were 16 studies in which the exposure of the animals was as adults. Nine studies were on mice and five studies were on rats. There was also one study on rabbits and one study on monkeys (common marmosets).

Only one study on mice was Tier 1 (Cetkovic-Cvrlje et al., 2017 [RefID 916]), which did not find any effects of two doses (160 and 1600 µg/kg bw) in males. The other eight mouse studies were Tier 2. Of these, four studies (Wyatt et al., 2016 [RefID 8080]; Kim MJ et al., 2014 [RefID 3534]; Ma et al., 2018 [RefID 12637] and Lin Y et al., 2017 [RefID 4338]) found no effects of BPA on the glucose level, of which two studies used a single dose (Kim MJ et al., 2014 [RefID 3534] and Lin Y et al., 2017 [RefID 4338]), and two studies used three doses (Wyatt et al., 2016 [RefID 8080] and Ma et al., 2018 [RefID 12637]). Kim MJ et al. (2014) [RefID 3534] used the apolipoprotein E knockout ($ApoE^{-/-}$) mouse model for atherosclerosis.

Four mouse studies found increasing effects of BPA on the glucose level. In Marmugi et al. (2014) [RefID 4884], male mice were exposed to 5, 50, 500 or 5000 µg/kg bw per day from 6 weeks of age for 8 months. The glucose level was increased with 500 and 5000 µg/kg bw, but not with the other doses. In Park et al. (2018) [RefID 12869], male mice were exposed to 10000 µg/kg bw by i.p. or gavage? (not clear in the study) from age 9 weeks to 21 weeks and terminated 24 hours later. The glucose level was increased. In Ahn et al. (2018) [RefID 11452], male mice were exposed to 5000 µg/kg bw for 5 days at 7 weeks of age and terminated 22 days after being given STZ to induce insulin deficiency. The glucose level was increased both in mice with and without treatment with STZ. In Sivashanmugan et al. (2017) [RefID 6773], male mice were exposed to 10000, 100000 or 400000

µg/kg per day for 30 days from 12–14 weeks of age. The glucose levels were increased with 100000 and 400000 µg/kg bw, but not with 10000 µg.

The doses 100000 and 400000 µg/kg bw per day inducing increasing effects in Sivashanmugan et al. (2017) [RefID 6773] were above the cut-off value of 10000 µg/kg bw per day.

Among the rat studies, two were in Tier 1 (Ding et al., 2014 [RefID 1620] and Vahdati Hassani et al., 2017 [RefID 2614]). In Ding et al. (2014) [RefID 1620], no effects at 0 or 21 weeks were found in the male offspring when the F0 sires were exposed to 50 µg/kg bw for 35 weeks and given a normal diet, whereas when the F0 sires were given a HFD, at 0 and 21 weeks, there were no effects in the male offspring, but at 35 weeks, the glucose level was increased. Female offspring were not studied. In Vahdati Hassani et al. (2017) [RefID 2614], when males were exposed to 500, 5000 or 50000 µg/kg bw per day for 30 days from 7 weeks of age and terminated 1 day later, there were no effects of any dose. Females were not studied. There were three Tier 2 studies on rats (Santos-Silva et al., 2018 [RefID 13047]; Amraoui et al., 2018 [RefID 11503] and Özaydin et al., 2018b [RefID 12854]). In Santos-Silva et al. (2018) [RefID 13047], BPA was given by subcutaneous injections and the doses were converted to oral doses. Of these studies, only Amraoui et al. (2018) [RefID 11503] found any effects of BPA on the glucose level. In male mice exposed to 10000 µg/kg bw per day for 3 weeks and terminated 24 hours later, the glucose level was increased. Females were not studied.

In the Tier 2 study on rabbits by Fang et al. (2014) [RefID 1914], no effects were found at 0–12 weeks when only males were exposed to 400 µg/kg bw for 12 weeks from 14 weeks of age.

In the Tier 2 study on monkeys (common marmosets) by Vijaykumar et al. (2017) [RefID 7477], no effects were found with any doses when only adult males were exposed to 2.5, 12.5 or 25 µg/kg bw per day for 70 days.

Ten studies observed no effects on glucose level (two Tier 1, eight Tier 2, in four species), whereas only six studies observed increasing effects (one Tier 1, five Tier 2, all six studies only in males and four of these only using a single dose). The CEP Panel considered this endpoint Not Likely.

For the endpoint insulin level, there were seven studies in which the exposure of the animals was as adults. Four studies were on mice and three studies were on rats.

All four studies on mice were in Tier 2. Three of the mouse studies found an effect of BPA on insulin level (Wyatt et al., 2016 [RefID 8080]; Ahn et al., 2018 [RefID 11452] and Lin Y et al., 2017 [RefID 4338]). In Wyatt et al. (2016) [RefID 8080], two strains of mice (C57BL/6J and DBA/2J) were exposed to 2.8, 28 or 280 µg/kg bw per day from 4 weeks to 11 weeks of age. In males, no effects on the insulin level were found of any dose in either strain. In females, the insulin level was increased in C57BL/6J mice only with the doses 2.8 and 280 µg/kg bw, and in DBA/2J mice with the doses 2.8, 28 and 280 µg/kg bw. In Ahn et al. (2018) [RefID 11452], male mice were exposed to 5000 µg/kg bw for 5 days at 7 weeks of age and terminated 22 days after being given STZ to induce insulin deficiency. The insulin level was increased both in mice with and without treatment with STZ. Females were not studied. In Lin Y et al. (2017) [RefID 4338], male mice were exposed to 50 µg/kg bw per day from 3 weeks of age for 90 days until ~16 weeks, and increased insulin level was observed. Females were not studied.

Of the three rat studies, one was Tier 1 (Ding et al., 2014 [RefID 1620]) and two were Tier 2 (Özaydin et al., 2018b [RefID 12854] and Santos-Silva et al., 2018 [RefID 13047]). In Santos-Silva et al. (2018) [RefID 13047], BPA was given by subcutaneous injections and the doses were converted to oral doses. Only one rat study found an effect on insulin level by BPA. In this Tier 1 study Ding et al. (2014) [RefID 1620], the F0 rat sires were exposed to 50 µg/kg bw for 35 weeks. On a normal diet, there were no effects on the insulin level at 21 weeks, whereas this was increased at 35 weeks. On a HFD, no effects were observed at either 21 or 35 weeks.

Approximately the same number of studies (three) found no effects on insulin level (three Tier 2 studies, one on mice and two on rats) or found increasing effects (four studies; one Tier 1 on rats and three Tier 2 on mice). The dose ranges overlapped in the two effect categories. Thus, the CEP Panel judged this endpoint as ALAN.

For the endpoint ipGTT, there were four studies in which the exposure of the animals was as adults. Three studies were on mice and one study was on rats.

All the three mouse studies were Tier 2. Kim MJ et al. (2014) [RefID 3534] used the apolipoprotein E knockout ($ApoE^{-/-}$) mouse model for atherosclerosis and found no effects of 50 µg/kg bw per day of BPA on a high-fat/high cholesterol diet. Two mouse studies found an effect in the ipGTT. In Marmugi et al. (2014) [RefID 4884], the mice were exposed to 5, 50, 500 or 5000 µg/kg bw per day from 6 weeks of age for 8 months. In males, at both 2 and 4½ months, glucose AUC was increased with 5000 µg, whereas there were no effects of the other doses. Females were not studied. In Susiarjo et al. (2015) [RefID 7022], the effects of BPA were studied both in F0 and F1 generation dams. F0 dams were exposed to 10 or 10000 µg/kg bw per day from 2 weeks before mating and through pregnancy and lactation. In F0 dams studied between E16.5-E17.5, glucose AUC was increased with both doses. When F1 dams were tested with only the dose 10000 µg/kg bw between E16.5-E17.5, glucose AUC was not affected.

In the Tier 1 rat study Ding et al. (2014) [RefID 1620], in F0 sires exposed to 50 µg/kg bw for 35 weeks on a normal diet, there was no effect on glucose AUC. However, on a HFD, glucose AUC was increased in the F0 sires.

Three studies of four found increasing effects on ipGTT (one Tier 1 study on rats and two Tier 2 studies on mice). However, all studies tested only females and the Tier 1 study found effect on HFD, but not on normal diet, and two of these studies used only one dose. Based on this limited evidence, the CEP Panel judged this endpoint as ALAN.

For the endpoints ivGTT, oGTT, GTT without known administration route, ipITT and pancreas weight there were no studies in which the exposure of the animals was as adults.

For the endpoint β-cell morphometry, there were two studies in which the exposure of the animals was as adults. Both studies were on rats.

In the Tier 1 study on male rats Ding et al. (2014) [RefID 1620], F0 sires were exposed to 50 µg/kg bw for 35 weeks and were found to have increased β-cell mass on a normal diet. On a HFD, no effects on β-cell mass were observed. In the Tier 2 study on rats by Özaydin et al. (2018b) [RefID 12854], males were exposed to 5, 50 or 500 µg/kg bw per day for 8 weeks from 8 weeks of age. For area of islet of Langerhans in pancreas, no effects of any doses were observed. Percentage of insulin-reactive cells in pancreas was increased with 500 µg, but no effects of the other two doses were observed. The H-score (taking degree of staining into account) was decreased with 5 and 50 µg, but no effects were seen of 500 µg/kg bw. Females were not studied.

Increasing effects were observed on β-cell morphometry in one Tier 1 and one Tier 2 study, both only in one sex of rats. In the Tier 2 study, the results were not consistent regarding the dose(s) having effects and the direction of the effects in related parameters. Based on this limited evidence, the CEP Panel judged this endpoint as ALAN.

For the endpoint α-cell morphometry, there were no studies in which the exposure of the animals was as adults.

There were no studies on ivGTT, oGTT, GTT with unknown administration route, ipITT, pancreas weight and α-cell morphometry. The likelihood level Not Likely was assigned for effects on glucose level, ALAN for insulin level, ALAN for ipGTT and ALAN for β-cell morphometry. In this exposure period, the majority, three of four, of the endpoints for which there were available studies were scored ALAN. The CEP Panel assigned a likelihood level of ALAN to the glucose regulation effects of BPA in the exposure period adult exposure, thus, none of the endpoints included in this cluster was taken forward for BMD analysis. However, the ALAN endpoints were considered in the uncertainty analysis (see Appendix D).

Indirect (germline) exposure

For the endpoint glucose level, there were three studies in which the exposure of the animals was via the germline. One study was on F2 mice and two studies were on F2 rats.

One Tier 2 study on male F2 mice with two doses (10 or 10000 µg/kg bw) (Susiarjo et al., 2015 [RefID 7022]) did not find any effects of either dose. F2 females were not studied.

Two Tier 2 rat studies using a single dose (both 40 µg/kg bw) (Li GQ et al., 2014 [RefID 4039] and Mao et al., 2015 [RefID 4864]) did not find any effects of BPA on the glucose level in the F2 offspring, sex not stated or in either sex, respectively.

None of the three available studies (all Tier 2, one on mice and two on rats) found any effects on glucose level. Based on this limited evidence, the CEP Panel considered this endpoint Not Likely.

For the endpoint insulin level, there were three studies in which the exposure of the animals was via the germline. One study was on mice and two studies were on rats, all three studies were Tier 2.

In the Tier 2 mouse study Susiarjo et al. (2015) [RefID 7022], F0 dams were exposed to 10 or 10000 µg/kg bw per day from 2 weeks before mating and through pregnancy and lactation. In F2 males, no effects were observed with either dose. F2 females were not studied.

The two rat studies found effects of BPA on insulin levels. In Li GQ et al. (2014) [RefID 4039], F0 dams were exposed to 40 µg/kg bw on GD0–PND21. In the F2 offspring (sex not stated) at 9 weeks, there were no effects, whereas at 20 weeks, the insulin level was increased. In Mao et al. (2015) [RefID 4864], F0 dams were exposed to 40 µg/kg bw on GD0–PND21. In the F2 females, no effects were observed on insulin level either when fasted or fed at 3 weeks or 21 weeks. In F2 males, the insulin level was increased when fasted or fed at 21 weeks, but not in either at 3 weeks.

Two of three Tier 2 studies found increasing effects on insulin level in rats, but only one study was tested on both sexes and found effects only on males, and both studies used only one dose. The third study showed no effects. Based on this limited evidence, the CEP Panel judged this endpoint as ALAN.

For the endpoint ipGTT, there were three studies in which the exposure of the animals was via the germline. One study was on mice and two studies were on rats.

In the Tier 2 mouse study by Susiarjo et al. (2015) [RefID 7022], F0 dams were exposed to 10 or 10000 µg/kg bw per day from 2 weeks before mating and through pregnancy and lactation. In F2 males, 10000 µg/kg bw increased glucose AUC at PND98–PND117, whereas 10 µg/kg bw did not. F2 females were not studied.

The two rat studies were Tier 2. In Li GQ et al. (2014) [RefID 4039], F0 dams were exposed to 40 µg/kg bw on GD0–PND21. In F2 offspring (sex not stated), glucose AUC was increased at 20 weeks, but not at 9 weeks. In Mao et al. (2015) [RefID 4864], F0 dams were exposed to 40 µg/kg bw on GD0–PND21. In F2 males and females, there were no effects on glucose AUC at 3 weeks, but glucose AUC was increased at 21 weeks in both sexes.

Three Tier 2 studies found increasing effects on the functional test ipGTT in two species. However, only one study tested both sexes and only one study used more than one dose. Based on this limited evidence, the CEP Panel judged this endpoint as Likely.

For the endpoints ivGTT, oGTT and GTT without know administration route there were no studies in which the exposure of the animals was via the germline.

For the endpoint ipITT, there was one study in which the exposure of the animals was via the germline. In this Tier 2 study on rats by Li GQ et al. (2014) [RefID 4039], F0 dams were exposed to 40 µg/kg bw on GD0–PND21. In F2 offspring (sex not stated), the glucose level was increased at 20 weeks at 15, 30 and 60 minutes, at 9 weeks, there were no effects. The CEP Panel considered this as Inadequate evidence.

For the endpoint pancreas weight, there was one study in which the exposure of the animals was via the germline. This Tier 2 single-dose study was on rats (Mao et al., 2015 [RefID 4864]). F0 dams were exposed to 40 µg/kg bw GD0–PND21. Increased pancreas weight to body weight ratio (%) at birth (both sexes together) and at 3 weeks and 21 weeks (both sexes separately) was observed in the F2 offspring. The CEP Panel considered this as Inadequate evidence.

For the endpoint β-cell morphometry, there were two studies in which the exposure of the animals was via the germline. One study was on mice and one study was on rats.

In the Tier 1 study on male mice by Bansal et al. (2017) [RefID 9499], the F0 dams were exposed to 10 or 10000 µg/kg bw per day from 2 weeks before mating until PND21. In the F2 males, relative β-cell mass was decreased with 10 µg, but not with 10000 µg. F2 females were not studied.

In the Tier 2 study on rats by Mao et al. (2015) [RefID 4864], F0 dams were exposed to 40 µg/kg bw on GD0–PND21. Absolute β-cell mass (mg) was decreased in F2 males, whereas in F2 females, no

effects were observed at 21 weeks. Also, the β-cell fraction (%) was decreased in F2 males, whereas in F2 females, no effects were observed at 21 weeks.

Two studies, one Tier 1 on mice and one Tier 2 on rats, found decreasing effects on one or two parameters for β-cell morphometry in males. However, only one study was tested in both sexes and only one study used more than one dose. Based on this limited evidence, the CEP Panel judged this endpoint as Likely.

For the endpoint α-cell morphometry, there were no studies in which the exposure was of the germline.

There were no studies on ivGTT, oGTT, GTT with unknown administration route and α-cell morphometry. The likelihood level Not Likely was assigned for effects on glucose level, ALAN for insulin level, Likely for ipGTT, Inadequate evidence for ipITT and pancreas weight and Likely for β-cell morphometry. In this exposure period, the majority, two of four, of the few endpoints for which there were available studies or adequate evidence were scored Likely. In addition, there was one endpoint scored Not Likely and one endpoint scored ALAN.

The CEP Panel assigned a likelihood level of ALAN to the glucose regulation effects of BPA in the exposure period germline exposure, thus, none of the endpoints included in this cluster was taken forward for BMD analysis. However, the Likely and ALAN endpoints were considered in the uncertainty analysis (Appendix D).

Overall cluster selection of the endpoints/studies for BMD analysis for glucose regulation

The endpoint insulin levels was scored Likely in the exposure period growth phase. The endpoints ipGTT and β-cell morphometry were both scored Likely in the germline exposure period. However, these effects were not observed in the exposure period adult exposure, in which they were all scored ALAN as endpoint and were all in a cluster scored ALAN. Thus, the increasing effects of BPA on insulin levels and ipGTT and the decreasing effect of BPA on β-cell morphometry that were indicated during the growth phase and the germline exposure periods, respectively, did not appear to be permanent effects into adult age.

Overall, the CEP Panel assigned a likelihood level to the Glucose regulation cluster of effects of BPA of ALAN in the exposure periods developmental until weaning, adulthood and germline exposure, and of Not Likely in the exposure periods developmental until adulthood and growth phase. The overall likelihood across all exposure periods, i.e. the highest likelihood given in the cluster glucose regulation, was ALAN.

The CEP Panel considered that the evidence from the studies available did not show a Likely or Very Likely effect of BPA on the cluster glucose regulation in any exposure period, therefore, none of the endpoints was taken forward for BMD analysis.

Blood lipids

The endpoints indicating a possible effect of BPA on metabolism of lipids were cholesterol, HDL cholesterol, LDL cholesterol, triglycerides and FFA. HDL cholesterol and LDL cholesterol should be assessed together as increased HDL-cholesterol levels are beneficial effects whereas increased LDL cholesterol levels are adverse effects and vice versa.

Within the cluster blood lipids, 27 studies were assessed. From these 27 studies, 92 results were obtained in total; 27 in mice, of which six results were obtained when exposure was during development, 9 in development and adult age, 3 in growth and young age, and 9 with exposure during adulthood. Of the 57 results in rats, 20 results were obtained when exposure was during development, 2 in development and adult age, 2 in growth and young age and 33 with exposure during adulthood. Four results were obtained in rabbits and four in monkeys in the exposure period adulthood.

Results of several of the endpoints were obtained from blood samples taken in the same study so that the number of studies is lower than the numbers of results. For example, the four results in rabbits and in monkeys were obtained in blood taken from the one study performed in this species.

Developmental exposure (pre-natal and/or post-natal until weaning)

For the endpoint cholesterol, in this exposure period, seven studies, five in rats (Lejonklou et al., 2017 [RefID 3975]; Dunder et al., 2018 [RefID 11866]; Altamirano et al., 2015 [RefID 155]; NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]; Santos-Silva et al., 2018 [RefID 13047]) and two in mice (van Esterik et al., 2014 [RefID 7393]; Rubin et al., 2017 [RefID 6319]) were identified. In the rat studies, doses between 0.5 and 1111000 µg/kg bw per day were tested with exposures from GD3 to PND21. In four of the rat studies, all studies in Tier 1 (Lejonklou et al., 2017 [RefID 3975]; Dunder et al., 2018 [RefID 11866]; Altamirano et al., 2015 [RefID 155]; NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]), no effect was observed with measurements taken on PND10, PND35 and at 1 year. In one study (Altamirano et al., 2015 [RefID 155]), females only were tested; in the three other studies both males and females were studied. In one study (Tier 2, Santos-Silva et al., 2018 [RefID 13047]) decreases of cholesterol levels were observed at 111100 µg/kg bw per day at PND180, in females only. In the mouse studies, doses between 0.5 and 55550 µg/kg bw per day were tested with exposure from premating until end of lactation. No effect was observed in the Tier 1 study with six doses (van Esterik et al., 2014 [RefID 7393]), while a decrease in the Tier 2 study with only one dose (0.5 mg/kg bw per day) (Santos-Silva et al., 2018 [RefID 13047]) was observed. The CEP Panel judged this endpoint as Not Likely.

For the endpoint HDL cholesterol, in this exposure period, four studies, three in rats (Lejonklou et al., 2017 [RefID 3975]; Dunder et al., 2018 [RefID 11866]; Santos-Silva et al., 2018 [RefID 13047]) and one in mice (van Esterik et al., 2014 [RefID 7393]), were identified. In the rat studies, doses between 0.5 and 1111000 µg/kg bw per day were tested with exposures from GD3 to PND21. In all rat studies, two in Tier 1 (Lejonklou et al., 2017 [RefID 3975]; Dunder et al., 2018 [RefID 11866]) and one in Tier 2 (Santos-Silva et al., 2018 [RefID 13047]), no effect has been observed with measurements taken on PND35 and 180 days and at 1 year (Tier 2, Santos-Silva et al., 2018 [RefID 13047]). Observations were made in both sexes (Lejonklou et al., 2017 [RefID 3975]; Dunder et al., 2018 [RefID 11866]) and in females only (Santos-Silva et al., 2018 [RefID 13047]). In the mouse study (van Esterik et al., 2014 [RefID 7393]), the doses of 3, 10, 30, 100, 300, 1000, 3000 µg/kg bw per day were tested with exposure from premating until end of lactation and no effect was observed. The CEP Panel judged this endpoint as Not Likely.

For the endpoint LDL cholesterol, in this exposure period, three studies, all in rats (Lejonklou et al., 2017 [RefID 3975]; Dunder et al., 2018 [RefID 11866]; Santos-Silva et al., 2018 [RefID 13047]), were identified. In the studies, doses between 0.5 and 1111000 µg/kg bw per day were tested with exposures from GD3 to PND21. In all studies, two in Tier 1 (Lejonklou et al., 2017 [RefID 3975]; Dunder et al., 2018 [RefID 11866]), one in Tier 2 (Santos-Silva et al., 2018 [RefID 13047]), no effect has been observed with measurements taken on PND35 and 180 day and at 1 year (Tier 2, Santos-Silva et al., 2018 [RefID 13047]). Observations were made in both sexes (Lejonklou et al., 2017 [RefID 3975]; Dunder et al., 2018 [RefID 11866]) and in females only (Santos-Silva et al., 2018 [RefID 13047]). The CEP Panel judged this endpoint as Not Likely.

For the endpoint triglycerides, in this exposure period, nine studies, seven in rats (Altamirano et al., 2015 [RefID 155]; Lejonklou et al., 2017 [RefID 3975]; Dunder et al., 2018 [RefID 11866]; NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]; Jiang et al., 2014 [RefID 3190]; Ding et al., 2014 [RefID 1620], experiments 1 and 2 and Santos-Silva et al., 2018 [RefID 13047]) and two in mice (van Esterik et al., 2014 [RefID 7393] and Rubin et al., 2017 [RefID 6319]), were identified. In the rat studies, doses between 0.5 and 25000 µg/kg bw per day were tested with exposures from GD3 to PND21. In five of the rat studies, all in Tier 1 (Altamirano et al., 2015 [RefID 155]; Dunder et al., 2018 [RefID 11866]; NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]; Ding et al., 2014 [RefID 1620], experiments 1 and 2), no effect has been observed with measurements taken on PND10, PND35 and at 1 year. In Altamirano et al. (2015) [RefID 155], females only were tested, the other studies were in males and females. In study Lejonklou et al. (2017) [RefID 3975] (Tier 1) with two doses (0.5 and 50 µg/kg bw per day), at 5 µg/kg bw per day no effect was seen in females but an increase in males, whereas an increase was seen with 50 µg/kg bw per day in females but no effect in males. In the study by Jiang et al. (2014) [RefID 3190] (Tier 1), with a single dose of 40 µg/kg bw per day, no effect was observed at 3 weeks, however, the level was increased at 15 and 26 weeks. In Santos-Silva et al. (2018) [RefID 13047] (Tier 2), for the measurement taken at 180 days, a decrease of triglycerides levels at 1110 µg/kg bw per day in females, but not in males, was observed; no effect was observed with the higher dose of 11110 µg/kg bw per day. In the mouse studies, doses between 3 and 55550

µg/kg bw per day were tested with exposure from premating until end of lactation. No effect was observed in the one Tier 1 study (van Esterik et al., 2014 [RefID 7393]) with six doses in males, but a decrease was seen in females. No effect was seen in a Tier 3 study (Rubin et al., 2017 [RefID 6319]) with doses between 55.5 and 555555 µg/kg bw per day in females only, males were not tested. The CEP Panel judged this endpoint as Not Likely.

For the endpoint FFA, in this exposure period, three studies, one in rats (Jiang et al., 2014 [RefID 3190]) and two in mice (van Esterik et al., 2014 [RefID 7393] and Rubin et al., 2017 [RefID 6319]), were identified. In these studies, doses between 3 and 55550 µg/kg bw per day were tested with exposures from GD0 to PND21. In the rat study (Jiang et al., 2014 [RefID 3190]) (Tier 1), with a single dose of 40 µg/kg bw per day, no effect has been observed at 3 weeks, and an elevated level of FFA at 15 and 26 weeks. In mice, from van Esterik et al. (2014) [RefID 7393] (Tier 1), no effect in males and a not adverse decrease in females were observed; measurements were taken at 23 weeks. No effect was observed in Rubin et al. (2017) [RefID 6319] (Tier 3), in both sexes. The CEP Panel judged this endpoint as Not Likely.

All endpoints in this cluster were considered Not Likely. The CEP Panel assigned a likelihood level of Not Likely to the cluster of blood lipids in Developmental (pre-natal and/or post-natal until weaning), thus, none of the endpoints included in this cluster was taken forward for BMD analysis.

Developmental and adult exposure (pre-natal and/or post-natal in pups until adulthood)

For the endpoint cholesterol, in this exposure period, four studies, one in rats (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) and three in mice (Ke et al., 2016 [RefID 3447]; Patel et al., 2014 [RefID 5695], experiments 1 and 2 were identified. In the rat study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]), doses between 2.5 and 25000 µg/kg bw per day were tested with exposure from GD6 until 1 year. No effect has been observed in males and females with measurements taken at 1 year. In the mouse studies, doses between 0.5 and 55,550 µg/kg bw per day were tested with exposure from premating until 43 weeks. No effect was observed in one Tier 2 study (Patel et al., 2014 [RefID 5695], experiment 2, high-fat diet) with one dose level (5 µg/kg bw per day during development and afterwards 0.6 µg/kg bw per day). In the Tier 2 study (Patel et al., 2014 [RefID 5695] experiment 1, normal diet) with one dose level (5 µg/kg bw per day during development and afterwards 0.6 µg/kg bw per day), a decrease in females and an increase in males was observed. In the single-dose study (Ke et al., 2016 [RefID 3447], Tier 2) with 0.5 µg/kg bw per day, an increase in males was seen. Since the Tier 1 study with five doses, one Tier 2 study and one Tier 3 study with four doses showed no effects, and in addition, one study gave divergent results with unexplained inconsistency and only one single-dose study was available within the dose range below the doses tested in the other studies, the CEP Panel judged this endpoint as Not Likely.

For the endpoint HDL cholesterol, in this exposure period, one study in mice (Ke et al., 2016 [RefID 3447]) was identified. In this study, a dose of 0.5 µg/kg bw per day was tested with exposure from PND1 to PND21 and afterwards until sacrifice at 8 weeks or 10 months. In this Tier 2 study, no effect has been observed at 8 weeks and a decrease at 10 months. Only males were tested. The CEP Panel judged this endpoint as ALAN.

For the endpoint LDL cholesterol, in this exposure period, one study in mice (Ke et al., 2016 [RefID 3447]) was identified. In this study, a dose of 0.5 µg/kg bw per day was tested with exposure from PND1 to PND21 and afterwards until sacrifice at 8 weeks or 10 months. In this Tier 2 study, no effect has been observed at 8 weeks and an increase at 10 months. Only males were tested. The CEP Panel judged this endpoint as ALAN.

For the endpoint triglycerides, in this exposure period, three studies, one in rats (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) and two in mice (Patel et al., 2014 [RefID 5695], experiments 1 and 2 and Ke et al., 2016 [RefID 3447]), were identified. In the rat study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370], Tier 1), doses between 2.5 and 25000 µg/kg bw per day were tested in males and females. In the two mouse studies, both in Tier 2 (Patel et al., 2014 [RefID 5695], experiments 1 and 2), single doses were tested, 0.5 µg/kg bw per day with exposure from PND1 to PND21 and afterwards until sacrifice at 8 weeks or 10 months, 5 µg/kg bw per day during development and 0.6 µg/kg bw per day afterwards. In Ke et al. (2016) [RefID 3447], the dose 0.5 µg/kg bw per day was administered for the same period as Patel et al. (2014) [RefID 5695]. No effect

has been observed in the study from Patel et al. (2014) [RefID 5695] experiments 1 and 2 and an increase was observed in the study from Ke et al. (2016) [RefID 3447]. The effect in this endpoint was judged as Not Likely.

All endpoints in this cluster were considered as Not Likely. The CEP Panel assigned a likelihood level of Not Likely to the cluster of blood lipids in the developmental and adult (pre-natal and/or post-natal in pups until adulthood) period, thus, none of the endpoints included in this cluster was taken forward for BMD analysis. However, the ALAN endpoints were considered in the uncertainty analysis (Appendix D).

Growth phase/young age exposure

For the endpoint cholesterol, in this exposure period, two studies, one in rats (Yang YJ et al., 2014 [RefID 10269]) and one in mice (Lin Y et al., 2017 [RefID 4338]), were identified. In the rat study (Yang YJ et al., 2014 [RefID 10269]), two doses (1 and 100 µg/kg bw per day) were tested with exposure from week 5 until week 10. No effect has been observed in males and females with measurements taken at 10 weeks. In the Tier 2 mouse study (Lin Y et al., 2017 [RefID 4338]), a dose of 50 µg/kg bw per day was tested with exposure for 90 days. No effect was observed in this study. The effect in this endpoint was judged as Not Likely.

For this exposure period (growth phase/young age), no studies were identified testing the endpoints HDL and LDL cholesterol.

For the endpoint triglycerides, in this exposure period, three studies, one in rats (Yang YJ et al., 2014 [RefID 10269]) and two in mice (Lin Y et al., 2017 [RefID 4338] and Rubin et al., 2017 [RefID 6319]), were identified. In the rat study (Yang YJ et al., 2014 [RefID 10269], Tier 2), two doses (1 and 100 µg/kg bw per day) were tested with exposure from week 5 until week 10. No effect has been observed in males and females with measurements taken at 10 weeks. In the study in mice (Rubin et al., 2017 [RefID 6319], Tier 3), doses of 55.5, 555.5, 5555 and 55550 µg/kg bw per day were given throughout the development and afterwards until week 43. No effect was reported in females (males were not studied). In the other mouse study (Lin Y et al., 2017 [RefID 4338], Tier 2), a dose of 50 µg/kg bw per day was tested with exposure for 90 days. An increase of triglycerides was observed in this study, where only males were tested. The Panelhe CEP Panel considered this endpoint as Not Likely, because one study (Yang YJ et al., 2014 [RefID 10269]) showed no effect with two doses encompassing the dose of the study from Lin Y et al. (2017) [RefID 4338], which showed an increase in triglycerides. Moreover, the second study without effect (Rubin et al., 2017 [RefID 6319]) included four doses, and the lowest dose was nearly identical with the dose of the study showing an effect.

For the endpoint FFA, in this exposure period, no studies on BPA effect were identified.

The CEP Panel assigned a likelihood level of Not Likely to the cluster of blood Lipids in the growth phase/young age period, thus, none of the endpoints included in this cluster was taken forward for BMD analysis.

Adult exposure (after puberty)

For the endpoint cholesterol, in this exposure period, 13 studies, one in monkeys (Vijakumar et al., 2017 [RefID 7477]), one in rabbits (Fang et al., 2014 [RefID 1914]), eight in rats (Özaydin et al., 2018b [RefID 12854]; Thilagavathi et al., 2017 [RefID 9247]; Ding et al., 2014 [RefID 1620], experiments 1 and 2; Ding et al., 2016 [RefID 1621], experiments 1 and 2; Vahdati Hassani et al., 2017 [RefID 2614]; Amraoui et al., 2018 [RefID 11503]; Mahmoudi et al., 2018 [RefID 12656]; Abdel-Rahman et al., 2018 [RefID 11426]), and three in mice (Marmugi et al., 2014 [RefID 4884]; Lin Y et al., 2017 [RefID 4338]; Kim MJ et al., 2014 [RefID 3534]) were identified. In the monkey study (Vijakumar et al., 2017 [RefID 7477]), doses between 2.4 and 125 µg/kg bw per day were administered for 70 days and no effect observed in males, the only sex tested. In the rabbit study (Fang et al., 2014 [RefID 1914]), a dose of 400 µg/kg bw per day was administered for 2 weeks and no effect was observed in males, the only sex tested. In six rat studies, one Tier 1 (Vahdati Hassani et al., 2017 [RefID 2614]), the others Tier 2 (Özaydin et al., 2018b [RefID 12854]; Thilagavathi et al., 2017 [RefID 9247]; Ding et al., 2014 [RefID 1620], experiments 1 and 2; Ding et al., 2016 [RefID 1621], experiments 1 and 2; Abdel-Rahman et al., 2018 [RefID 11426]), no effect was observed, the tested doses ranging between 5 and 50000 µg/kg bw per day encompassing exposure periods from 3 weeks to 35 weeks. In two rat studies (Amraoui et al., 2018 [RefID 11503] and Mahmoudi et al., 2018 [RefID 12656]), both Tier 2, the cholesterol levels

were increased in males, the only sex tested, at the only dose tested of 10000 µg/kg bw per day. In one of the mouse studies (Lin Y et al., 2017 [RefID 4338]), no effect was observed with single doses of 50 µg/kg bw per day and in two of the mouse studies (Marmugi et al., 2014 [RefID 4884] and Kim MJ et al., 2014 [RefID 3534]) increased cholesterol levels were noted. In Marmugi et al. (2014) [RefID 4884] (Tier 2), elevated levels were seen at all doses tested, between 5 and 5000 µg/kg bw per day, without a clear dose–response. In Lin Y et al. (2017) [RefID 4338], increased levels were seen in males, the only sex tested, at the only dose tested of 10000 µg/kg bw per day. This endpoint was scored as ALAN because only four out of the 13 studies showed an increase at doses which were tested also in the studies not showing an effect, which were the majority.

For the endpoint HDL cholesterol, in this exposure period, nine studies, one in monkeys (Vijaykumar et al., 2017 [RefID 7477]), one in rabbits (Fang et al., 2014 [RefID 1914]), six in rats (Özaydin et al., 2018b [RefID 12854]; Thilagavathi et al., 2017 [RefID 9247]; Vahdati Hassani et al., 2017 [RefID 2614]; Mahmoudi et al., 2018 [RefID 12656]; Abdel-Rahman et al., 2018 [RefID 11426]; Amraoui et al., 2018 [RefID 11503]) and one in mice (Kim MJ et al., 2014 [RefID 3534]), were identified. In the monkey study (Vijaykumar et al., 2017 [RefID 7477]), doses between 2.4 and 125 µg/kg bw per day were administered for 70 days and no effect was observed in males, the only sex tested. In the rabbit study (Fang et al., 2014 [RefID 1914]), a dose of 400 µg/kg bw per day was administered for 2 weeks and no effect was observed in males, the only sex tested. In four rat studies, one Tier 1 (Vahdati Hassani et al., 2017 [RefID 2614]), the others Tier 2 (Özaydin et al., 2018b [RefID 12854]; Mahmoudi et al., 2018 [RefID 12656]; Abdel-Rahman et al., 2018 [RefID 11426]), no effect was observed, the tested doses ranging between 5 and 50000 µg/kg bw per day encompassing exposure periods from 3 weeks to 35 weeks. In two rat studies, the HDL-cholesterol levels were decreased in males and unchanged in females (Amraoui et al., 2018 [RefID 11503], Tier 2) at the only dose tested of 10000 µg/kg bw per day, or marginally decreased in females, the only sex tested (Thilagavathi et al., 2017 [RefID 9247], Tier 1). In one of the mouse studies (Kim MJ et al., 2014 [RefID 3534], Tier 2), increased levels were observed with single doses of 50 µg/kg bw per day in male animals, the only sex tested. In Thilagavathi et al. (2017) [RefID 9247] (Tier2), decreased levels were seen at all doses tested, between 5 and 5000 µg/kg bw per day, without a clear dose–response. This endpoint was considered as Not Likely because only in two studies a decrease was shown (in one study in females, in one study only in males and not in females) at doses which were tested also in the studies without an effect, whereas in six studies no effect and in one study even an increase was observed.

For the endpoint HDL cholesterol, in this exposure period, 10 studies, one in monkeys (Vijakumar et al., 2017 [RefID 7477]), one in rabbits (Fang et al., 2014 [RefID 1914]), six in rats (Özaydin et al., 2018b [RefID 12854]; Vahdati Hassani et al., 2017 [RefID 2614]; Thilagavathi et al., 2017 [RefID 9247]; Amraoui et al., 2018 [RefID 11503]; Mahmoudi et al., 2018 [RefID 12656]; Abdel-Rahman et al., 2018 [RefID 11426]) and two in mice (Kim MJ et al., 2014 [RefID 3534] and Marmugi et al., 2014 [RefID 4884]), were identified. In the monkey study (Vijakumar et al., 2017 [RefID 7477]), doses between 2.4 and 125 µg/kg bw per day were administered for 70 days and an increase at 25 µg/kg bw per day was observed in males, the only sex tested. In the rabbit study (Fang et al., 2014 [RefID 1914]), a dose of 400 µg/kg bw per day was administered for 2 weeks and no effect was observed in males, the only sex tested. In two Tier 2 rat studies (Özaydin et al., 2018b [RefID 12854] and Vahdati Hassani et al., 2017 [RefID 2614]), no effect was observed and the tested doses (ranging between 5 and 50000 µg/kg bw per day) encompassed exposure periods from 3 weeks to 35 weeks. In four rat studies, the LDL cholesterol levels were increased in males and unchanged in females (Abdel-Rahman et al., 2018 [RefID 11426], Tier 2) at the only dose tested of 10000 µg/kg bw per day, marginally increased in females, the only sex tested (Thilagavathi et al., 2017 [RefID 9247], Tier 1), or increased at the only dose tested of 10000 µg/kg bw per day (Mahmoudi et al., 2018 [RefID 12656], Tier 2, and Amraoui et al., 2018 [RefID 11503], Tier 2). In one of the mouse studies (Kim MJ et al., 2014 [RefID 3534], Tier 2), increased levels were observed with single doses of 50 µg/kg bw per day in male animals, the only sex tested. In Thilagavathi et al. (2017) [RefID 9247] (Tier 2), increased levels were seen at all doses tested, between 5 and 5,000 µg/kg bw per day, without a clear dose–response.

Overall, in three out of 10 studies no effect was observed. In one study, only marginal effects were seen, in one study unexplained sex differences were observed and in two studies no clear dose–responses were seen. In three studies, only one dose was tested, of these in two studies the only dose tested was the dose of the cut-off value. Hence, the effect in this endpoint was judged as ALAN.

For the endpoint triglycerides, in this exposure period, 14 studies, one in monkeys (Vijakumar et al., 2017 [RefID 7477]), one in rabbits (Fang et al., 2014 [RefID 1914]), nine in rats (Özaydin et al., 2018b [RefID 12854]; Thilagavathi et al., 2017 [RefID 9247]; Ding et al., 2014 [RefID 1620], experiments 1 and 2; Lejonklou et al., 2017 [RefID 3975]; Ding et al., 2016 [RefID 1621], experiments 1 and 2; Vahdati Hassani et al., 2017 [RefID 2614]; Amraoui et al., 2018 [RefID 11503]; Mahmoudi et al., 2018 [RefID 12656]; Abdel-Rahman et al., 2018 [RefID 11426]) and three in mice (Kim MJ et al., 2014 [RefID 3534]; Lin Y et al., 2017 [RefID 4338]; Ma et al., 2018 [RefID 12637]), were identified. In the monkey study (Vijakumar et al., 2017 [RefID 7477]), doses between 2.4 and 125 µg/kg bw per day were administered for 70 days and no effect was observed in males, the only sex tested. In the rabbit study (Fang et al., 2014 [RefID 1914]), a dose of 400 µg/kg bw per day was administered for 2 weeks and no effect was observed in males, the only sex tested. In the rat studies, doses between 0.5 and 50000 µg/kg bw per day were tested. In four rat studies, two Tier 1 (Ding et al., 2014 [RefID 1620], experiments 1 and 2; Lejonklou et al., 2017 [RefID 3975]) and two Tier 2 (Özaydin et al., 2018b [RefID 12854] and Ding et al.,2016 [RefID 1621], experiments 1 and 2), no effect was observed, and the tested doses ranging between 5 and 500 µg/kg bw per day. In four rat studies, the triglyceride level was increased in females (Abdel-Rahman et al., 2018 [RefID 11426], Tier 2) at the only dose tested of 10000 µg/kg bw per day, marginally increased in females, the only sex tested (Thilagavathi et al., 2017 [RefID 9247], Tier 1), or increased at the only dose tested of 10000 µg/kg bw per day (Mahmoudi et al., 2018 [RefID 12656], Tier 2 and Amraoui et al., 2018 [RefID 11503], Tier 2). Inconclusive results with increased levels without a dose–response were seen in Vahdati Hassani et al. (2017) [RefID 2614]. In all mouse studies (Kim MJ et al., 2014 [RefID 3534]; Lin Y et al., 2017 [RefID 4338]; Ma et al., 2018 [RefID 12637], all Tier 2), no effect was observed with single doses of 50 µg/kg bw per day (two studies) and 5 to 500 µg/kg bw per day (one study).

Overall, from 14 studies, nine studies showed no effect, in one study the effect was marginal, in one study the effects were inconsistent, in three studies the increase was seen at the dose of 10000 µg/kg bw per day. Hence, this endpoint was judged as ALAN.

For the endpoint FFA, in this exposure period, no studies on BPA effects were identified.

The CEP Panel assigned a likelihood level of ALAN to the cluster of blood Lipids in the adult exposure period, thus, none of the endpoints included in this cluster was taken forward for BMD analysis. However, the ALAN endpoints were considered in the uncertainty analysis (see Appendix D).

Indirect (germline) exposure

For the endpoint cholesterol, in this exposure period, one Tier 1 study in rats with two arms, high-fat diet and normal diet, was performed. The paternal animals were treated with 50 µg/kg bw per day for 21 weeks before mating and then for further 14 weeks; treatment was 35 weeks in total. Maternal animals and offspring were not treated. Neither in F1 nor in F2 animals, BPA had an effect on cholesterol levels.

For this exposure period, no studies were identified testing the endpoints HDL cholesterol and LDL cholesterol, as well as FFA and triglycerides.

The CEP Panel assigned a likelihood level of Not Likely to the cluster of blood Lipids in the indirect (germline) exposure period, thus, none of the endpoints included in this cluster was taken forward for BMD analysis.

Overall cluster selection of the endpoints/studies for BMD analysis for blood lipids

Overall, the CEP Panel assigned a likelihood level to effects of BPA on the blood lipids of Not Likely in the exposure periods developmental until weaning, developmental until adulthood, growth phase/young age and indirect (germline), and of ALAN in the adult exposure.

The overall likelihood across all exposure periods, i.e. the highest likelihood given in the cluster blood lipids, was ALAN.

The CEP Panel considered that the evidence from the studies available did not show a Likely or Very Likely effect of BPA in any exposure period, therefore, none of the endpoints was taken forward for BMD analysis.

Uric acid

The effects of BPA on uric acid in serum, urine or liver were assessed as a measure of the influence of BPA on purine metabolism. Within the cluster uric acid, two studies were performed in mice, of which one study had exposure during development and one had exposure during adulthood. The only study in rats was performed during the adult phase.

Developmental exposure (pre-natal and/or post-natal until weaning)

For this exposure period, one study in mice (Bodin et al., 2014 [RefID 623]) was identified. In this study, a dose of 397.5 µg/kg bw per day was given once. No effect has been observed on uric acid concentration in serum and urine.

The CEP Panel considered that there was Inadequate Evidence on the effect of BPA on uric acid in the developmental exposure period and, thus, this endpoint was not taken forward for BMD analysis.

Adult exposure (after puberty)

For this exposure period, one study in two strains of male mice, CD-1 and C57BL/6 (Ma et al., 2018 [RefID 12637] (experiment 1), Tier 2), was identified. In this study, doses of 5, 50 and 500 µg/kg bw per day were given for 8 weeks from week 6. A dose-dependent increase in serum concentration of uric acid was observed in both strains and the increases were statistically significant at 50 and 500 µg/kg bw per day. A dose-dependent increase in liver concentration of uric acid was observed in CD1 mice, the increases were statistically significant at 50 and 500 µg/kg bw per day.

Doses of 5, 50 and 500 µg/kg bw per day were given for 8 weeks from week 6 in rats (Ma et al., 2018 [RefID 12637] (experiment 2), Tier 2). A statistically significant increase of uric acid was shown at 500 µg/kg bw per day. No results for 5 and 50 µg/kg bw per day were presented.

The CEP Panel assigned a likelihood level of Likely to the uric Acid effect of BPA in the adult exposure period. Since the likelihood level for this cluster is Likely for the endpoints uric acid concentration in serum and in liver in mice strain CD-1, and for uric acid concentration in serum in mice strain C57BL/6 (Ma et al., 2018 [RefID 12637]), these endpoints were taken forward for BMD analysis (see Chapter 3.2.1) and uncertainty analysis (see Appendix D).

Developmental and adult, growth phase/young age and indirect (germline) exposure periods

In these exposure periods, no studies on BPA effects on uric acid were identified.

Overall cluster selection of the endpoints/studies for BMD analysis for uric acid

No effect was seen in one mouse study with a single-dose of BPA in the developmental exposure period. A dose-dependent increase of uric acid concentration in serum in two mice strains and in liver in one mice strain was observed after 8 weeks exposure in the adult age. In one rat study, uric acid concentration in serum was also statistical significantly increased at the highest dose of 500 µg/kg bw per day after 8 weeks exposure in the adult age.

Overall, the CEP panel considered that there was Inadequate Evidence for concluding on the likelihood of effects of BPA on Uric acid in the developmental until weaning exposure period, whereas the CEP Panel assigned a likelihood level of Likely to effects of BPA on Uric Acid in the adult exposure period.

The overall likelihood across all exposure periods, i.e. the highest likelihood given in the cluster uric acid, was Likely.

The CEP Panel considered that the evidence from the studies available showed a Likely effect of BPA in the exposure period adult for the endpoint uric acid (Ma et al., 2018 [RefID 12637]), therefore, this endpoint was taken forward for BMD analysis (see Chapter 3.2.1).

Type 1 diabetes mellitus (T1DM)

The incidence of T1DM was studied in a mouse model which spontaneously develops TD1M (the NOD mouse) (Bodin et al., 2014 [RefID 623]) and in an experimental model in which β-cells of the pancreas were destroyed by a β-cell-specific toxin (streptozotocin) (Cetkovic-Cvrlje et al., 2017 [RefID 916]). Both are mouse models commonly used in experimental animal studies of T1DM.

Within the cluster T1DM, two studies were performed in mice, of which one study had exposure during development, and one had exposure both during growth phase/young age and during adulthood. No studies in other species were available.

Developmental exposure (pre-natal and/or post-natal until weaning)

For this exposure period, one study in mice was identified (Bodin et al., 2014 [RefID 623], Tier 3). This study used a special mouse strain (diabetes type 1 prone NOD) and BPA doses of 30, 300 and 3,000 µg/kg bw per day were given from mating until weaning. In the F1 generation, no effect was seen at 7 weeks. Above 20 weeks, the incidence of diabetes increased at the dose of 3,000 µg/kg bw per day in females only. Because an effect was seen only in the highest dose tested and only in females in a Tier 3 study, the CEP considered that there was Inadequate Evidence for concluding on the likelihood of effects of BPA on the incidence of T1DM in this special mouse strain, in the developmental exposure period and, thus, none of the endpoints included in this cluster was taken forward for BMD analysis. However, the ALAN endpoints were considered in the uncertainty analysis (see Appendix D).

Developmental and adult exposure (pre-natal and/or post-natal in pups until adulthood)

In this exposure period, no studies on effects of BPA on T1DM were identified.

Growth phase/young age exposure

In this exposure period, one study in mice (Cetkovic-Cvrlje et al., 2017 [RefID 916], Tier 1) was identified. In this study, only male animals were tested at BPA doses of 160 and 1600 µg/kg bw per day, starting at 4 weeks. T1DM was initiated in the mice by five streptozotocin i.p. injections at 9 weeks. Both doses increased the T1DM incidence statistically significantly compared with the control but without a statistically significant difference between the two dose groups. The higher dose showed a trend towards higher glycaemic levels, even if not statistically significant.

Because an effect was seen at both dose levels without a dose–response relationship and only in males, the CEP Panel assigned a likelihood of ALAN to the effects of BPA on the incidence of T1DM in a streptozotocin model in the growth phase/young age exposure period and, thus, none of the endpoints included in this cluster was taken forward for BMD analysis.

Adult exposure (after puberty)

In this exposure period, one study in mice (Cetkovic-Cvrlje et al., 2017 [RefID 916], Tier 1) was identified, which is the same study as described under Growth phase/young age.

In the mouse study, only male animals were tested, at doses of 160 and 1600 µg/kg bw per day, starting at 4 weeks. T1DM was initiated in the mice by five streptozotocin i.p. injections at 9 weeks. Both doses increased the T1DM incidence statistically significantly compared with the control, but without statistically significant difference between the two dose groups. The higher dose showed a trend towards higher glycaemic levels, even if not statistically significant.

Because an effect was seen in both dose levels without a dose–response relationship and only in males, the CEP Panel assigned a likelihood of ALAN to effects of BPA on the incidence of T1DM in the adult exposure period and, thus, none of the endpoints included in this cluster was taken forward for BMD analysis.

Indirect (germline) exposure

No studies were available on BPA effects on T1DM in this exposure period.

Overall cluster selection of the endpoints/studies for BMD analysis for type I diabetes mellitus (TIDM)

Two models of initiation of T1DM in mice were used to study the influence of BPA on the development of T1DM. In the mouse model which spontaneously develops TD1M (the NOD mouse), an increase in the incidence of T1DM was seen only in the highest dose tested and only in females in a Tier 3 study with developmental exposure. No effect was observed with BPA exposure during development and in adulthood. Overall, the BPA effect on the incidence of T1DM was judged as ALAN.

In the experimental model in which β-cells of the pancreas were destroyed by streptozotocin, a β-cell-specific toxin, one study with exposure encompassing growth phase/young age and adulthood was

performed only in males. A higher incidence of T1DM was seen at both dose levels without a dose–response relationship and the effect was judged as ALAN.

The CEP Panel considered that there was Inadequate Evidence for concluding on the likelihood of BPA effects on T1DM in the developmental exposure period, while assigned a likelihood level of ALAN in the growth phase/young age and adult exposure periods.
The overall likelihood across all exposure periods, i.e. the highest likelihood given in T1DM, was ALAN.

The CEP Panel considered that the evidence from the studies available did not show a Likely or Very Likely effect of BPA in any exposure period, therefore, none of the endpoints was taken forward for BMD analysis.

Other metabolic hormones

Adiponectin, leptin, glucagon and resistin in plasma were included as metabolic endpoints because they are hormones related to obesity, glucose regulation and lipid metabolism. These endpoints therefore formed the cluster other metabolic hormones.

Within this cluster, the hormones were measured in five mouse studies, two of them with exposure during development until weaning period, one during development and adulthood, three during growth phase/young age, two during adulthood, whereas no studies were available with a germline exposure. In 10 studies, the metabolic hormone measurements were performed in rats, seven of them when exposure was during development until weaning, two during developmental and adulthood, one during growth phase/young age, one during adulthood and one with a germline exposure.

Developmental exposure (pre-natal and/or post-natal until weaning)

For the endpoint adiponectin, in this exposure period, six studies, five in rats (Dunder et al., 2018 [RefID 11866]; Lejonklou et al., 2017 [RefID 3975]; Zhang et al., 2013 [RefID 8798]; Song SZ et al., 2014 [RefID 6829]; Leung et al., 2017 [RefID 3990]), and one in mice (van Esterik et al., 2014 [RefID 7393]), were identified.

In the rat studies, doses between 0.5 and 2500 µg/kg bw per day were tested. In three of the rat studies (two studies Tier 1, Dunder et al., 2018 [RefID 11866] and Lejonklou et al., 2017 [RefID 3975] and one in Tier 3, Leung et al., 2017 [RefID 3990]), no effect has been observed with measurements taken on PND21, PND35, PND50 and at 1 year. In two studies (Song SZ et al., 2014 [RefID 6829], Tier 1 and Zhang et al., 2013 [RefID 8798], Tier 2), decreases of adiponectin concentrations were observed without dose–response at doses between 90 and 900 µg/kg bw per day, one study in males only and the other study in females only.

In the mouse study, with doses between 2 and 3000 µg/kg bw per day, no effect has been observed at week 23 in males and no dose–response was seen in females.

For the endpoint leptin, in this exposure period, six studies, five in rats (Dunder et al., 2018 [RefID 11866]; Lejonklou et al., 2017 [RefID 3975]; NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]; Santos-Silva et al., 2018 [RefID 13047]; Leung et al., 2017 [RefID 3990], experiments 1 and 2) and one in mice (van Esterik et al., 2014 [RefID 7393]), were identified.

In the rat studies, doses between 0.5 and 2,500 µg/kg bw per day were tested. In four of the rat studies (three studies Tier 1, Dunder et al., 2018 [RefID 11866]; Lejonklou et al., 2017 [RefID 3975]; NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) and one in Tier 3, Leung et al., 2017 [RefID 3990], experiments 1 and 2), no effect has been observed with measurements taken on PND15, PND21, PND50 and at 1 year. In one study (Santos-Silva et al., 2018 [RefID 13047], Tier 2), no effects were seen in females, whereas in males, increase on PND15 and decrease on PND21 at 1,785 µg/kg bw per day was observed and a decrease was seen also at 178,500 µg/kg bw per day on PND21.

In the mouse study, with doses between 2 and 3000 µg/kg bw per day no effect has been observed at week 23 in males and no dose–response was seen in females.

For the endpoint glucagon, in this exposure period, one study in mice (van Esterik et al., 2014 [RefID 7393], Tier 1) was identified. Six doses between 2 and 3000 µg/kg bw per day were tested with exposure between GD0 and PND22 and showed no effect.

In this exposure period, 14 studies in total were available, in rats 11 studies and in mice three studies. Adiponectin was measured in six studies, leptin in seven studies and glucagon in one study, resistin was not measured. Most of the results showed no effect.

Since most of the studies showed no effect, the CEP Panel assigned a likelihood level of Not Likely to the effect of BPA during developmental until weaning exposure period on adiponectin, leptin and glucagon, thus, none of these endpoints was taken forward for BMD analysis.

Developmental and adult exposure (pre-natal and post-natal in pups until adulthood)

For the endpoint adiponectin, in this exposure period, two studies, one in rats (Ben-Jonathan et al., 2018 (NTP Grantee study) [RefID 13786]) and one in mice (Patel et al., 2014 [RefID 5695], experiments 1 and 2), were identified.

In the rat study (Ben-Jonathan et al., 2018 (NTP Grantee study) [RefID 13786], Tier 1), doses between 0.05 and 25000 µg/kg bw per day were tested and no effect has been observed with measurements taken at 1 year.

In the mouse study (Patel et al., 2014 [RefID 5695], experiments 1 and 2, Tier 2), a dose of 5 µg/kg bw per day was tested during gestation and afterwards a dose of 0.6 µg/kg bw per day until four months with and without a high-fat diet. No effect has been observed at four months.

For the endpoint leptin, in this exposure period, three studies, two in rats (Ben-Jonathan et al., 2018 (NTP Grantee study) [RefID 13786] and NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) and one in mice (Patel et al., 2014 [RefID 5695], experiments 1 and 2), were identified.

In the rat studies (both Tier 1), doses between 0.05 and 25,000 µg/kg bw per day were tested and no effect has been observed with measurements taken at 1 year.

In the mouse study (Patel et al., 2014 [RefID 5695], experiments 1 and 2, Tier 2), a dose of 5 µg/kg bw per day was tested during gestation and afterwards a dose of 0.6 µg/kg bw per day was given until four months with and without a high-fat diet. No effect has been observed at four months in females and a decrease was observed in males.

In this exposure period, three studies in total were available, in rats two studies and in mice one study. Adiponectin was measured in two studies, leptin in three studies, whereas glucagon and resistin were not measured. Most of the studies showed no effect.

The CEP Panel assigned a likelihood level of Not Likely to the effect of BPA exposure during development and in adulthood on adiponectin (because all studies showed no effect) and on leptin (because both Tier 1 studies showed no effect and in the mouse study with and without high-fat diet an unexplained sex difference was observed) and, thus, none of these endpoints was taken forward for BMD analysis.

Growth phase/young age exposure

For the endpoint adiponectin, in this exposure period, two studies, one in rats (Yang YJ et al., 2014 [RefID 10269]) and one in mice (Wyatt et al., 2016 [RefID 8080], experiments 1 and 2), were identified.

In the rat study (Ben-Jonathan et al., 2018 (NTP Grantee study) [RefID 13786], Tier 2), doses between 1 and 100 µg/kg bw per day were tested and no effect was observed with measurements taken at week 10.

In the mouse study (Wyatt et al., 2016 [RefID 8080], experiments 1 and 2, Tier 2), doses of 1.71 to 1171 µg/kg bw per day were tested from 4 weeks to 11 weeks in two strains. A decrease was observed at 17.1 µg/kg bw per day only in one strain.

For the endpoint leptin, in this exposure period, two studies, one in rats (Yang YJ et al., 2014 [RefID 10269]) and one in mice (Wyatt et al., 2016 [RefID 8080], experiments 1 and 2), were identified.

In the rat study (Ben-Jonathan et al., 2018 (NTP Grantee study) [RefID 13786], Tier 2), doses between 1 and 100 µg/kg bw per day were tested and no effect has been observed with measurements taken at week 10.

In the mouse study (Wyatt et al., 2016 [RefID 8080], experiments 1 and 2, Tier 2), doses of 1.71 to 1171 µg/kg bw per day were tested from 4 weeks to 11 weeks in two strains. A decrease was observed at 17.1 µg/kg bw per day only in one strain.

For the endpoint resistin, in this exposure period, two studies in mice (Wyatt et al., 2016 [RefID 8080], experiments 1 and 2 and Yang et al., 2016 [RefID 8375], experiments 1 and 2), were identified.

In the mouse study from Yang et al. (2016) [RefID 8375], experiments 1 and 2, Tier 1, doses between 5 and 5000 µg/kg be per day were tested. In the group with a high-fat diet, no adverse effect has been observed in males and females, whereas in the group with normal diet, increased levels were seen at both 50 and 500 µg/kg bw per day both in males and females. In another mouse study (Wyatt et al., 2016 [RefID 8080], experiments 1 and 2, Tier 2), doses of 1.71 to 1,171 µg/kg bw per day were tested from 4 weeks to 11 weeks in two strains and no effect was seen in males and females.

In this exposure period, 4 studies in total were available, in rats one study and in mice three studies. Adiponectin was measured in two studies, leptin in three studies and resistin in two studies, whereas glucagon was not measured. Most of the results showed no effect.

The CEP Panel assigned a likelihood level of Not Likely to the effect of BPA exposure during growth phase/young age on adiponectin and leptin (because only in one strain at one dose an effect was observed) and on resistin (because only in one mouse study with normal diet an effect was observed without dose–response and in the other three studies no effect was observed) and, thus, none of these endpoints was taken forward for BMD analysis.

Adult exposure (after puberty)

For the endpoint adiponectin, in this exposure period, one Tier 2 study in mice (Wyatt et al., 2016 [RefID 8080]) was identified. Doses between 1.71 and 1171 µg/kg bw per day were tested and no adverse effect has been observed at week 11.

For the endpoint leptin, in this exposure period, three studies, one in rats (Santos-Silva et al., 2018 [RefID 13047]) and two in mice (Wyatt et al., 2016 [RefID 8080], experiments 1 and 2 and Yang et al., 2016 [RefID 8375], experiments 1 and 2), were identified.

In the rat study (Santos-Silva et al., 2018 [RefID 13047], Tier 2), doses of 1785 and 178,500 were tested and no effects were observed in males and females.

In the mouse study from Wyatt et al. (2016) [RefID 8080], experiments 1 and 2, Tier 2, doses between 1.71 and 1171 µg/kg bw per day were tested and no adverse effect has been observed with measurements taken at week 11 in males and females in the two strains tested. In the mouse study from Yang et al. (2016) [RefID 8375], experiments 1 and 2, Tier 1, doses between 5 and 5000 µg/kg be per day were tested. In the group with a high-fat diet, no adverse effect has been observed in males and females, whereas in the group with normal diet, increased levels were seen at all four doses tested in males and females, without dose–response.

For the endpoint resistin, in this exposure period, two studies in mice (Wyatt et al., 2016 [RefID 8080], experiments 1 and 2 and Yang et al., 2016 [RefID 8375], experiments 1 and 2), were identified.

In the mouse study from Yang et al. (2016) [RefID 8375], experiments 1 and 2, Tier 1, doses between 5 and 5000 µg/kg bw per day were tested. In the group with a high-fat diet, no adverse effect has been observed in males and females, whereas in the group with normal diet increased levels were seen at both 50 and 500 µg/kg bw per day both in males and females. In the mouse study from Wyatt et al. (2016) [RefID 8080], experiments 1 and 2, Tier 2, doses of 1.71 to 1171 µg/kg bw per day were tested from 4 weeks to 11 weeks in two strains and no effect was seen in males and females.

In this exposure period, 10 studies in total were available, in rats one study and in mice two studies. Adiponectin was measured in one study, leptin in three studies and resistin in one study, whereas glucagon was not measured. Most of the results showed no effect.

The CEP Panel assigned a likelihood level of Not Likely to the effect of BPA during adult exposure period on adiponectin and leptin (because only in one mouse study with normal diet an effect was observed at all dose levels without dose–response and in the other four studies no effect was observed) and on resistin (because only in one mouse study with normal diet an effect was observed without dose–

response and in the other three studies no effect was observed) and, thus, none of these endpoints was taken forward for BMD analysis.

Indirect (germline) exposure

For the endpoint adiponectin, in this exposure period, one rat study (Li GQ et al., 2014 [RefID 4039], Tier 2) with exposure during gestation (GD0) and lactation (PND21), at 40 µg/kg bw per day and measurements in the F2 generation at 20 weeks was identified. No effects in the pups or adult rats were observed.

For the endpoint leptin, in this exposure period, one rat study (Li GQ et al., 2014 [RefID 4039], Tier 2) with exposure during gestation (GD0) and lactation (PND21), at 40 µg/kg bw per day and measurements taken in the F2 generation at 20 weeks was identified. No effects in the pups or adult rats were observed.

The CEP Panel considered that there was Inadequate Evidence for concluding on the likelihood of effects of BPA on adiponectin and leptin during the indirect (germline) exposure period and, thus, none of these endpoints was taken forward for BMD analysis.

Overall cluster selection of the endpoints/studies for BMD analysis for other metabolic hormones

Overall, the CEP Panel assigned a likelihood level of Not Likely to the effect of BPA exposure on other metabolic hormones (adiponectin, leptin, resistin and glucagon), during the developmental until weaning, developmental until adulthood, growth phase/young age and adult exposure periods. The CEP Panel considered that there was Inadequate Evidence for concluding on the likelihood of effects of BPA during indirect (germline) exposure period.

The overall likelihood across all exposure periods, i.e. the highest likelihood given in the cluster other metabolic hormones, was Not Likely.

The CEP Panel considered that the evidence from the studies available did not show a Likely or Very Likely effect of BPA in any exposure period, therefore, none of the endpoints was taken forward for BMD analysis.

Thyroid hormones

Hormone measurements (T_3, TT_3, FT_3, T_4, TT_4, FT_4 and reverse T_3/TT_4 (rT_3/TT_4)) were available for the assessment of the function of the thyroid.

Thyroid hormones were measured in studies with exposure during development until weaning period (one Tier 1 study in sheep (Guignard et al., 2017 [RefID 2451]), two Tier 1 studies in rats (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370] and Bansal and Zoeller, 2019 [RefID 13783]) and one Tier 3 study in mice (Bodin et al., 2014 [RefID 623]), during developmental and adulthood (one Tier 1 study in rats (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) and during adulthood (one Tier 1 study in sheep (Guignard et al., 2017 [RefID 2451]) and one Tier 2 study in rats (Zhang J et al., 2017 [RefID 8770]).

Developmental exposure (pre-natal and/or post-natal until weaning)

For the endpoints T_3, TT_3, FT_3, in a rat study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]), doses between 2.5 µg/kg bw per day and 25000 µg/kg bw per day were tested in males and females, with exposure from GD6 until PND21. Measurements were done at 1 year. No effects were seen. In sheep (Guignard et al., 2017 [RefID 2451]), exposure was from GD28 until GD132 (134) at doses between 185 µg/kg bw per day and 625,000 µg/kg bw per day and measurement was done on PND132 (134) in males and females. No effects were seen.

For the endpoints T_4, TT_4, FT_4, in a rat study, doses between 2.5 µg/kg bw per day and 25000 µg/kg bw per day were tested in males and females with exposure from GD6 until PND15 (or PND21). Measurements were done at PND15 (Bansal and Zoeller, 2019 [RefID 13783]) or 1 year (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]). No effects were seen in any of the studies. In sheep (Guignard et al., 2017 [RefID 2451]), exposure was from GD28 until GD132 (134) and measurement was done on PND132 (134) in males and females. No effects were seen.

The CEP Panel assigned a likelihood level of Not Likely to the effect of BPA in the developmental exposure period on the function of thyroid, thus, none of the endpoints was taken forward for BMD analysis.

Developmental and adult exposure (pre-natal and/or post-natal in pups until adulthood)

For the endpoints T_3, total T_3 (TT_3), free T_3 (FT_3), in this exposure period one Tier 1 rat study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) was identified. No effect was seen on T3 in this study. Male and female rats were dosed with 2.5, 25, 250, 2500 or 25000 µg/kg bw per day.

For the endpoints T_4, TT_4, FT_4, in this exposure period, one Tier 1 rat study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) was identified. Rats were dosed with 2.5, 25, 250, 2500 or 25000 µg/kg bw per day; No effect was seen on T_4 in this study in females. No significant changes at any dose tested in males; Trend analysis by the authors is significant but the nature of the trend is not evident from inspection of the data.

The CEP Panel assigned a likelihood level of Not Likely to the effect of BPA exposure in the developmental phase until adulthood on the function of thyroid, thus, none of the endpoints was taken forward for BMD analysis.

Growth phase/young age exposure

No studies were available on BPA effects on thyroid hormones for this exposure period.

Adult exposure (after puberty)

For the endpoints T_3, TT_3, FT_3, in a rat study (Zhang J et al., 2017 [RefID 8770]), doses between 250 µg/kg bw per day and 1000 µg/kg bw per day were tested in female rats. Measurements of FT_3 were done. No effects were seen.

In sheep (Guignard et al., 2017 [RefID 2451]), exposure was with 5, 50 and 5000 µg/kg bw per day and from GD28 to GD132(134). Measurement (T3) was done on GD132 (134) in females. Reduced TT3 was observed only at 50 µg/kg bw per day. The ratio reverse T_3/TT_4 was increased, but this endpoint does not contribute to the assessment of the thyroid function (Smith and Wassner, 2020).

For the endpoints T_4, TT_4, FT_4, in a rat study (Zhang J et al., 2017 [RefID 8770]), doses between 250 µg/kg bw per day and 1000 µg/kg bw per day were tested in female rats. Measurements of FT_4 were done. No effects were seen.

In sheep (Guignard et al., 2017 [RefID 2451]), exposure was with 5, 50 and 5000 µg/kg bw per day and from GD28 to GD132 (134). Measurement (FT_4) was done on PND132 (134) in females. Reduced FT_4 was not dose dependent.

The CEP Panel assigned a likelihood level of Not Likely to the effect of BPA exposure in adulthood on the function of thyroid, thus, none of the endpoints was taken forward for BMD analysis.

Indirect (germline) exposure

No studies were available on BPA effects on thyroid hormones for this exposure period.

Overall cluster selection of the endpoints/studies for BMD analysis for thyroid hormones

Overall, the CEP Panel assigned a likelihood level of Not Likely to the effect of BPA exposure on the function of thyroid, in the developmental, developmental and adult, and adult exposure periods.

The overall likelihood across all exposure periods, i.e. the highest likelihood given in the cluster thyroid hormones effects, was Not Likely.

The CEP Panel considered that the evidence from the studies available did not show a Likely or Very Likely effect of BPA on thyroid hormones in any exposure period, therefore, none of the endpoints was taken forward for BMD analysis.

3.1.4.3. Integration of likelihoods from human and animal studies

Table 10 presents the overall likelihood per cluster, across all exposure periods, from the human and animal studies separately, as well as the integration of the likelihood per cluster from both the human and animal studies when available, for the Metabolic HOCs.

The division into clusters was done according to what was considered the best way of clustering the endpoints from each of the two streams of evidence (human and animal). There was partial overlap in the endpoints included in some of the human and animal clusters. Some endpoints were relevant in more than one HOC, such as both for obesity and cardiovascular effects. For instance, levels of leptin and adiponectin hormones were included in the human cluster obesity and in the animal cluster other metabolic hormones. Other endpoints in the human cluster cardiometabolic effects (cholesterol (total), HDL cholesterol, LDL cholesterol and triglycerides) were included in the animal cluster blood Lipids. The reasoning behind the inclusion of the endpoints in the specific clusters can be found in Chapter 2.2.2. for human evidence and in Chapter 3.1.4.2 for the animal evidence.

Table 10: Integration of the human and animal studies for Metabolic effects.

Human stream		Animal stream		Integrated likelihood
Cluster: Obesity		**Cluster: Obesity**		
Exposure during Pregnancy	Not Likely	Developmental (pre-natal and/or post-natal until weaning)	ALAN	
Exposure during Childhood	Not Likely	Developmental and adult (pre-natal and/or postnatal in pups until adulthood)	ALAN	
Exposure during adulthood	ALAN	Growth phase/young age	ALAN	
		Adult exposure (after puberty)	Not Likely	
		Indirect (germline) exposure	Not Likely	
Overall likelihood:	*ALAN*	*Overall likelihood:*	*ALAN*	*ALAN*
Cluster: Thyroid effects		**Cluster: Thyroid hormones**		
Exposure during Pregnancy	Not Likely	Developmental (pre-natal and/or post-natal until weaning)	Not Likely	
		Developmental and adult (pre-natal and/or postnatal in pups until adulthood)	Not Likely	
		Adult exposure (after puberty)	Not Likely	
Overall likelihood:	*Not Likely*	*Overall likelihood:*	*Not Likely*	*Not Likely*
Cluster: Cardiometabolic effects				
Exposure during pregnancy	Not Likely	Not applicable		
Overall likelihood:	*Not Likely*			*Not Likely*
Cluster: Type 2 Diabetes Mellitus				
Exposure during Adulthood	ALAN	Not applicable		
Overall likelihood:	*ALAN*			*ALAN*
Cluster: Gestational Diabetes Mellitus				
Exposure during Adulthood	Not Likely	Not applicable		
Overall likelihood:	*Not Likely*			*Not Likely*
Cluster:		**Uric Acid**		
Not applicable		Developmental (pre-natal and/or post-natal until weaning)	Inadequate evidence	

	Adult exposure (after puberty)	Likely	
	Overall likelihood:	*Likely*	*Likely*
Cluster:	**Type 1 Diabetes Mellitus**		
Not applicable	Developmental (pre-natal and/or post-natal until weaning)	Inadequate evidence	
	Growth phase/ young age	ALAN	
	Adult exposure (after puberty)	ALAN	
	Overall likelihood:	*ALAN*	*ALAN*
Cluster:	**Fat deposition in the liver**		
Not applicable	Developmental (pre-natal and/or post-natal until weaning)	ALAN	
	Developmental and adult (pre-natal and/or postnatal in pups until adulthood)	Inadequate evidence	
	Growth phase/ young age	ALAN	
	Adult exposure (after puberty)	ALAN	
	Overall likelihood:	*ALAN*	*ALAN*
Cluster:	**Glucose regulation**		
Not applicable	Developmental (pre-natal and/or post-natal until weaning)	ALAN	
	Developmental and adult (pre-natal and/or postnatal in pups until adulthood)	Not Likely	
	Growth phase/ young age	Not Likely	
	Adult exposure (after puberty)	ALAN	
	Indirect (germline) exposure	ALAN	
	Overall likelihood:	*ALAN*	*ALAN*
Cluster:	**Blood lipids**		
Not applicable	Developmental (pre-natal and/or post-natal until weaning)	Not Likely	
	Developmental and adult (pre-natal and/or postnatal in pups until adulthood)	Not Likely	
	Growth phase/ young age	Not Likely	
	Adult exposure (after puberty)	ALAN	
	Indirect (germline) exposure	Not Likely	
	Overall likelihood:	*ALAN*	*ALAN*
Cluster:	**Other metabolic hormones**		
Not applicable	Developmental (pre-natal and/or post-natal until weaning)	Not Likely	
	Developmental and adult (pre-natal and/or postnatal in pups until adulthood)	Not Likely	
	Growth phase/ young age	Not Likely	
	Adult exposure (after puberty)	Not Likely	
	Indirect (germline) exposure	Inadequate evidence	
Overall likelihood:	*Overall likelihood:*	*Not Likely*	*Not Likely*

3.1.4.4. In vitro and mechanistic studies

Regarding scoring of likelihood of effects in the WoE for the HOC Metabolic effects, no clusters were scored Very Likely for any exposure periods, neither in animals nor humans. A few clusters and endpoints were scored Likely and several were scored ALAN. In the following, MoA studies for the Likely and ALAN clusters among the HOC Metabolic effects are described.

There is some overlap in the various parts of the text on MoA, since often the included studies have examined several MoAs which may belong to more than one cluster.

Obesity

After the integration of the human and animal evidence, the overall likelihood of effects of BPA for the cluster obesity was scored ALAN.

A MoA for obesity studied in many publications in experimental animals involves BPA-induced increased expression of adipogenic genes and their proteins, increasing adipogenesis, i.e. the generation of mature adipocytes. Adipogenesis is a tightly controlled differentiation process sensitive to hormones such as insulin, which is driven by key regulators such as CCAATT enhancer binding proteins (C/EBP) α, β and δ and peroxisome proliferator-activated receptor gamma (PPARγ), which induce the expression of genes that lead to the development of the adipocyte phenotype, including formation of lipid droplets and adipokine release (Boucher et al., 2014 [RefID 667]). One such gene which is subsequently upregulated is adipocyte protein 2 (aP2), also known as fatty acid binding protein 4 (FABP4), coding for a protein involved in insulin sensitivity and glucose metabolism as well as lipid metabolism (Atlas et al., 2014 [RefID 291]).

In a variety of experiments, Yang et al. (2016) [RefID 8375] investigated if BPA promoted adiposity and inflammation. In a human study of 228 subjects (mean age 62–63 years, around 39–40% men) from a Chinese population, it was found that total urinary BPA concentrations were associated with increased circulating inflammatory factors such as TNF-α, as well as leptin, in lean female subjects (BMI < 23.0 kg/m^2) but not in lean males or in both sexes of overweight/obese subjects (BMI > 25.0 kg/m^2). In a mouse *in vivo* experiment, 5-week-old mice of both sexes were exposed to BPA (5, 50, 500 or 5000 µg/kg bw per day) orally for 30 days and showed increased body weight and fat mass in a non-monotonic dose-dependent manner when fed a normal diet, even observed at the lowest dose. On a HFD, increased body weight was only in seen in males with 50, 500 and 5000 µg/kg bw per day and no difference in fat mass was observed in either sex of mice, suggesting that BPA may interact with diet in affecting obesity. In this mouse *in vivo* experiment, BPA increased mRNA expression of genes involved in adipogenesis [*C/ebp-α* and *Ppar-γ* (with 5, 50, 500 and 5000 µg/kg bw) and *Ap2* (50, 500 and 5000 µg/kg bw] and lipogenesis [fatty acid synthase (*Fas*) (50, 500 and 5000 µg/kg bw), *Srebp-1c* and *Scd-1* (5 and 500 µg/kg bw), whereas there were no effects on thermogenesis genes (uncoupling protein 1 (*Ucp1*) and PPARγ coactivator (*Pgc*)*-1α*), in male mice on a normal diet. Increased gene expression of *F4/80*, *Cd11c* and *Mcp-1* in WAT from male mice given BPA (only seen with 500 and/or 5000 µg/kg bw) on a normal diet, indicated macrophage WAT infiltration. mRNA of cytokines related to inflammation including *IL-6*, *IL-1β*, *TNF-α*, *IFN-Y* and *iNos2* were also increased in WAT of male mice with BPA (500 and/or 5,000 µg/kg bw) on a normal diet. In an *in vitro* study with differentiated adipocytes isolated from the stromal vascular fraction of adipose tissue, mRNA expression of *C/ebp-α*, *Ppar-γ* and *Ap2* was only increased with 10 and/or 50 µM BPA, not with the lower concentrations. Based on considerations of all data derived by this study, BPA elevated adipogenesis and lipid biosynthesis, probably by a direct effect upon differentiation of adipose progenitor cells to white adipocytes, possibly via GR activation, and BPA may have a role in chronic inflammation.

Overall, the available evidence from human studies was limited in the number of available studies, the study sample size, was focused on obesity, and lacked sensitivity and specificity for the remaining metabolic-related biomarkers under study.

Three relevant *in vivo* studies were performed in rats. In primary adipose progenitor stem cells from newborn male rats maternally exposed to 5 mg/L of BPA in drinking water (converted oral dose 250 µg/kg bw per day) from 2 weeks before mating and during pregnancy and lactation, significantly increased protein expression of the adipogenic transcription factor PPARγ, but not transcription factor C/EBPα or the lipogenic factor sterol regulatory element-binding protein SREBP-1, was observed at

PND1 (Desai et al., 2018 [RefID 11817]). At PND21, increased protein expression of C/EBPα, but not PPARγ, was observed, while SREBP-1 was increased in conjunction with evidence of hypertrophic adipocytes. Consistent with increased adipose tissue mass and hypertrophic adipocytes in the BPA-exposed males, protein expression of the macrophage marker cluster of differentiation 68 (CD68) and pro-inflammatory cytokine tumour necrosis factor (TNF)-α was significantly increased at 3 weeks of age.

Perinatal BPA exposure on GD6–PND21 to 1 or 10 µg/mL in drinking water (converted oral doses 50 or 500 µg/kg bw per day) had long-term adverse effects on body weight and glucose metabolism, probably associated with the downregulated expression of zinc-α2-glycoprotein (ZAG) and adiponectin genes in adolescent female rats (Zhang et al., 2013 [RefID 8798]). In a subsequent study on offspring of female rats, changes in fat metabolism induced by the same BPA exposure were associated with downregulated ZAG gene expression caused by decreased PPARγ mRNA expression (Zhang et al., 2014 [RefID 8797]).

Compared with controls, male and female rat offspring developmentally exposed from GD3.5 to PND22 to 0.5 and 50 µg/kg bw per day of BPA had differentially expressed mRNA of genes central to lipogenesis and adipocyte adiponectin signalling in adipose tissue depending on dose, tissue and sex (Lejonklou et al., 2017 [RefID 3975]). In gonadal white adipose tissue (gWAT) from males, both BPA doses decreased the mRNA expression of adiponectin receptor 2 (AdipoR2) and acetyl-CoA carboxylase (ACC), and the 50 µg/kg bw dose decreased stearoyl-CoA desaturase (SCD1), whereas in females, the 50 µg/kg bw dose increased AdipoR1 and the 0.5 µg/kg bw dose decreased SREBP-1c expression. In iWAT from males, 0.5 µg/kg bw dose decreased AdipoR1 and SCD1 expression, whereas there were no effects on these genes in females.

Among the *in vitro* studies, several used human primary adipocytes or human mesenchymal stem cells (hMSC), which can differentiate into either adipocytes or osteoblasts. A study examined whether BPA (1, 10 and 100 nM) could interfere with the endocrine function that regulates metabolism in mature human adipocytes from pre-pubertal, non-obese children (7–10 years old) (Menale et al., 2015 [RefID 5027]). They found that at 24 h, ERα mRNA was upregulated by 10 and 100 nM BPA, *ERβ* mRNA levels were not affected by any dose of BPA and oestrogen-related receptor-γ (ERRγ) was downregulated by 10 nM BPA. G protein-coupled receptor (GPR30) was downregulated with 10 nM BPA and leptin mRNA was upregulated by 10 and 100 nM BPA. Microarray results showed that at 24 h 10 nM BPA increased the expression of pro-inflammatory cytokines (chemokine (C-C motif) ligand 20 (CCL20), interleukin 18 (IL-18) and interleukin 1β (IL-1β), and their increased secretion was confirmed by enzyme-linked immunosorbent assay (ELISA). BPA upregulated the expression of fatty acid binding protein 4 (FABP4) (1 and 10 nM BPA) and cluster of differentiation 36 (CD36) (10 nM BPA), genes involved in lipid binding and transport, which was confirmed by mean lipid area and triglyceride measurement in the adipocytes.

The results from a study on hMSC indicated that 1 and 10 nM concentrations of BPA, which did not induce any alterations on cell viability, self-renewal and oxidative stress, could enhance adipogenic differentiation seen as changes in gene expression and increased osteogenesis (Dong et al., 2018 [RefID 11840]). There was a need for pre-differentiation exposure to BPA to demonstrate marked differentiation, indicating that other mechanisms, such as epigenetic alterations, not only regulation of PPARγ, might be involved.

Cultured human adipose stromal/stem cells (ASC), the precursors to mature adipocytes, treated with 1 nM, 100 nM and 1 µM BPA had an increase in adipogenesis after 21 days, with a maximal response at 1 µM BPA and with cytotoxicity observed at 10 µM BPA (Ohlstein et al., 2014 [RefID 5491]). BPA for 14 days increased adipogenesis with concentrations of 10 nM, 100 nM and 1 µM, with a maximal response at 100 nM. The overall proposed mechanism of BPA suggested that BPA induced adipogenesis through the upregulation of adipogenic genes in an ER-dependent manner. The expression of the adipogenesis-associated genes dual leucine zipper-bearing kinase (*DLK* (*MAP3K12*)), insulin-like growth factor 1 (*IGF1*), *C/EBPα*, *PPARγ* and lipoprotein lipase (*LPL*) occurred earlier and was increased by 1 µM BPA (the only concentration tested).

In primary human subcutaneous pre-adipocytes *in vitro*, BPA concentrations (10 nM to 50 µM) did not induce significant changes in gene or protein expression of the adipogenic markers aP2 and perilipin below 100 nM (Boucher et al., 2014 [RefID 667]). However, there was a non-significant dose–response in aP2 protein throughout the dose range, with significant levels reached at 25 and 50 µM BPA. Both the ER and the GR can influence adipogenesis and lipid metabolism. BPA has been linked to modulation

of both of these nuclear receptors. However, based on the results from the whole dose range used in this study, BPA induced adipocyte differentiation through a non-classical ER-mediated mechanism rather than through GR activation, and could contribute to the final maturation of cells committed to the adipocyte lineage, supporting that BPA may act as an obesogen.

BPA in 1 and 100 nM concentrations impaired insulin sensitivity and 1 nM induced the release of inflammatory factors in human subcutaneous adipocytes and murine 3T3-L1 embryonic fibroblasts (Valentino et al., 2013 [RefID 7377]). A suggested mechanism was that BPA activated ERK, phospho-Thr183/Tyr185c-Jun N-terminal kinase (JNK), via TLRs or ERs, which might directly impair insulin action. Alternatively, the release of cytokines, interleukin 6 (IL-6) and interferon gamma (IFN-γ), may contribute to JNK-activation in the adipocytes and either directly or indirectly downregulate insulin-stimulated glucose uptake.

The potency of BPA (0.1 nM to 0.03 µM) to act as an ER agonist using both a sensitive oestrogen-responsive luciferase reporter assay in human T47D-KBluc breast cancer cells, and by assessing ER-mediated signals, i.e. ERK phosphorylation, mitochondrial respiration and glycolytic function, in murine 3T3-L1 cells, was examined by Tsou et al. (2017) [RefID 7318]. The results indicated that BPA exposure decreased both mitochondrial respiration and glycolytic function, followed by a rapid and transient activation of ERK via ER, suggesting that BPA may affect the regulation of body fat.

In murine 3T3-L1 adipocytes, dose-dependent lipid accumulation was seen with BPA, with a five-fold higher accumulation with 25 µM versus 1.2–1.8-fold for 0.01–10 µM BPA (Pomatto et al., 2018 [RefID 12911]). No difference in lipidogenesis was seen when BPA exposure occurred at early or mid–late differentiation. *In silico* molecular docking studies of BPA to PPARγ and retinoid-X-receptor-alpha (RXRα) indicated that BPA was capable of binding with these receptors, however, the binding of BPA to PPARγ was weak.

BPA (1 nM for 3 weeks) increased both adipocyte number and lipid content by affecting murine 3T3-L1 pre-adipocytic cell growth and altering timing and expression of master genes involved in adipocyte differentiation and adipose tissue development, e.g. PPARγ, FABP4/AP2 and C/EBPα (Ariemma et al., 2016 [RefID 251]). Additionally, BPA may generate metabolic dysfunctional 3T3-L1 adipocytes with insulin resistance, indicated by downregulation of insulin signalling and reduction of glucose utilisation.

Low concentrations (0.1–10 nM) of BPA dose dependently increased differentiation of murine 3T3-L1 cells into adipocytes in the absence of glucocorticoids and caused the upregulation of the adipogenic marker aP2, through a potentiation of a transcriptional complex containing GR and C/EBPδ specifically on the aP2 promoter (Atlas et al., 2014 [RefID 291]). Further, BPA did not activate the nuclear GR on the mouse mammary tumour virus (MMTV) or the C/EBPα promoters, indicating that BPA was unlikely to act as a PPARγ agonist.

In porcine ovarian follicles *in vitro*, BPA (20 ng/mL (87.6 nM)) directly stimulated adiponectin secretion and expression of adiponectin and its receptors AdipoR1 and AdipoR2 (Rak et al., 2017 [RefID 6083]). It also increased adiponectin-stimulated E2 secretion, but decreased adiponectin-stimulated testosterone secretion. In addition, BPA decreased protein expression of cytochrome P450 19 (CYP19) but did not affect protein expression of 11β-hydroxysteroid dehydrogenase type 2 (17β-HSD) in adiponectin-stimulated ovarian follicular cells.

Regulation of gene expression through epigenetic programming may lead to numerous developmental disturbances, which may contribute to or cause metabolic disorders. Epigenetic effects, especially DNA methylation, have been suggested to be involved in BPA-induced obesity.

When studying the role of pre-natal BPA exposure in childhood obesity by combining epidemiological data with experimental mouse models and BPA-dependent DNA methylation changes *in vitro*, Junge et al. (2018) [RefID 12262] suggested that pre-natal BPA exposure was associated with BMI Z-scores at age 1 and 6 years, and was linked to cord blood mesoderm specific transcript (MEST) promoter methylation and MEST expression. These effects in children were confirmed in mice in which pre-natal BPA exposure (5 µg/L in drinking water, converted oral dose 0.45 µg/kg bw per day) from 1 week before mating to birth altered *Mest* promoter methylation and transcription with an accompanying increase in the body weight of the juvenile offspring.

Other studies on epigenetic effects and obesity were also performed in mice. Perinatal BPA exposure from 2 weeks before mating and through gestation and lactation to 3, 10, 30, 100, 300, 1000 or 3000 µg/kg bw per day in mice demonstrated some dose-dependent metabolic effects (van Esterik et al., 2014 [RefID 7393]). These included increased body weight and liver weights, but no effects on fat pad weights, and decreased glucagon in male offspring. Female offspring had decreased body weight, liver, muscle and fat pad weights, adipocyte size, serum lipids and serum leptin and adiponectin. These effects persisted into adulthood after termination of BPA exposure at weaning, suggesting that BPA initiated permanent functional changes when exposed during early development, affecting energy homeostasis. However, the data did not support BPA as a specific obesogen. In contrast, in a related study with the same BPA doses and exposure period as well as a high-fat diet (HFD), the observed altered metabolic phenotypes seen in their earlier work were not found to be associated with liver DNA methylation changes (van Esterik et al., 2015 [RefID 7393]).

Studies on DNA methylation of hepatic genes using an epigenome-wide discovery platform were performed in adult female mice offspring after perinatal BPA exposure from 2 weeks before mating through gestation and weaning to 50 ng, 50 µg and 50 mg/kg diet of BPA (converted oral doses 0.008, 7.5 and 7500 µg/kg bw) per day by Anderson et al. (2017) [RefID 187]. Genes related to energy expenditure, body weight and body fat phenotypes, Regulatory factor X-associated protein (*Rfxap*), transmembrane protein 238 (*Tmem238*), Janus kinase 2 (*Jak-2*) and Retinoid-X receptor (*Rxr)*, were identified.

Pre-natal exposure on GD9–GD18 to BPA (5 or 500 µg/kg bw per day) disrupted the bimodal nature of epigenetic regulation of the FGGY carbohydrate kinase domain containing (*fggy*) gene in gonadal gWAT of mouse offspring shown by genome-wide DNA methylation and messenger RNA (mRNA) expression, which may possibly contribute to adult-onset obesity (Taylor et al., 2018 [RefID 13239]).

In differentiated rat primary hypothalamic neuroprogenitor cells (NPCs) from newborn offspring of dams administered BPA (5 mg/L) in drinking water (converted oral dose 250 µg/kg bw per day) from 2 weeks before mating and throughout pregnancy, BPA increased appetite peptide and reduced satiety peptide expression (Desai et al., 2018 [RefID 11816]). The mechanisms for the BPA-induced enhanced neuroprogenitor cell proliferation and differentiation may involve epigenetic modifications, particularly altered DNA methylation of the basic helix–loop–helix (bHLH) gene proliferative factor (Hes1). No change in the enzyme DNA methyltransferase 3a (Dnmt3a) was seen, however, BPA increased protein expression of lysine (K)-specific histone demethylase 1A (LSD1), indicating an epigenetically-mediated shift toward neurogenesis.

In addition to DNA methylation, BPA could also affect other epigenetic mechanisms. BPA interacted with different families of non-coding RNAs (microRNAs, long non-coding RNAs and small nucleolar RNAs), suggesting that BPA could affect the regulation of gene transcription and various aspects of post-transcriptional mRNA processing (Verbanck et al., 2017 [RefID 7443]). Using genome-wide gene expression in differentiating human primary subcutaneous pre-adipocytes at low (10 nM) and high (10 µM) BPA concentrations, microarray data indicated that chronic exposure to BPA had adverse effects on the transcriptome of human primary adipocytes during differentiation, even at the 10 nM dose. However, subsequent pathway analysis indicated that the most highlighted pathways were related to oncogenesis but not adipogenesis or inflammation.

Other MoAs for an obesogenic effect of BPA have been suggested in individual studies. In mice exposed to 50 µg/kg bw per day of BPA from GD15 to PND21, it was observed that BPA induced intestinal and systemic immune imbalances at PND45, through a decrease of Th1/Th17 cell frequencies in the lamina propria concomitant to an increase of splenic Th1/Th17 immune responses (Malaisé et al., 2017 [RefID 4815]). Relative to control mice, these early effects were associated with altered glucose sensitivity, defect IgA secretion into faeces and a decrease in faecal bifidobacteria. The BPA-mediated disturbed immune homeostasis and gut dysbiosis preceded infiltration of pro-inflammatory M1 macrophages in gWAT appearing with ageing, together with a decreased insulin sensitivity and an increased weight gain.

Exposure of dams to BPA (10 µg/mL in drinking water, 900 µg/kg bw per day) at GD10–PND30 reduced the expression of the cannabinoid receptor 1 (CB1) and induced gene expression of cocaine and amphetamine regulated transcript-1 in the hypothalamus of male offspring at 78 days of age (Suglia et

al., 2016 [RefID 6943]). This suggested that BPA induced activation of anorexigenic signals, causing loss of appetite, via downregulation of CB1, reducing food intake.

In conclusion, the CEP Panel considered that there is substantial evidence supporting that BPA is a potential environmental obesogen based on the available studies on developmentally exposed animals as well as on cell models, although there is also evidence available against this hypothesis. Several MoAs for increased adipogenesis have been put forward in these studies, including changes in expression of adipogenic genes and their proteins (with 0.5–5000 µg/kg bw per day of BPA) leading to induced differentiation of pre-adipocytes into mature, lipid-accumulating adipocytes. BPA was considered unlikely to act as a classical oestrogen due to the high concentrations required for the transactivation of the ER by BPA, but BPA induced adipocyte differentiation through a non-classical ER-mediated mechanism. BPA does not seem to act as a GR agonist in differentiation of murine and human adipocytes. The increased gene expression may be regulated via epigenetic effects, of which DNA methylation is mostly studied (with 0.008–7500 µg/kg bw of BPA). Single studies also suggested the effect of BPA on obesity to be caused by disturbed immune homeostasis and gut dysbiosis (50 µg/kg bw) or via effects on the signalling systems of the brain regulating appetite and food intake (900 µg/kg bw). However, the underlying mechanisms resulting in adverse apical effects in humans, such as increased body weight or BMI, need further investigation.

Fat deposition in the liver

There was no human evidence available for the cluster fat deposition in the liver. Thus, the overall likelihood of effects of BPA for this cluster was scored ALAN, based on the animal evidence.

Lipid metabolism and excess fat accumulation (steatosis) in the liver can be a result of abnormal synthesis, retention or breakdown of lipid. In addition to *in vivo* animal studies, *in vitro* studies on lipid metabolism often used hepatoma cell lines to examine modulation of expression of relevant genes and/or proteins.

Development of hepatic steatosis in offspring of rats after exposure to BPA (40 µg/kg bw per day) during gestation and lactation (GD0–PND21) was mediated through impaired hepatic mitochondrial function, subsequent oxidative stress and upregulated hepatic lipid metabolism (Jiang et al., 2014 [RefID 3190]).

Hepatic steatosis in offspring of pregnant rats exposed to 100 µg/kg bw per day of BPA on GD6–PND21 was associated with increased and modified hepatic triglyceride and FFA compositions in males only (Strakovsky et al., 2015 [RefID 6914]). In males, BPA increased the hepatic expression of the FFA uptake gene fatty acid translocase/cluster determinant 36 (*Fat/Cd36*) and decreased the expression of triglyceride synthesis-related and β-oxidation-related genes (diacylglycerol O-acyltransferase 1 (*Dgat1*), 1-acylglycerol-3-phosphate *O*-acyltransferase 6 (*Agpat6*), C/EBPα (*Cebpa*), C/EBPβ (*Cebpβ*), phosphoenolpyruvate carboxykinase 1 (*Pck1*), phosphoenolpyruvate carboxykinase 1 (*Acox1*), carnitine palmitoyltransferase 1a (*Cpt1a*) and cytochrome *b*-245, β polypeptide (*Cybb*)). BPA altered DNA methylation and histone marks (acetylated histone H3 or H4 (H3Ac or H4Ac), di-methylated histone H3 at lysine residue 4 (H3Me2K4), tri-methylated histone H3 at lysine residue 36 (H3Me3K36)) and decreased the binding of several transcription factors (Pol II, C/EBPβ and SREBP-1) within the gene for the key β-oxidation enzyme *Cpt1a*. In females, BPA only increased the expression of genes involved in FFA uptake and triglyceride synthesis (*LPL*, fatty acid synthase (*Fasn*) and *Dgat*). The study indicated that developmental BPA exposure altered and reprogrammed hepatic β-oxidation capacity in males, potentially through the epigenetic regulation of genes.

In adult male rats administered 10000 µg/kg bw per day of BPA via drinking water for 60 days, BPA increased plasma levels of triglycerides, total cholesterol, LDL-cholesterol, AST, alanine transaminase (ALT), LDH and TNF-α (Mahmoudi et al., 2018 [RefID 12656]). Immunohistochemical analysis showed increased expression of cyclooxygenase-2 (COX-2) and tumour protein p53 (p53) and decreased expression of B-cell lymphoma 2 (Bcl-2), related to liver inflammation. Thus, BPA may contribute to hepatotoxicity via oxidative stress, lipid metabolism disruption and degenerative changes in hepatic cells.

Experiments were performed on adult male mice exposed to 5, 50 or 500 µg/kg bw per day of BPA for eight weeks and *in vitro* experiments in primary murine hepatocytes and the human HepG2 cell line

with 0.001–10 µM BPA (Yang SM et al., 2017 [RefID 8398]). Based on effects in mice and *in vitro* with 10 µM BPA, it was shown that BPA had dual inhibitory effects on the autophagy-lysosomal pathway, suppressing the initiation of autophagy and inhibiting autophagic degradation, thus, affecting hepatic lipid accumulation. The likely major mechanism for the inhibition of autophagy initiation was activation of the serine/threonine protein kinase mTOR, whereas to elucidate how BPA inhibits autophagic degradation will need further studies.

Studies of induction of steatosis by BPA (0.2, 2, 20, 200 or 2000 nM) in the developing liver model human (female) hepatoma HepaRG cell line indicated that BPA increased lipid accumulation with a non-monotonic dose–response, with significant effects on neutral lipids and triglycerides at 2 nM only (Bucher et al., 2017 [RefID 743]). The gene expression of many enzymes involved in lipid and carbohydrate homeostasis and oxidative stress was not changed. Further, the expression of different known targets of ERRγ and pregnane X receptor (PXR), enzymes involved in BPA biotransformation, was not changed, suggesting that these nuclear receptors were not involved in BPA-induced steatosis.

The overall conclusions on effects of BPA (0.3, 3, 30 or 300 ng/mL (1.3, 13.1, 131.4 or 1314.1 nM)) in rat hepatoma FaO cells were that BPA could directly induce lipid accumulation by interfering with the pathways involved in lipid oxidation and secretion, without affecting those involved in lipogenesis (Grasselli et al., 2013 [RefID 2386]). Significant increased levels of triacylglycerol (TAG) in these cells were observed dose dependently with 131.4 and 1314.1 nM BPA only. The mechanisms of action appeared to be ER-independent and involved activation of kinase-mediated pathways, in particular the phosphatidyl inositol-3 kinase (PI-3K) pathway.

Several of the available studies focused on two specific MoAs for fat deposition in the liver, by measuring how BPA could modulate PPARs and SREBPs genes and proteins. PPARs are ligand-activated transcription factors and function as regulators of lipid and lipoprotein metabolism and glucose homeostasis, and also influence cellular proliferation, differentiation and apoptosis (Grygiel-Górniak, 2014). PPARα is highly expressed in tissues such as liver, muscle, kidneys and heart, where it stimulates the β-oxidative degradation of fatty acids, lowering lipid levels. PPARγ is mostly involved in the regulation of the adipogenesis, energy balance and lipid biosynthesis. SREBPs are a family of transcription factors that regulate lipid biosynthesis and adipogenesis by controlling the expression of several enzymes required for cholesterol, fatty acid, TAG and phospholipid synthesis (Shimano and Sato, 2017).

Exposure to 50 µg/kg bw per day of BPA throughout gestation and lactation (GD0–PND21) predisposed rat offspring to fatty liver disease when fed a standard diet or a HFD after weaning (Wei et al., 2014 [RefID 7890]). Perinatal exposure to BPA worsened the hepatic damage caused by the HFD. The additive effects of BPA were correlated with higher levels of hepatic oxidative stress. The expression of mRNA and protein of SREBf1 was significantly increased in the liver regardless of diet. Similarly, mRNA expression of Fasn was co-ordinately upregulated. The mRNA expressions of PPARγ and Cd36 were elevated in BPA-exposed offspring on HFD only. The mRNA levels of PPARα and one of its targets Cpt1a were increased on a standard diet, whereas expression of both genes was decreased on a HFD with BPA compared with HFD controls.

After exposure to BPA (10 µg/kg bw per day s.c., converted oral dose 2222 µg/kg bw per day) during GD9–GD16 of pregnancy, hepatic triglyceride levels were increased in male mice offspring compared with controls (García-Arévalo et al., 2014 [RefID 2193]). BPA altered the expression of important genes involved in fatty acid uptake in the liver, i.e. upregulated *Ppary* and the 5'-adenosine monophosphate-activated protein kinase (AMPK) gene protein kinase AMP-activated catalytic subunit α 1 (*Prkaa1*). AMPK is a fuel sensor protein that inhibits anabolic pathways and activates catabolic pathways in the liver. BPA also decreased the expression of the *Cd36* gene, coding for a fatty acid transport protein.

Perinatal (GD8–PND16 and PND21–PND35) BPA exposure (25 µg/kg bw per day) of mice offspring induced persistent fat accumulation via hypomethylation of lipogenic genes and increased nuclear factor erythroid 2-related factor 2 (Nrf2) recruitment to the *Srebp-1c* promoter in the liver (Shimpi et al., 2017 [RefID 6685]).

BPA (0.5 µg/kg bw per day) exposure of mice from birth to 10 months of age influenced the expression of genes involved in hepatic lipid metabolism, thereby leading to increased hepatic accumulation of cholesterol and triglycerides in middle-aged males (Ke et al., 2016 [RefID 3447]). Based on this *in vivo*

study and murine hepatocyte Hepa1–6 cell line studies, downregulation of DNA methyltransferases and upregulation of the transcription factors *Srebf1* and *Srebf2*, and *Fasn* and 3-hydroxy-3-methyl glutaryl-coenzyme A reductase (*Hmgcr*) genes, key enzymes in fatty acid and cholesterol synthesis, respectively, indicated that BPA epigenetically reprogrammed DNA methylation patterns of genes involved in hepatic lipid synthesis, likely caused by disrupted expression of DNA methyltransferases.

The role of KC polarisation in hepatosteatosis induced in adult male mice exposed to BPA at 5, 50 or 500 µg/kg bw per day for eight weeks, or *in vitro* in primary KC cultures from the mice and the human hepatocellular carcinoma cell line HepG2 exposed to 10 µM BPA, was studied by Lv Q et al. (2017) [RefID 4697]. The effects of BPA on mRNA levels of key components involved in hepatic lipid metabolism and transport were evaluated, including SREBP-1, FAS, PPARα, microsomal triglyceride transfer protein (MTP), adipose triglyceride lipase (ATGL), CD36 and fatty acid transport protein 1 (FATP1). Overall, BPA promoted hepatic lipid synthesis without changing lipid transport at the transcription level. The results also demonstrated that pro-inflammatory M1 KC polarisation was involved in BPA-induced hepatic fat deposition, possibly associated with the ER signalling pathway.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR) performed on adult mouse hepatic mRNA after exposure to BPA (5, 50, 500 or 5000 µg/kg bw per day) for 8 months demonstrated an overexpression of key genes involved in cholesterol biosynthesis; *Mvd* (mevalonate diphosphodecarboxylase), *Lss* (lanosterol synthase), *Hmgcr* and *Sqle* (squalene epoxidase) (Marmugi et al., 2014 [RefID 4884]). BPA also induced the expression of *Srebp-2*, a master regulator of hepatic cholesterol biosynthesis.

BPA exposure (100 µg/kg bw per day) on GD7–PND21 promoted expression of hepatic lipid synthesis and fatty acid accumulation genes in female adolescent mice offspring (Meng Z et al., 2018 [RefID 12708]). Fasn and Cd36 mRNA increased significantly, whereas the mRNA expression of Scd1, a key enzyme regulating formation of monounsaturated fatty acids (MUFA), decreased, resulting in a significant increase in two FFA (saturated fatty acid (SFA) C20:0 and MUFA C20:1n9).

The effects of BPA on lipid metabolism were studied using both adult C57BL/6 mice exposed to 50 µg/kg bw per day for 90 days and the human hepatocellular carcinoma cell line HepG2 (Lin Y et al., 2017 [RefID 4338]). Overall, it was concluded that BPA-induced NAFLD was caused by downregulation of the microRNA (miR)-192 both *in vivo* and *in vitro* resulting in increased SREBf1 expression in hepatocytes, thus, increasing triglyceride synthesis. However, these *in vitro* data were based on significant effects seen only above 100 nM (with 200 and 2000 nM).

Human liver HepG2 cells exposed to BPA (1 fM to 1 µM) showed an increase in triglyceride storage only with 1 pM but no change in glucose uptake (Hélies-Toussaint et al., 2014 [RefID 2676]). The effects were potentially linked to increased expression of early differentiation genes SREB1c, PPARγ and aP2, as well as ERRα and ERRγ genes.

In conclusion, the CEP Panel considered that there is substantial evidence supporting that BPA may increase fat accumulation in the liver based on the available studies on animals after developmental exposure or as adults as well as in cell models. The MoAs for increased fat deposition in the liver being put forward in these studies are that BPA is causing abnormal synthesis, retention or breakdown of lipid (with 5–10000 µg/kg bw per day of BPA), often via modulation of the PPARs and SREBPs genes and proteins (with 0.5–5000 µg/kg bw per day of BPA).

Glucose regulation

There was no human evidence available for the cluster glucose regulation. Thus, the overall likelihood of effects of BPA for this cluster was scored ALAN, based on the animal evidence.

The main function of β-cells in the islets of pancreas is to produce and secrete insulin, the hormone responsible for regulating levels of glucose in the blood. Several studies were concerned with BPA-induced β-cell dysfunction and disturbance of glucose homeostasis, with the underlying mechanisms possibly being loss of β-cell mass or modulation of insulin secretion from the β-cells.

After exposure of mice to BPA at 10 or 100 µg/kg bw per day s.c. (converted oral doses 2222 or 22220 µg/kg bw per day) during pregnancy (GD9–GD16), the offspring had decreased insulin secretion and reduced pancreatic β-cell mass (Alonso-Magdalena et al., 2015 [RefID 139]). Proliferation was

decreased together with decreased expression of the cell cycle activators cyclin D2 (*Ccnd2*) and cyclin-dependent kinase-4 (*Cdk-4*). In addition, β-cell apoptosis level and expression of the cell cycle inhibitors p16 and p53 were increased. In contrast, when female non-pregnant mice were treated with BPA at the same doses no effects on glucose metabolism or insulin sensitivity were observed.

BPA exposure (10 or 100 µg/kg bw per day s.c., converted oral doses 2222 or 22220 µg/kg bw per day) during pregnancy (GD9–GD16) affected pancreatic β-cell growth and function in mice offspring during early life (García-Arévalo et al., 2016 [RefID 2194]). BPA increased β-cell mass/area with hyperinsulinemia without insulin resistance and insulin over-secretion. Microarray analysis showed that BPA altered the expression of genes in the islets involved in β-cell growth regulation and β-cell proliferation. Excess of insulin signalling during early life may contribute to impaired glucose tolerance during adulthood and also obesity.

Pancreatic islets of adult F1 and F2 mice offspring from F0 dams exposed to doses of 10 or 10000 µg/kg bw per day of BPA in the diet from two weeks before mating and during gestation and lactation showed dose-specific and sex-specific effects (Bansal et al., 2017 [RefID 9499]). The high BPA dose impaired mitochondrial function, whereas the low dose reduced β-cell mass and increased β-cell death that persisted into the F2 generation. Increased insulin-like growth factor 2 (Igf2) expression persisted in the islets of male F1 and F2 offspring and was associated with altered DNA methylation. Thus, BPA exposure induced impaired glucose homeostasis in the F2 offspring through the transmission of sperm, possibly via either mitochondrial dysfunction and/or epigenetic modifications.

After BPA exposure at 100 µg/kg bw per day from GD7 to PND21, adolescent female mice offspring showed changes in expression of genes related to hepatic glucose metabolism, glycolysis and glucose transport [farnesoid X receptor (*Fxr*), small heterodimer partner (*Shp*), glucose-6-phosphatase (*G6Pase*), pyruvate kinase isozymes R/L (*Pklr*) and glycogen transporter gene (*Glut2*), and severely disturbed glucose homeostasis (Meng Z et al., 2018 [RefID 12708])].

In mouse pancreatic β-cells from wild-type and oestrogen receptor beta $(Erβ)^{-/-}$ mice, exposure to low BPA concentrations (100 pM and 1 nM) *in vitro* decreased Ca^{2+} entry via an ERβ-dependent pathway involving the transcriptional regulation of Cav2.3 ion channels, whereas high BPA concentrations (10 and 100 nM) had no such effects (Villar-Pazos et al., 2017 [RefID 13316]). Higher BPA concentrations (100 nM to 1 µM) involved both ERβ and Erα, which counteracted each other. ERα increased Ca^{2+} entry only in response to higher BPA concentrations (100 nM and 1 µM) after increasing Ca^{2+} currents in a pathway involving phosphoinositide 3-kinase (PI3K). Based on these results, BPA affected the pancreatic β-cell insulin content and secretion via oestrogen receptors ERα and Erβ actions outside the nucleus. The combined opposing effect of ERα and ERβ upon Ca^{2+} entry was hypothesised by the authors to potentially explain the NMDR relationship observed for BPA effects.

A link between BPA exposure and the potential risk of diabetes was reported by Perreault et al. (2013) [RefID 5797], who performed two separate experiments on BPA and effects on hepatic glucokinase activity, a glucose sensor in the body. In the first experiment, adult male mice were exposed by gavage once with 50 µg/kg bw of BPA. In the second experiment, adult male mice were exposed via drinking water to 50 µg/kg bw of BPA once daily for two weeks. The results showed that both a single dose and exposure over a two-week period to BPA reduced hepatic glucokinase activity over a range of tested glucose concentrations (0–20 mmol/L).

Whether BPA exposure at 40 µg/kg bw per day during gestation and lactation could disrupt glucose homeostasis was studied in F2 rat offspring (Li GQ et al., 2014a [RefID 4039]). The glucokinase (*Gck*) promoter in F2 hepatic tissue was completely methylated in all CpG sites compared with five unmethylated sites in controls. In the F1 sperm, the global DNA methylation was decreased. However, there was only one CpG site (−314) that was differently methylated between BPA and controls in sperm. The study supported the hypothesis that glucose metabolism disorders can be induced trans-generationally through epigenetic alterations, especially DNA methylation changes in sperm from as early as during paternal development.

There were also several studies that investigated the effect of BPA on insulin resistance and insulin signalling. Epigenetic effects from exposure of BPA (50 µg/kg bw per day) throughout gestation and lactation (GD0–PND21) in male rat F1 offspring were investigated by Ma et al. (2013) [RefID 4748]. The results showed insulin resistance caused by decreased global hepatic methylation accompanied by

overexpression of DNA methyltransferase 3B mRNA, in addition to promoter hypermethylation and reduction of expression of the hepatic *Gck* gene.

In four-week-old to six-week-old mice given 50 µg/kg bw per day of BPA for 12 weeks, serum insulin levels did not increase but glucose intolerance tended to increase in the growing males fed a HFD (Moon et al., 2015 [RefID 5195]). Altered serum adipocytokine level causing decreased phosphorylation of Akt, an important factor in the insulin signalling pathway, in skeletal muscle, was suggested as one mechanism by which BPA induced glucose intolerance and insulin resistance.

Treatment of human liver HepG2 cells with 100 nM BPA induced significantly decreased glucose consumption, impaired insulin signalling, increased pro-inflammatory cytokines and oxidative stress, and activated signalling pathways (Geng et al., 2017 [RefID 2242]). Inhibition of JNK and p38 pathways, but not ERK nor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathways, improved glucose consumption and insulin signalling, indicating a role of JNK/p38 activation in BPA-induced insulin resistance. In a later study with normal human hepatocyte LO2 cells treated with 100 nM BPA, inhibition of the JNK pathway, but not the p38 nor NF-κB pathways, improved glucose consumption and insulin signalling (Geng et al., 2018 [RefID 11987]).

The human pancreatic cell line (PANC-1) treated with 10 nM BPA had decreased secretion of active insulin and decreased mRNA expression of PCSK1, pro-protein convertase subtilisin/kexin type 1 (PC1/3), a pro-insulin-processing enzyme that regulates insulin biosynthesis (Menale et al., 2015 [RefID 5027]).

Carchia et al. (2015) [RefID 852] used a toxicogenomic approach to investigate the mechanisms of effects of 1 nM of BPA in cultured *ex vivo* murine primary hepatocytes and pancreatic islets and further investigated pancreatic islet mitochondrial dysfunction, cellular pathways and response to glucose stress. *In vivo* studies on mice involved the induction of hyperglycaemia via a single dose of STZ and was used to confirm *in vitro* findings. Twenty-nine inhibited genes were identified in the islets and none in the hepatocytes. Although their expression was slightly altered, their impaired cellular level as a whole resulted in specific phenotypic changes. It was concluded that BPA affected two complementary mechanisms on mitochondria: enhancement of oxidative stress and decreased ROS scavenging systems, promoting apoptosis. The data suggested a multifactorial mechanism for BPA toxicity in pancreatic islets involving mitochondria dysfunction and NF-κB activation.

Effects of BPA on metabolism and metabolomics were examined in several publications. In a study in pre-adipocytes and differentiated adipocytes obtained from non-obese children (mean age of 10.5 ± 2.3 years), Menale et al. (2017) [RefID 5026] showed that BPA could alter adipocyte metabolism contributing to metabolic dysfunction, such as insulin resistance, by altering expression of adiponectin (mRNA significantly decreased with 10 and 100 nM) and resistin (induced with 1, 10 and 100 nM).

BPA exposure (1 or 10 µg/mL, converted oral doses 35.7 or 357 µg/kg bw per day) in drinking water from GD6 to PND21 had long-term deleterious effects on body weight and glucose metabolism in female adolescent rats, which appeared to be associated with the downregulated expression of ZAG and adiponectin mRNA and proteins (Zhang et al., 2013 [RefID 8798]). In a further study with the same experimental design, abnormal glucose metabolism and insulin resistance were seen with both doses at PND100, but only with the high dose at PND50 (Song SZ et al., 2014 [RefID 6829]). The insulin resistance was associated with decreased adiponectin production and increased oxidative damage.

After short-term (20 min or 48 h, 100–1000 nM) and long-term (four weeks, 250 nM) exposure to BPA in the oestrogen-dependent human breast cancer cell line MCF-7, BPA increased glucose uptake only with 250 and 500 nM after 20 min and had no effects after 48 h, and decreased glucose with 250 nM after long-term exposure. BPA did not affect the level of lactate, a measure of glucose oxidation efficiency, at any exposure times (Norberto et al., 2017 [RefID 5440]).

Low concentrations of BPA (0.1 nM to 100 µM) reduced adipocyte-specific gene expression, but did not accelerate adipogenesis, in human adipose-derived stromal cells or murine 3T3-L1 or C3H10T1/2 cells (De Filippis et al., 2018 [RefID 11796]). The data indicated that chronic BPA exposure was unlikely to directly cause the increase in fat tissue, at least mediated through direct adipocyte differentiation. BPA exposure during differentiation led to generation of dysfunctional adipocytes with reduced insulin-stimulated glucose uptake and protein kinase B (Akt) phosphorylation associated with increased expression of pro-inflammatory cytokines such as TNF-α and IL-6, without affecting expression of

adiponectin. Thus, chronic BPA exposure appeared to contribute to chronic, low grade inflammation, which subsequently may affect insulin sensitivity in adipose tissue independent of adipogenesis. This suggest that BPA may actually be a more potent diabetogen than obesogen.

A metabolomics study with ^{1}H-nuclear magnetic resonance (NMR)-based spectroscopy examined metabolic shifts induced by perinatal (GD8–LD16) exposure to BPA (0.025, 0.25 or 25 µg/kg bw per day s.c., converted oral doses 5.6, 55.6 or 5555 µg/kg bw per day) in male mice offspring (Cabaton et al., 2013 [RefID 776]). Variations in glucose, pyruvate, some amino acids and neurotransmitters (γ-aminobutyric acid and GLU) were found, mainly affecting energy metabolism and brain function. The shift observed for glucose would likely be involved in the disruption of pyruvate biosynthesis through glycolysis. In a later *in vitro* study, Cabaton et al. (2018) [RefID 11650] showed by bioinformatics (metabolic network modelling) that BPA could modulate major metabolic routes involved in cellular functioning and detoxification processes in human hepatoblastoma HepG2 cells. While having some commonality with E2, BPA had a distinct activity possibly reflecting a ligand-specific conformation change of the activated ER. The results also indicated that metabolism of branched-chain amino acids might be modulated by BPA with possible consequences in the promotion of protein synthesis and turnover, signalling pathways and the metabolism of glucose.

Serum ^{1}H-NMR metabolomics studies of effects of BPA exposure at 10 µg/kg bw per day s.c. (converted oral doses 2222 µg/kg bw per day) during gestation (GD10–GD18) on male mice offspring generally did not change expression of genes involved in glucose homeostasis compared with controls, except for an upregulation of hepatic glycogen synthase 2 (Gys2) (Wang D et al., 2018 [RefID 13341]).

Using ^{1}H-NMR-based metabolomics, with F0 rat dams exposed to low doses of BPA (0.25, 2.5, 25 and 250 µg/kg bw s.c., converted oral doses 8.9, 89.3, 892.5 and 8925 µg/kg bw per day) from GD9 to PND16, Tremblay-Franco et al. (2015) [RefID 7285] demonstrated that metabolism of glucose, lactate and fatty acids was modified over time in BPA-exposed female offspring. The data showed that BPA may modulate energy metabolism and neurotransmitter signalling.

More specific MoAss by BPA, such as modulation of the homeodomain-containing transcription factor pancreatic and duodenal homeobox 1 (Pdx-1), were demonstrated. Pdx-1 is a major regulator of transcription in pancreatic cells. Glucose regulates insulin gene transcription through Pdx-1. Thus, the modulation of Pdx-1 may disturb the glucose homeostasis (Chang et al., 2016 [RefID 965]).

Maternal exposure to BPA at 10 µg/kg bw per day during gestation and lactation reduced pancreatic β-cell mass at birth in rat offspring (Chang et al., 2016 [RefID 965]). This was possibly caused by reduction of Pdx-1^{+} progenitor cells during fetal development by alteration of histone modifications (histones H3 and H4 deacetylation, demethylation of histone 3 lysine 4 (H3K4) and methylation of histone 3 lysine 9 (H3K9)) in the promoter of Pdx-1, potentially increasing susceptibility to glucose intolerance in later life.

Insulin-like growth factor 2 (Igf2) is a protein hormone that is structurally similar to insulin and is acting as a growth-regulating and mitogenic factor. Several studies examined changes in expression of Igf2 after BPA exposure, possibly involving epigenetic mechanisms, which may lead to glucose intolerance and β-cell dysfunction.

Early-life BPA exposure from two weeks before mating and through gestation and lactation to 10 or 10000 µg/kg bw per day perturbed metabolic health across F1 and F2 generations in mice through stable inheritance of increased DNA methylation at the *Igf2* differentially methylated region 1 (Susiarjo et al., 2015 [RefID 7022]).

Exposure of F0 mice to 10 or 10000 µg/kg bw per day of BPA from two weeks before mating and during gestation and lactation lead to induced islet inflammation in male F1 offspring that persisted into the F2 generation (Bansal et al., 2017 [RefID 9499]). The higher dose impaired mitochondrial function, whereas the lower dose reduced β-cell mass and increased β-cell death. Increased Igf2 expression persisted in the islets of male F1 and F2 offspring and was associated with altered DNA methylation.

Exposure of rats to 40 µg/kg bw per day of BPA during gestation and lactation (GD0–PND21) resulted in generational transmission of glucose intolerance and β-cell dysfunction in rat offspring through the male germline from F1 to F2 generation (Mao et al., 2015 [RefID 4864]). This transfer was associated with hypermethylation of Igf2 in islets. In a subsequent study with the same experimental design, Mao

et al. (2017) [RefID 4865] showed that BPA-induced pancreatic impairments in the rat offspring were associated with DNA hypermethylation and low expression of Igf2 in the islets, and that maternal dietary folate supplementation counteracted these effects, upregulating Igf2 expression.

Some available studies also suggested that endoplasmic reticulum stress contributes to pancreatic β-cell loss and insulin resistance, and thus, may have a role in the pathogenesis of diabetes.

In pancreatic β-cells, which produce and secrete insulin, Ca^{2+} signals contribute to insulin production and secretion. After BPA exposure (5,000 µg/kg bw) for five days of adult male mice using the STZ-induced T1DM model, Ahn et al. (2018) [RefID 11452] found that expression of genes involved in transporting Ca^{2+} to the cytosol and endoplasmic reticulum decreased while the expression of genes affecting the removal of Ca^{2+} from the cytosol and endoplasmic reticulum increased. Depletion of calcium from the endoplasmic reticulum by BPA lead to endoplasmic reticulum stress and could induce insulin resistance in this model, confirmed by decreased expression of insulin-responsive genes, such as glucose transporter 4 (*Glut4*) and insulin response gene (*Irs2*).

Long-term (35 weeks) paternal BPA exposure (50 µg/kg per day) via the diet disrupted glucose homeostasis and pancreatic function in rat sires and offspring (Ding et al., 2014 [RefID 1620]). A HFD aggravated the adverse effects. The results indicated that autophagy was activated as a defensive response for survival during endoplasmic reticulum stress. Upregulated autophagy in the islets may deal with accumulation of misfolded and aggravated proteins and remove the abnormal unnecessary cellular components in β-cells. Increased protein expression of microtubule-associated proteins 1A/1B light chain 3 (LC3) in the pancreas indicated that autophagy was essential for maintaining the function of pancreas and that pancreatic β-cells may be protected by upregulated autophagy under insulin-resistant states caused by BPA and HFD. Thus, BPA may cause β-cell dysfunction via endoplasmic reticulum stress and enhanced autophagy.

In conclusion, the CEP Panel considered that there is substantial evidence supporting that BPA may negatively affect glucose regulation based on the available studies on animals exposed during development, as adolescents or as adults, as well as in cell models. Some effects were also shown to be transferred across generations from F1 to F2 via the male germline. One MoA for disturbed glucose regulation being put forward in these studies was effects on β-cell dysfunction and disturbance of glucose homeostasis, either via loss of β-cell mass or modulation of insulin secretion (with 10–22,220 µg/kg bw of BPA). Several studies indicated effects of BPA on insulin resistance and insulin signalling, sometimes shown to include circulation of cytokines, which may lead to disruption of glucose transport, uptake and metabolism, or enhanced oxidative stress (with 50 µg/kg bw of BPA). Some metabolomics and other studies found various effects of BPA (5.6–8,925 µg/kg bw) on metabolism. More specific effects of BPA were modulation of the homeodomain-containing transcription factor pancreatic and duodenal homeobox 1 (Pdx-1) (10 µg/kg bw), which plays a key role in pancreatic development and β-cell function, changes in expression of insulin-like growth factor 2 (Igf2) (10–10000 µg/kg bw), which may lead to glucose intolerance and β-cell dysfunction, or via endoplasmic reticulum stress (with 50–5000 µg/kg bw of BPA). Thus, apparently BPA may disturb glucose regulation via several different mechanisms, and some evidence may suggest that BPA may be a more potent diabetogen than obesogen. Unfortunately, only very limited evidence was available in the WoE for type 1 diabetes mellitus (see chapter on the cluster T1DM)) and none for type 2 diabetes mellitus, the last being the most common form of diabetes in humans.

Blood lipids

There was no human evidence available for the cluster Blood Lipids. Thus, the overall likelihood of effects of BPA for this cluster was scored ALAN, based on the animal evidence.

Hypercholesterolaemia is a condition which is thought to be caused by a susceptible genotype. However, the involved genes are not yet identified. The condition is aggravated by one or more factors, including a so-called atherogenic diet, characterised by higher than needed intake of saturated fat, trans-fat and, to a lesser extent, cholesterol, obesity and sedentary lifestyle. Triglycerides in the blood (serum, plasma) are a mixture of triglycerides that are taken up from the diet, by higher than needed intake of saturated fat and trans-fat and triglycerides which are synthesised in several organs (liver, kidney, muscle and adipose tissue). Hypertriglyceridemia is often caused or worsened by the same

factors as hypercholesterolaemia, e.g. sedentary lifestyle, obesity and type 2 diabetes mellitus (Mach et al., 2020).

Feng et al. (2017) [RefID 1959] investigated the effect of BPA (0.1–10 nM) on the absorption of cholesterol in an *in vitro* Caco-2 intestinal cell model. BPA at 1 and 10 nM increased the absorption of cholesterol which might be related to increased Niemann–Pick C1-like 1 (NPC1L1) protein. A slight increase of sterol regulatory element-binding protein-2 (SREBP-2) which regulates the expression of NPC1L1 protein might be responsible for this finding. However, the mechanism by which BPA would influence SREBP-2 was not elucidated.

In the study of Marmugi et al. (2014) [RefID 4884], a wide array of gene expression was investigated in male mice (dose range 5, 50, 500 or 5000 µg/kg bw per day of BPA for 31 weeks) (described above in the cluster fat deposition in the liver). The results of Feng et al. (2017) [RefID 1959] hint to an increased absorption of cholesterol due to BPA in µg/kg bw per day concentrations higher than 1 nM (effects found with 1 and 10 nM, but not with 0.1 nM). The study of Marmugi et al. (2014) [RefID 4884] gives some indications on elevated expression of genes involved in cholesterol synthesis. Whereas mRNA expression of Hmgcr, the classic control point in the synthesis, was increased, results of the second rate-limiting enzyme, squalene monooxygenase, were not reported (Sharpe et al., 2020). Thus, there is some indication for an influence of BPA on the mRNA expression of enzymes involved in cholesterol synthesis.

Regarding triglycerides, the only mechanistic study by Bucher et al. (2017) [RefID 743] could not demonstrate an effect on gene expression of eight enzymes involved in lipid homeostasis and thus, could not contribute to provide a MoA for elevated triglycerides.

In conclusion, increased absorption of cholesterol under the influence of BPA in an *in vitro* model for absorption was demonstrated, involving NPC1L1 protein and SREBP-2. However, the initiating molecular event is not identified. Regarding triglycerides, a convincing MoA was not demonstrated.

Uric acid

There was no human evidence available for the cluster uric acid. Thus, the overall likelihood of effects of BPA for this cluster was scored Likely, based on the animal evidence. The evaluation was based on three measurements reported in the same publication, scored Tier 2, in which the results of studies in two mice strains and in rats were reported. The studies were performed in males only. Uric acid concentrations were measured in liver, (in one strain) and serum (in two strains) of mice showing a monotonic dose–response (MDR) for three doses. One study was on rats (measured only in serum with results of only the highest dose reported).

In the same publication (Ma et al., 2018 [RefID 12637]), the authors had examined the MoA for increased levels of uric acid. The effect of BPA on the xanthine oxidase (XO) enzyme was studied *ex vivo* in mouse primary hepatocytes and homogenised liver (at concentrations of 100 or 1000 nM BPA), *in vitro* using a transfected human embryonic kidney cell line HEK-293FT and *in silico* by modelling BPA binding sites. The overall conclusion was that BPA increased the formation of hepatic uric acid via increasing the activity of xanthinoxidase. Xanthinoxidase is involved in purine metabolism, catalysing the formation of hypoxanthine, which is a product in the catabolism of adenosine to xanthine. Xanthine is itself a product in the catabolism of guanosine. In a next step, xanthinoxidase catalyses the oxidation of xanthine to uric acid. By molecular modelling, it was made probable that BPA may bind to the enzyme, thus, leading to changes in enzyme conformity with resulting increased activity. Thus, this was considered a plausible MoA for how BPA may increase the levels of uric acid in the liver.

Type 1 diabetes mellitus (*T1DM*)

There was no human evidence available for the cluster T1DM. Thus, the overall likelihood of effects of BPA for this cluster was scored ALAN, based on the animal evidence.

In the study by Cetkovic-Cvrlje et al. (2017) [RefID 916], doses of 160 and 1,600 µg/kg bw per day for 11 and 50 days to adult mice, respectively, were studied for effects on splenic cells *ex vivo*. On day 11, only for the low dose, a decrease in $CD3^+$, $CD4^+$ and $CD8^+$ cells was observed and no effects of the high dose, whereas the total cell counts and response to stimulation were not impaired in either dose groups. Inconsistent effects on cytokines (IL-6, IFN-γ, TNF-α) with increase in the low dose and

decrease in the high dose were noted. On day 50, proliferation of unstimulated T cells was increased at the high dose only, and after stimulation with the mitogen concanavalin A, T-cell proliferation was decreased with the low dose only. No effects on cytokines were noted.

In the study by Ahn et al. (2018) [RefID 11452], BPA at 5 mg/kg bw per day given to adult male mice lead to survival of pancreatic β-cells against the effect of STZ, showed increased insulin secretion and mitigated STZ-induced oxidative stress. Conversely, BPA induced endoplasmic reticulum stress and lead to insulin resistance especially in the STZ-induced T1DM model by disrupting calcium homeostasis.

The pathogenesis of type 1 diabetes mellitus is known to be the results of T-cell-mediated autoimmune destruction of pancreatic β-cells (Atkinson et al., 2011). Given that background, the results from the two MoA studies do not shed light on the mechanism by which BPA might enhance the effect of STZ. In the study of Ahn et al. (2018) [RefID 11452], BPA led to survival of pancreatic β-cells although STZ was given, and in the study of Cetkovic-Cvrlje et al. (2017) [RefID 916], no consistent effect on T cells was shown, although a higher incidence of type 1 diabetes was already observed on day 12 in both dose group indicating the effect of STZ.

Bodin et al. (2014) [RefID 623] investigated if trans-maternal BPA exposure accelerated T1DM development in NOD mice exposed to 100, 1000 or 10000 µg BPA/L in drinking water throughout mating, gestation and lactation (estimated doses approximately 30, 300 or 3000 µg/kg bw per day during gestation, 4,500 µg/kg bw per day for the highest dose during lactation). No effects of BPA were found on serum autoantibodies against insulin, GAD65 or HSP60, or on testosterone levels. With the highest dose, increased number of apoptotic cells, reduction in tissue resident macrophages and increase in regulatory T cells were observed in islets before insulitis development in the trans-maternally exposed offspring. The detectable apoptotic cells were identified as mostly glucagon producing α-cells (increased with the two highest doses) but also tissue resident macrophages and β-cells (both increased with the highest dose). In the local (pancreatic) lymph node neither regulatory T cells nor natural killer T cells were affected by maternal BPA exposure. Maternal BPA exposure also induced systemic immune changes in offspring, as evidenced by alterations in lipopolysaccharide-induced (increased IL-2, IL-10 and IL-17 with the highest dose) and concanavalin A-induced (reduced IL-2 with highest dose) cytokine secretion in splenocytes. Thus, trans-maternal BPA exposure, *in utero* and through lactation, increased the severity of insulitis at 11 weeks of age and accelerated the spontaneous development of diabetes at 20 weeks of age in female NOD mice, which appeared to be related to early-life modulatory effects on the immune system.

In conclusion, a convincing MoA of BPA's contribution to the development of T1DM in the STZ-induced T1DM model was not demonstrated, lowering the probability of BPA to elicit type 1 diabetes mellitus. However, in the NOD mouse model, trans-maternal BPA exposure, *in utero* and through lactation, appeared to be related to early-life modulatory effects on the immune system.

Type 2 diabetes mellitus (*T2DM*)

There was no animal evidence available for the cluster T2DM. Thus, the overall likelihood of effects of BPA for this cluster was scored ALAN, based on one human study. Bi et al. (2016) [RefID 571] conducted a cohort study in 2009, using a Chinese population of 2209 non-diabetic middle-aged and elderly subjects. The study sought to prospectively investigate associations of urinary BPA with incident T2DM risk and the longitudinal changes in glycaemic traits, particularly examining the interaction between genetic background and BPA exposure on the associations. Four years after the baseline study, the subjects were followed up and the population was found to include 242 diabetes cases. A genetic risk score for T2DM based on 34 common variants of single-nucleotide polymorphisms (SNPs) that was identified in genome-wide association studies and performed or validated in East Asians was created. The study found that T2DM genetic susceptibility significantly modulated the association of BPA exposure with longitudinal increase in fasting plasma glucose levels.

3.1.4.5. Conclusion on hazard identification for Metabolic effects of BPA

In EFSA 2015 opinion (EFSA CEF Panel, 2015), it was stated regarding human studies that, because of the limitations of using urinary BPA concentrations as a surrogate of exposure, the problems of interrelated dietary exposures, the mostly cross-sectional study designs and inconsistency of the results between cross-sectional and prospective studies, limited conclusions could be drawn concerning the

relationship between BPA exposure and the reported findings. Notwithstanding, there were indications from cross-sectional studies that higher BPA may be associated with increased body mass in children and indication from a prospective study that pre-natal 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 were no indications of note for other hormonal or metabolic endpoints.

The overall conclusion on likelihood of Metabolic effects on animals exposed post-natally was that the evidence for associations between BPA exposure and these metabolic effects was inconsistent. The CEF Panel concluded further that there was 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 was causing diabetes, insulin resistance and increases in weight (obesogenic effect) longer term. The overall conclusion on likelihood for metabolic effects and the scoring of influence on likelihood in animals exposed pre-natally were the same as for post-natal exposure.

The CEF Panel assigned a likelihood level of ALAN in the WoE of metabolic effects of BPA based on the human and animal evidence, and, thus, the CEF Panel did not take any metabolic effects forward for risk characterisation (EFSA CEF Panel, 2015).

In accordance with the results from 2015, in the current risk assessment of BPA, no clear causal association (scored ALAN) was found for effects of BPA on obesity based on WoE of human and animal data, including data on effects of BPA on body weight, BMI, body fat mass, visceral and non-visceral adipocyte size. However, substantial amounts of supporting evidence for plausible effects of BPA on obesity were available from MoA data, mostly from animal and *in vitro* studies. This included changes in expression of adipogenic genes and their proteins, leading to induced differentiation of pre-adipocytes into mature, lipid-accumulating adipocytes, often regulated via epigenetic effects such as DNA methylation. In addition, single studies indicated that BPA may affect obesity by disturbing immune homeostasis and gut dysbiosis or may have effects on the signalling systems of the brain regulating appetite and food intake.

Increased insulin level was considered a likely effect of BPA in the growth phase of animals. For ipGTT and β-cell morphometry, the evidence was also considered likely after indirect (germline) exposure in animals. However, when considering also the effects of BPA on glucose level, glucose tolerance tests via several other administration routes (i.v., oral or unknown), ipITT, pancreas weight and α-cell and β-cell morphometry (indicating glucagon and insulin production, respectively), based on the overall WoE in animals, no clear causal association (scored ALAN) was found for effects of BPA on glucose regulation. However, also for glucose regulation, substantial amounts of supporting evidence based on MoA studies in animals and *in vitro* were available, which included BPA-induced β-cell dysfunction and disturbance of glucose homeostasis, either via loss of β-cell mass or modulation of insulin secretion, effects on insulin resistance and insulin signalling, sometimes including circulation of cytokines, which may lead to disruption of glucose transport, uptake and metabolism, or enhanced oxidative stress, and effects on metabolism and metabolomics. In addition, more specific MoAs of BPA, such as modulation of the homeodomain-containing transcription factor pancreatic and duodenal homeobox 1 (Pdx-1), changed expression of insulin-like growth factor 2 (Igf2) or endoplasmic reticulum stress, were indicated.

Based on the overall WoE in animals for effects of BPA on liver cholesterol and liver triglycerides, no clear causal association (scored ALAN) was found for effects of BPA on fat deposition in the liver. Substantial supportive MoA data was available from animals and *in vitro* studies, involving abnormal synthesis, retention or breakdown of lipid, often via PPARs and SREBPs.

For effects of BPA on blood lipids and T1DM, based on the WoE in animals no clear causal association (scored ALAN) was found. T2DM was also scored ALAN, based on human evidence. The MoA data were very limited and the results on T1DM depended on the animal model used.

Based on the WoE of the data available, causal effects of BPA on the thyroid (human and animal data), gestational diabetes mellitus (human data), cardiometabolic effects (human data) and other metabolic hormones (animal data) were judged as Not Likely.

Thus, the results in the present opinion are quite consistent with the results in the BPA opinion from 2015 for the same endpoints, even when a much higher number of metabolic studies are included in the present assessment.

In the 2015 EFSA opinion (EFSA CEF Panel, 2015), the endpoint uric acid was not identified in the available literature. In the current risk assessment, increased uric acid measured in liver and serum in adult animals was judged as Likely. The MoA data in the animals showed that BPA could increase the formation of hepatic uric acid by increasing the activity of the enzyme xanthinoxidase, which catalyses the conversion of purines hypoxanthine and xanthine into uric acid.

Overall, the CEP Panel considers that a hazard exists for effect of BPA on increasing uric acid level. Using a WoE approach, the CEP Panel assigned a likelihood level of Likely to the effect of BPA on this parameter. Therefore, this endpoint was brought forward for BMD analysis (see Chapter 3.2.1).

3.1.5. Neurotoxicity and developmental neurotoxicity

3.1.5.1. Epidemiological studies

For the HOC Neurotoxicity and developmental neurotoxicity, a total of 18 studies was appraised by the CEP Panel. The details of the appraisals (internal validity) are reported in Annex B.

Identification of the clusters to be considered for WoE

On the basis on the approach described in Chapter 2.3.2 'Definition of Health Outcome Categories and Clusters ', the following Clusters (C) and Exposure periods (Exp) were brought forward to WoE analysis:

- C: Neurodevelopment
  - Exp: Pregnancy and Childhood.

WoE of the relevant clusters

The main information extracted from the studies included in relevant clusters in the HOC Neurotoxicity and developmental neurotoxicity are summarised in Annex C. The outcome of the weight of the evidence is described in the text below and presented in a tabulated format in Annex D.

Cluster Neurodevelopment

Exposure during pregnancy and childhood

A large number of endpoints related to neurodevelopment were studied in the 18 publications pertaining to 14 longitudinal studies all assessing exposure during pregnancy (Braun et al., 2014a [RefID 707]; Evans et al., 2014 [RefID 1862]; Casas et al., 2015 [RefID 878]; Roen et al., 2015 [RefID 6253]; Perera et al., 2016 [RefID 5773]; Braun et al., 2017a [RefID 704]; Braun et al., 2017c [RefID 710][18]; Braun et al., 2017d [RefID 712]; Giesbrecht et al., 2017 [RefID 2272]; Lim et al., 2017 [RefID 4285]; Lin CC et al., 2017 [RefID 4292]; Minatoya et al., 2017a [RefID 5104]; Philippat et al., 2017 [RefID 5822]; Stacy et al., 2017 [RefID 6876]; Ghassabian et al., 2018 [RefID 11997]; Kim et al., 2018a [RefID 11136]; Minatoya et al., 2018 [RefID 12732]; Nakiwala et al., 2018 [RefID 12789]).

BPA exposure was measured via a single spot urine sample in all studies and varied between studies. The populations under study were of comparable sample size but varied in their characteristics; there were eight studies including a European-descent population. The observed heterogeneity for endpoint definitions was considerable including a large number of questionnaires evaluating cognition, behaviour, intellectual ability and psychomotor development. No statistically significant associations were observed in more than one study. Thus, the currently available longitudinal epidemiological evidence is characterised by a small number of studies, suboptimal exposure assessment and considerable heterogeneity in the assessed populations, exposure levels and endpoints. In summary, the currently

[18] This study considered as a cross-sectional study in the EFSA supporting publication 'Implementation of the evidence-based risk assessment for the re-evaluation of Bisphenol A: preparatory work on cross-sectional studies' (AERU, University of Hertfordshire, 2020) was recategorized by the EFSA WG on BPA re-evaluation as a cohort study and therefore considered in the WoE assessment.

available epidemiological evidence does not put forward an endpoint related to neurodevelopment as a critical one for risk assessment.

In the text below, the assessed longitudinal studies are described in brief. Their detailed description and risk of bias assessment are provided in Annexes C and D.

The HOME study is a birth cohort conducted in the USA (Braun et al., 2017b). A total of 389 pregnant women were enrolled between March 2003 and February 2006 in Cincinnati, Ohio, who delivered live singleton infants. Pre-natal exposure assessment was done at around 16 weeks of gestation. Post-natal exposure assessment was done at 1, 2, 3, 4 and 5 years of age. Median maternal urinary BPA concentrations during pregnancy were 2.0 mg/g creatinine (range: 0.4–49). Children were followed up at 1, 2, 3, 4 and 5 years of age for neurodevelopment, physical growth and health conditions. Three publications report on the results of various associations pertinent to the present Opinion. Braun et al. (2014a) [RefID 707] investigated the association between pre-natal exposure to BPA (n = 175) and autistic behaviours at 4 and 5 years (Social Responsiveness Scale, SRS). No statistically significant association was observed. Braun et al. (2017d) [RefID 712] assessed the association between pre-natal BPA exposure (16, 26 weeks) and neurodevelopment until 8 years old (n = 229). The included neurodevelopment endpoints pertained to behaviour (BASC-2), mental and psychomotor development (Bayley Scales of Infant Development-II, BSID-II) and child cognitive abilities (Wechsler primary and preschool scale of intelligence–III (WPPSI-III), Wechsler intelligence scale for children IV (WISC-IV)). Overall, no statistically significant associations were observed. In girls, each 10-fold increase in maternal urinary BPA concentrations was associated with a 5.9-point increase (95% CI: 1.1, 11) in BASC-2 externalising scores and nearly 6-times the risk of having a score 60 (RR = 5.8; 95% CI: 1.7, 20). Finally, Braun et al. (2017a) [RefID 704] assessed children's visual-spatial abilities at 8 years of age using the Virtual Morris Water Maze (VMWM), a computerised version of the rodent Morris water maze (MWM). Pre-natal BPA exposure was not associated with VMWM performance.

Braun et al. (2017c) [RefID 710] in the MIREC birth cohort assessed the association between pre-natal BPA exposure at 12 weeks of gestation and child neurobehaviour at 3 years of age (n = 812; WPPSI-III, BRIEF-P, BASC-2, Social responsiveness scaleE–2/SRS-2). Overall, while BPA exposure was not associated with WPPSI-III scores, the BASC-2 or BRIEF-P scales, a statistically significant association was observed for the SRS-2 scores (b = 0:3; 95% CI: 0, 0.7).

The EDEN study (Philippat et al., 2017 [RefID 5822]) is another birth cohort conducted in France between February 2003 and January 2006 (n = 529). BPA and 19 additional phthalate metabolites and phenols (four parabens, benzophenone-3, two dichlorophenols, triclosan) were measured in spot urine samples collected during pregnancy among mothers who delivered a boy. Behaviour was investigated using the Strength and Difficulties Questionnaire (SDQ) at 3 and 5 years of age. No overall statistically significant association was observed.

Nakiwala et al. (2018) [RefID 12789] assessed verbal and performance IQ at 5–6 years (WPPSI). No statistically significant association was observed.

The Columbia Center for Children's Environmental Health (CCCEH, n = 727) is a birth cohort study recruiting African-American and Dominican pregnant women between 1998 and 2006 in USA. BPA was quantified in maternal urine collected during the third trimester of pregnancy and in child urine collected at ages 3 and 5 years. Neurodevelopment was assessed periodically using the Revised Children's Manifest Anxiety Scale (RCMAS) and Children's Depression Rating Scale-Revised (CDRS) at 10–12 years. Two CCCEH publications are included in the present opinion. Roen et al. (2015) [RefID 6253] and Perera et al. (2016) [RefID 5773] included 239 children in their assessment and found no statistically significant associations between pre-natal BPA and the scales' overall score for depression and anxiety. Among the numerous analyses performed, statistically significant associations were found for boys for the overall score results.

Evans et al. (2014) [RefID 1862] in the SSF II study in USA evaluated pre-natal BPA exposure in relation to child behaviour at 6–10 years (Child Behaviour Checklist (CBCL), n = 153). No statistically significant association was observed.

Casas et al. (2015) [RefID 878] reporting on the INMA birth cohort in Spain evaluated the association between pre-natal BPA and cognitive and psychomotor development at 1 and 4 years (Bayley Scales of Infant Development BSID, MCSA) and attention deficit hyperactivity disorder (ADHD) symptoms

(ADHD-DSM-IV) and other behavioural problems (CPRS, SDQ) at 4 and 7 years. At 1 year of age, exposure in the highest BPA tertile was associated with a reduction of psychomotor scores (BSID, T3 vs T1, β = −4.28 points, 95% CI −8.15 to −0.41). At 4 years, BPA exposure was associated with an increased risk of ADHD hyperactivity symptoms (IRR per log10 BPA increase 1.72; 95% CI 1.08, 2.73) both overall and in boys. No statistically significant association was observed for the remaining endpoints.

Lim et al. (2017) [RefID 4285] in the Environment and Development of Children study in Korea evaluated pre-natal BPA exposure and neurobehaviour at 4 years (K-SCQ, n = 304). No statistically significant association was observed for the prospective component of the study.

Lin CC et al. (2017) [RefID 4292] in the TBP birth cohort in China assessed pre-natal BPA (cord blood, n = 208) in relation to neurodevelopment at 2 and 7 years (Comprehensive Developmental Inventory for Infants and Toddlers (CDIIT), WISC-IV). In the WISC-IV neurocognitive assessment, a significant negative association was found for the overall scale as well as for various scale domains.

Minatoya et al. (2017a) [RefID 5104] in the Hokkaido Study on Environment and Children's Health Study in Japan investigated the association between pre-natal BPA levels (cord blood) and neurodevelopment up until 3.5 years (CBCL, K-ABC, BSID-II, n = 285). Although no overall statistically significant findings were reported among the numerous analyses performed, cord blood BPA concentration was statistically significantly positively associated only with CBCL development problems score (β = 2.60, 95% CI: 0.15, 5.06). In another publication of the same study, (Minatoya et al., 2018 [RefID 12732]) examined the association between maternal (1st trimester) BPA exposure and behaviour at 5 years using the Strengths and Difficulties Questionnaire (SDQ) in 458 children. The median concentration of BPA was 0.062 ng/ml and no overall statistically significant findings were reported. Among the numerous analyses performed, BPA levels were associated with an increased risk of prosocial behaviour (OR = 1.46, 95% CI 1.04–2.06).

Kim et al. (2018a) [RefID 11136] in the Korean CHECK birth cohort (n = 140) investigated the association between four phthalates, BPA, three heavy metals, 19 polychlorinated biphenyls (PCBs), 19 organochlorine pesticides and 19 polybrominated diphenyl ethers, and early neurodevelopment (13–24 months of age). For the endpoint assessment the following tools were used: BSID-II, Social maturity scale (SMS) and CBCL. No statistically significant associations were observed for BPA in the overall cohort.

Ghassabian et al. (2018) [RefID 11997] in the Upstate KIDS birth cohort investigated the association between perfluorooctane sulfonic acid (PFOS), perfluorooctanoic acid (PFOA) and BPA (Gathrie cards) and behaviour at age 7 (Strengths and Difficulties Questionnaire, n = 650 singletons to 138 twins). The median (interquartile range) of BPA was 7.93 ng/ml (10.79). No statistically significant association was observed for BPA and total behavioural difficulties (continuous and categorical analyses).

Giesbrecht et al. (2017) [RefID 2272] in the Alberta Pregnancy Outcomes and Nutrition study (birth cohort, n = 132) examined the association between pre-natal maternal urinary BPA concentration and cortisol and cortisol reactivity at age 3 months. The association between maternal total BPA concentration and baseline infant cortisol (β = 0.13, 95% CI: −0.01, 0.28) or cortisol reactivity (−18% decrease per hour for females per 10-fold increase in BPA, 95% CI: −35, 3) was not significant when infant sex and creatinine was considered in the model.

Four studies also assessed BPA exposure during childhood (Roen et al., 2015 [RefID 6253]; Perera et al., 2016 [RefID 5773]; Stacy et al., 2017 [RefID 6876]; Kim et al., 2018a [RefID 11136]).

Perera et al. (2016) [RefID 5773] included 239 children in their assessment and found no statistically significant associations between post-natal BPA and the scales' overall score for depression and anxiety. Roen et al. (2015) [RefID 6253] included 250 children in their assessment and found statistically significant associations between post-natal BPA and internalising and externalising scores on the CBCL. Girls and boys were respectively showing increasing and decreasing problems. Stacy et al. (2017) [RefID 6876] in a HOME study publication attempted to extend these findings and to identify potential windows of vulnerability using repeated measures of pre-natal BPA exposures. Among all children, there was not strong evidence that the associations between BPA and neurobehaviour varied by the timing of exposure (Visit × BPA p-values ≥ 0.16). Finally, Kim et al. (2018a) [RefID 11136] in the Korean CHECK birth cohort investigated the association between post-natal BPA exposure via breast milk

sampling at 30 days after delivery (n = 73) and early neurodevelopment (13–24 months of age; BSID-II, SMS, CBCL). No statistically significant associations were observed for BPA in the overall cohort.

Overall conclusions

On the basis of the above, the CEP Panel concluded that the evidence for an association between BPA exposure and impaired neurodevelopment is Not Likely.

Cross-sectional studies

Ten cross-sectional studies investigated BPA exposure and childhood behaviour, learning disabilities and autism (Findlay and Kohen, 2015 [RefID 2029]; Stein et al., 2015 [RefID 6899]; Arbuckle et al., 2016 [RefID 237]; Kardas et al., 2016 [RefID 3383]; Kondolot et al., 2016 [RefID 3646]; Perez-Lobato et al., 2016 [RefID 5786]; Tewar et al., 2016 [RefID 7178]; Rahbar et al., 2017 [RefID 6058]; Li Y et al., 2018c [RefID 11160]; Metwally et al., 2018 [RefID 11177]). All but one (Rahbar et al., 2017 [RefID 6058]) yielded statistically significant results pertaining to various clinical endpoints (autism, n = 3 (Stein et al., 2015 [RefID 6899]; Kardas et al., 2016 [RefID 3383]; Metwally et al., 2018 [RefID 11177]); ADHD = 3 (Arbuckle et al., 2016 [RefID 237]; Tewar et al., 2016 [RefID 7178]; Li Y et al., 2018c [RefID 11160]); Pervasive Developmental Disorder-Not Otherwise Specified (PDD-NOS), n = 1 (Kondolot et al., 2016 [RefID 3646])) and scales ((SDQ), n = 2 (Findlay and Kohen, 2015 [RefID 2029]; Arbuckle et al., 2016 [RefID 237]); CBCL, n = 1 (Perez-Lobato et al., 2016 [RefID 5786])) and across different populations and studies of varying power. The available cross-sectional evidence is not aligned with the available longitudinal evidence where most associations neither reached statistical significance, nor were replicated by subsequent research.

3.1.5.2. Animal studies

For the HOC Neurotoxicity and developmental neurotoxicity a total of 94 studies was appraised by the CEP Panel. The details of the appraisals (internal and external validity) are reported in Annex E.

The endpoints for each study identified as relevant in this opinion are reported in Annex F. Effects on behaviour were already key endpoints in the uncertainty analysis in the 2015 EFSA opinion (EFSA CEF Panel, 2015, Section 4.3.2). For more details see Annex A, Section 2.5.

Identification of clusters of relevant endpoints

Endpoints for which statistically significant changes were reported were extracted from the available literature and grouped into several clusters related to brain morphology and function. These clusters have been evaluated in a WoE approach and the results are described here.

Rather than identifying all the multiple pathways these endpoints can interact with, the CEP Panel decided to group them into three empirical clusters that represent different aspects of brain function: neuromorphology, nervous system functionality and behaviour. However, it is important to note that they are more or less interrelated and are not independent of each other; for example, dendritic spine plasticity as a driver or consequence of behaviour (Gipson and Olive, 2017). They all contribute to perception, cognition and integrated responses that enable an organism to cope with its changing environment. Potential connections between these clusters will be explored in the section on hazard identification:

1) Neuromorphology (including neuronal dendrite morphology, dendritic spine density (hippocampus, neocortex), brain nucleus size/volume, number of neurons/glia in various brain regions).
2) Nervous system functionality (neurochemistry, electrophysiology, brain regional neurotransmitter/receptor/hormone content, neuronal responsiveness).
3) Behaviour (anxiety-related behaviour, learning and memory, social behaviour, taste preference, locomotor activity, sensory/motor coordination) as the most apical outcome of brain function.

Table 11: Clusters of relevant endpoints.

Clusters	Relevant endpoints

Neuromorphology	Dendrite branching Dendrite intersections Dendrite length Hypothalamic histology (kisspeptin-ir cells in anteroventral periventricular nucleus (AVPV), rostral periventricular area (rPen), caudal periventricular nucleus (cPen)) Number of dopamine (DA) neurons in neocortex Number of DA neurons in ventral mesencephalon (VM) Number of glia within medial prefrontal cortex (mPFC) Pro-opiomelanocortin (POMC) projection into the paraventricular nucleus (PVN) POMC projections to hypothalamus Dendritic spine density in CA1 pyramidal cells Dendritic spine density in in hippocampus Dendritic spine density in CA1 region of hippocampus Dendritic spine synapses in CA1 region Dendritic spine density in hippocampal dentate gyrus Dendritic spine head size of dentate gyrus neurons Dendrite length (hippocampus) Number of hippocampal CA1 neurons Number of hippocampal CA3 neurons Dendritic spine density on CA1 pyramidal cells Dendritic spine density in CA1 regions Dendritic spine density on layer II/III on mPFC pyramidal cells Dendritic spine density in layers 2/3 of dorsolateral prefrontal cortex (DLPFC) Hypothalamic histology (number of kisspeptin-ir cells in AVPV) Spine synapses in CA1 regions Spine synapses in layers 2/3 of DLPFC
Nervous system functionality	Gamma amino butyric acid (GABA) (hippocampus) GABA (cortex) Aspartate (hippocampus) Aspartate (cortex) Glutamine (GLN) (hippocampus) GLN (cortex) Noradrenaline (NA) (hippocampus) Dopamine and metabolite (hippocampus) Serotonin (hippocampus) Oxytocin receptor (OTR) density (in posterior bed nucleus of stria terminalis ($BNST_p$), ventromedial hypothalamus (VMH), PVN and dorsolateral bed nucleus of the stria terminalis ($BNST_{dl}$)) Excitatory and inhibitory responsiveness of recorded medial amygdala neurons to neurochemical signals Leptin blood concentration Leptin sensitivity Serum corticosterone Corticosterone production Acetylcholinesterase (AChE) activity (in prefrontal cortex, hypothalamus, cerebellum and hippocampus) GLU (cortex) GLU (hippocampus) Glycine (GLY) (cortex) GLY (hippocampus) Monoamino-oxidase (MAO) activity Serum corticosterone Taurine (cortex) Taurine (hippocampus)
Behaviour	Anxiety/emotionality (avoidance of predator odour, elevated plus maze (EPM), elevated zero maze (EZM), forced swimming test (FST), open field test (OFT), dark light test (DLT)) Learning and memory (radial arm maze (RAM), reference memory, Barnes maze, Morris water maze (MWM), object recognition, fixed interval reinforcement, object placement, Y maze; working memory, spatial memory)

	Locomotor activity (exploratory behaviour, open field test (OFT), running activity, spontaneous activity, hole poke test, tremor activity (electronic balance test), EPM, EZM, mirrored maze) Preference behaviour (sodium salt intake; sweet preference; water intake) Social behaviour (maternal behaviour; female sexual behaviour; male sexual behaviour; interaction) Sensory-motor coordination (rotarod experiment; string test)

Neuromorphology

The first cluster comprises endpoints related to brain development, including effects on neurogenesis and on the morphology of the various brain regions. It includes the number of cells, the dendritic spine density and the degree of connectivity between cells, as well as brain volume and growth. Such relevant endpoints can be considered at the level of the whole brain or a specific brain area.

Nervous system functionality

The second cluster considers endpoints related to brain function, including several systems of neurotransmission (GLU, GABA, serotonin, noradrenaline, dopamine) within the brain and hormone communication (noradrenaline, steroid and peptide hormones) with the whole body. Electrophysiological neuronal responsiveness is also considered within this cluster as neurons propagate chemical signals by generating electric potentials.

Behaviour

The third cluster comprises behavioural endpoints including anxiety-related behaviour, learning and memory, social behaviour and sensory-motor function, which were considered by EFSA in 2015 (EFSA CEF Panel, 2015) to be ALAN related to BPA exposure. Behaviour represents the highest level of integrated function between the brain, the organism and the environment. Behavioural disturbances may cause problems for human health and be indicative of neurodevelopmental syndromes such as attention deficits, autism, schizophrenia and neurodegenerative processes later in life such as dementia, Alzheimer's or Parkinson's disease. However, even subtle effects can potentially impact all stages of human life from birth to old age, starting with mother-child bonding and parenting, the education process and the interaction with peers.

For adversity to human, endpoints of the clusters Neuromorphology and Nervous system functionality are highly interrelated, representing anatomical signalling pathways, and signal generation and transduction, respectively.

Depending on the study, endpoints of the clusters of Neuromorphology and Nervous system functionality were studied at the level of the whole brain or a specific brain region. A neurofunctional or neuroanatomical effects with the same causal mechanism but occurring in different parts of the brain may have different outcomes depending on the brain region. As a consequence, measurement done at the level of the whole brain is less indicative of specific BPA-related changes. In addition, effects observed in any of the three clusters may not be identical for males and females due to the known sex-specific development of the brain. These gender-related aspects of brain function may necessitate separate evaluations in males and females to determine if sex could influence the neurotoxicity of BPA.

Finally, brain weight, which is a general endpoint used in neurotoxicity, does not seem to be specifically relevant due to a lack of significant effects of BPA in any of the reported studies. The lack of significant effects could be explained by the fact that the brain is a well protected organ against most insults and the brain weight is an endpoint that remains very difficult to disrupt.

WoE of the clusters of relevant endpoints

The main information extracted from the studies addressing relevant endpoints in the HOC Neurotoxicity and developmental neurotoxicity are summarised in Annex G. The outcome of the WoE is described in the text below and presented in a tabulated format in Annex H.

The clusters of the effects of BPA on Neurotoxicity and developmental neurotoxicity considered for this assessment were the following:

- Neuromorphology
- Nervous system functionality
- Behaviour

Neuromorphology

The cluster Neuromorphology includes various endpoints which relate to different types of actions or responses that can be observed in animals or humans. For the sake of clarity, the results of the WoE exercise will be presented by endpoint to demonstrate which neuromorphological measurements were found to be affected by BPA exposure of a certain age group and which were not. The specific measurements that were included for the effects of BPA on neuromorphology concern the cell number and/or volume (mostly concerning dopaminergic neurons), the dendritic morphology and/or density, and the number of spine synapses. It has to be noticed that most studies report such measurements in various parts of the brain, cover more than one endpoint and assess such endpoints relatively to specific behavioural performances.

The complete cluster Neuromorphology includes 15 studies, eight being conducted in rats, six in mice, one in rhesus monkeys and one in vervet monkeys. Animals were exposed during development in seven studies (two in rats, four in mice and one in rhesus monkeys) or during the growth phase/young age in six studies (four in rats, one in mice and one in rhesus monkeys). Two studies were conducted at the adult stage, one in mice and one in vervet monkeys.

Developmental exposure (pre-natal and/or post-natal until weaning)

Seven studies were identified concerning this period of exposure. Two studies were performed in rats, four in mice, and one in rhesus monkeys (*Macaca mulatta*). One of the rat studies was allocated to Tier 2 (Liu ZH et al., 2014 [RefID 10411]) and one to Tier 3 (Liu ZH et al., 2015 [RefID 4535]). In mice, two studies were allocated to Tier 1 (Komada et al., 2014 [RefID 3641]; MacKay et al., 2017 [RefID 4767]) and two to Tier 2 (Kimura et al., 2016 [RefID 3566]; Naule et al., 2014 [RefID 5335]). The study performed in rhesus in monkeys was allocated to Tier 1 (Elsworth et al., 2013 [RefID 1810]).

Four studies reported effects of BPA on dendritic spine density measured in two highly plastic parts of the brain, namely hippocampus and PFC. The Tier 1 study from Elsworth et al. (2013) [RefID 1810]) reported that a gestational exposure to BPA for 50 days using subcutaneous implants in rhesus monkeys (*Macaca mulatta*), delivering an estimated level of exposure of 550 µg/kg bw per day, was associated with a significant loss of spine synapses in CA1 hippocampus, but not in the dorsolateral part of PFC. Two Tier 2 studies revealed similar results in the same brain region, one in mice (Kimura et al., 2016 [RefID 3566]) and one in rats (Liu ZH et al., 2014 [RefID 10411]). In mice (Kimura et al., 2016 [RefID 3566]) BPA exposure from GD8.5 to GD18.5 at the highest dose of 400 µg/kg bw per day induced a significant reduction in the three different endpoints measured at PND21, all related to the synaptic connectivity in the hippocampal CA1 area. These endpoints are the number of 5th order branching, the number of intersections >40 µm from cell body and the dendrite length. No changes were observed at the lowest dose level (40 µg/kg bw per day). Both reductions were observed only in basal dendrites at the dose of 400 µg/kg bw per day, whereas the apical part of dendrites remained unaffected at any dose. Only males were studied. In rats (Liu ZH et al., 2014 [RefID 10411]) a 7-day post-natal exposure from PND7 to PND14 induced a significant BPA dose-related decrease in the dendritic spine density of dentate gyrus neurons (−5% at 50 µg/kg bw per day, −12% at 250 µg/kg bw per day and −15% at the dose of 500 µg/kg bw per day). A concomitant 25% reduction in the dendritic spine head size of the same neurons was observed in the same region at all three dose levels.

The Tier 3 study from Liu ZH et al. (2015) [RefID 4535] revealed a dose-related reduction in the dendritic spine density of CA1 hippocampal neurons (0%, −4% and −13%) in 12-week-old rats post-natally exposed to BPA (50, 250 or 500 µg/kg bw per day from PND7 to PND14, i.p. administration). A more important reduction has been measured in the same study in 12-week-old rats after chronic exposure to BPA via oral maternal dosing (GD0–PND21) and post-natal dosing (PND21–12 weeks of age) through drinking water. Two levels of exposure were applied (0.15 and 7.5 µg/kg bw per day) leading to 14% and 21% decreases in the dendritic spine density, respectively. Only males were tested in both experiments. This study is reported here despite its allocation to Tier 3 because it revealed comparable results related to the same endpoints as reported in other Tier 1 or Tier 2 studies (Elsworth

et al., 2013 [RefID 1810]; Kimura et al., 2016 [RefID 3566]; Liu ZH et al., 2014 [RefID 10411]) and then highly contributes to the final likelihood per endpoint (Likely) and per cluster (Likely).The effect of BPA on the dendritic spine density in the same brain region in three different species was judged as Likely.

A Tier 3 study conducted in rats (Sadowski et al., 2014b [RefID 6362]) reported a significant increase in the number of neurons (+15%) and glial cells (+19%) in layers 5–6 of the medial PFC at PND140 only in males early exposed to the highest dose of BPA (GD0–PND9, 400 µg/kg bw per day). No effects were observed in males at the low and mid doses studied (4 and 40 µg/kg bw per day) or in females at any dose. No effect was observed in layers 2–3 of the same part of the brain in males and females. This Tier 3 study is the only one available exploring the number of glial cells in medial PFC. The evidence was considered Inadequate to conclude on the likelihood of the effect.

Two studies investigated the plasticity of the dopaminergic system in various parts of the brain, one in mice (Komada et al., 2014 [RefID 3641]) and one in monkeys (Elsworth et al., 2013 [RefID 1810]).

The Tier 1 study (Komada et al., 2014 [RefID 3641]) was performed in mice and showed at PND3 a significant reduction in the projection of dopaminergic neurons in the neocortex (from layer 1 to 6) of pre-natally BPA-exposed newborns at the two doses tested. BPA was administered orally from GD6 to GD18 at a dose of 20 or 200 µg/kg bw per day. Effects of BPA on this endpoint was judged as ALAN for concluding on the likelihood of the BPA effects.

The other Tier 1 study related to the dopaminergic system (Elsworth et al., 2013 [RefID 1810]) investigated the effects of a single-dose of BPA on the same endpoint considered by Komada et al. (2014) [RefID 3641] but in rhesus monkeys (*Macaca mulatta*). Results showed a significant reduction in the number of dopaminergic TH-immunoreactive neurons in VM, a part of the brain including dopaminergic regions like substantia nigra and ventral tegmental area, in newborn monkeys gestationally exposed to BPA (400 µg/kg bw per day, GD100–GD165, oral administration by food). The evidence was considered Inadequate to conclude on the likelihood of the effect.

The Tier 2 study from Naule et al. (2014) [RefID 5335] investigated the number of kisspeptin-immunoreactive cells in three brain areas, the AVPV and the rostral and caudal parts of the periventricular nucleus. Results showed a significant increase in the number of kisspeptin-immunoreactive cells at both doses of BPA 50 and 5000 µg/kg bw per day (+25% and +35%, respectively) limited to the rostral part of the periventricular nucleus. This endpoint was judged as ALAN for concluding on the likelihood of the BPA effects.

A Tier 1 study (MacKay et al., 2017 [RefID 4767]) assessed the effects of a perinatal exposure to BPA (3 µg/kg bw per day through the diet) from GD0 to PND21 on hypothalamic feeding circuitry in mice pups. At PND21, BPA induced a significant reduction in density of POMC immunolabeled fibres in the periventricular nucleus in males and females whereas the counting of POMC neurons in the arcuate nucleus was unchanged in both sexes, suggesting that BPA did not alter the embryonic neurogenesis of POMC neurons. The evidence was considered to be Inadequate to conclude on the likelihood of the effect.

The CEP Panel assigned a likelihood level of Likely to the Neuromorphology effect of BPA in the developmental exposure period, based on Likely effects for dendritic spine density (Kimura et al., 2016 [RefID 3566]; Liu ZH et al., 2014 [RefID 10411]). Therefore, these studies were taken forward for BMD analysis (see Chapter 3.2.1). Moreover, the Likely and ALAN endpoints were also considered in the uncertainty analysis (see Appendix D)

Developmental and adult exposure (pre-natal and/or post-natal in pups until adulthood)

No studies were available for this exposure period.

Growth phase/young age exposure

Six studies were identified in this exposure period, all of them allocated to Tier 1. Four studies were conducted in rats (Chen Z et al., 2018 [RefID 11734]; Wise et al., 2016 [RefID 7970]; Bowman et al., 2014 [RefID 687]; Bowman et al., 2015 [RefID 688]), one in mice (Zhou YX et al., 2017 [RefID 9083]) and one in vervet monkeys (*African green monkeys*, *Chlorocebus aethiops sabaeus*) (Elsworth et al., 2013 [RefID 1810]).

These studies explored the neuro-morphological effects of BPA in three different brain regions, namely hippocampus, PFC and mesencephalon.

In hippocampus, the Tier 1 study from Zhou YX et al. (2017) [RefID 9083] revealed a reduced number of neurons in hippocampal CA1 and CA3 areas) of juvenile male rats (PND56) exposed orally to BPA for 8 weeks from birth to post-natal week (PNW) 8 at three doses, 0.5, 50 and 5000 µg/kg bw per day. In the CA1 area, the number of neurons was significantly lower (about 10%) compared with controls at the highest dose of BPA (5000 µg/kg bw per day). In the CA3 area, the number of neurons was significantly lower than in controls (also about 10%) at 0.5 and 5000 µg/kg bw per day. Only males were tested. The evidence for these endpoints was judged as Likely.

A Tier 1 study in rats (Chen Z et al., 2018 [RefID 11734]) reported a decrease in the hippocampal CA1 dendritic spine density in animals exposed to 40, 400 and 4000 µg/kg bw per day of BPA after weaning (PND21–PND49) but it was significant only at the highest dose compared with controls. No effect of BPA on the dendritic length was observed in the same region. Only males were tested.

Two Tier 1 studies (Bowman et al., 2014 [RefID 687]; Bowman et al., 2015 [RefID 688]) investigated the dendritic spine density of hippocampal CA1 pyramidal neurons in both basal and apical parts of the cell at two post-natal ages (PND49 or PND91) in rats exposed subcutaneously to 40 µg/kg bw per day of BPA, from PND42 to PND49. At PND49, spine density was lower in the BPA group in both basal (−24%) and apical (−15%) parts of the CA1 pyramidal cells (Bowman et al., 2014 [RefID 687]). Males, both controls and BPA-treated animals, had fewer spines in CA1 basal dendrites (−9%) than control and BPA-exposed females, with no significant interaction between treatment and sex. At adulthood (PND91), a reduction in basal and apical CA1 dendritic spine densities was observed in both males and females compared with control animals (−19% and −21%, respectively). No significant interaction between treatment and sex was noted. Considering the above evidence, the endpoint dendritic spine density of pyramidal cells in hippocampal CA1 area was judged as Likely.

The Tier 1 study of Elsworth et al. (2013) [RefID 1810], reported no difference in the hippocampal CA1 dendric spine density regions measured at the end of dosing in vervet monkeys (*African green monkeys*, *Chlorocebus aethiops sabaeus*, 14–18 months of age) continuously exposed to BPA (550 µg/kg bw per day) through subcutaneous implants for 30 days during the pre-pubertal period. The evidence was considered to be Inadequate to conclude on the likelihood of the effect

Regarding the PFC, the Tier 1 study from Wise et al. (2016) [RefID 7970] explored the total number of glial cells in the medial part of the PFC in both adult male and female rats (PND150) exposed during the brain growth phase (PND27–PND46) to BPA at a dose of 4, 40 or 400 µg/kg bw per day. Results showed similar increases in the number of glial cells in females, rather microglial cells than astrocytes, and similar decreases in males. However, such differences were statistically significant referred to controls only at the dose of 40 µg/kg bw per day in females and 4 µg/kg bw per day in males. No effects on the number of neurons were observed at any dose in males and females. This endpoint was judged ALAN for the number of glial cells.

The two Tier 1 studies from Bowman et al. (2014) [RefID 687] and Bowman et al. (2015) [RefID 688] investigated at two post-natal ages (PND77 or PND91) the dendritic spine density of pyramidal neurons (basal and apical parts of the cell) in the medial part of PFC of rats exposed subcutaneously to 40 µg/kg bw per day of BPA from PND42 to PND49. At PND77, the BPA group had lower spine density in both basal (−21%) and apical (−10%) parts of the prefrontal pyramidal cells (Bowman et al., 2014 [RefID 687]). Both sexes showed the same effect on this endpoint in this part of the brain. At adulthood (PND91), no effect on basal and apical dendritic spine densities was observed in either males or females. The endpoint dendritic spine density of pyramidal cells in PFC was judged as Likely.

The Tier 1 single-dose study of Elsworth et al. (2013) [RefID 1810] reported no difference in the prefrontal dendric spine synapses measured in the cortical layers 2 and 3 at the end of the period of exposure in vervet monkeys (African green monkeys, *Chlorocebus aethiops sabaeus*, 14–18 months of age) continuously exposed to BPA through subcutaneous implants for 30 days during the pre-pubertal period (plasma levels 13.1 ± 1.4 ng/mL at termination after 30 days exposure; equivalent to 4500 µg/kg bw per day oral). The same study reported also the same absence of effect of BPA on the number of dopaminergic neurons measured in the VM of the same animals.

The evidence for dendritic spine density of pyramidal cells in PFC and the number of dopaminergic neurons in mesencephalon was judged Inadequate to conclude on the likelihood of the effect.

Overall, the CEP Panel assigned a likelihood level of Likely to the Neuromorphology effects of BPA in the growth phase/young age exposure period. Since the likelihood for Neuromorphology is Likely for the endpoints Number of hippocampal CA1 neurons (Zhou YX et al., 2017 [RefID 9083]), Number of hippocampal CA3 neurons (Zhou YX et al., 2017 [RefID 9083]) and dendritic spine density on CA1 pyramidal cells (Chen Z et al., 2018 [RefID 11734]), these studies were taken forward for BMD analysis (see Chapter 3.2.1). The endpoint dendritic spine density on layer II/III pyramidal cells in the PFC, also Likely, was not taken forward for BMD analysis because only single-dose studies were identified for this endpoint. However, the Likely and ALAN endpoints were considered in the uncertainty analysis (see Appendix D).

Adult exposure (after puberty)

Only two studies from two different species were identified in this exposure period, one in mice and one in vervet monkeys.

The study performed in mice (Wang XL et al., 2014 [RefID 7784]) was allocated to Tier 2 and reported a lack of changes in the number of kisspeptin-immunoreactive cells in the anteroventral part of the periventricular nucleus of females 6 hours after a unique oral administration of 20 µg/kg bw per day of BPA. The second study (Elsworth et al., 2015 [RefID 1809]) is a Tier 1 study performed in adult male vervet monkeys (*Chlorocebus sabaeus*) that consisted in administrating BPA subcutaneously using an osmotic minipump to achieve a dose of 50 µg/kg bw per day for 30 days (this level of dose was calculated to correspond to an equivalent oral dose of 5556 µg/kg bw per day). The results showed a significant reduction in the number of spine synapses measured at the end of exposure in hippocampus (CA1 stratum radiatum) and PFC (layers 2/3 of the dorsolateral part of PFC) compared with controls. It is noted that measurements done 4 weeks after the removal of the minipump and the end of BPA exposure showed a partial recovery of the number of synapses into the same regions.

These studies were considered to be of Inadequate evidence for the endpoint related to the hypothalamic histology (Wang XL et al., 2014 [RefID 7784]) and for the endpoints number of spine synapses in hippocampus and PFC (Elsworth et al., 2015 [RefID 1809]).

The CEP Panel considered the evidence Inadequate to assign a likelihood level to the Neuromorphology effects of BPA in the adult exposure period. Therefore, none of the endpoints was taken forward for BMD analysis.

Indirect (germline) exposure

No studies were available for this exposure period.

Overall cluster selection of the endpoints/studies for BMD analysis for Neuromorphology

Overall, the CEP Panel assigned a likelihood level of 'Likely' to the effects of BPA on neuromorphology in the exposure periods developmental (pre-natal and/or post-natal until weaning) and growth phase/young age, and Inadequate evidence in the period adult exposure (after puberty).

The CEP Panel considered that the evidence from the studies available showed a Likely effect of BPA in the exposure period developmental (pre-natal and/or post-natal until weaning) for the endpoint 'Dendritic spine density of pyramidal cells in hippocampus (CA1 and dentate gyrus areas)' (Kimura et al., 2016 [RefID 3566]; Liu ZH et al., 2015 [RefID 4535]; Elsworth et al., 2013 [RefID 1810]; Liu ZH et al., 2014 [RefID 10411]). The endpoint 'Dendritic spine density of pyramidal cells in hippocampus (CA1 and dentate gyrus areas)' (Kimura et al., 2016 [RefID 3566]; Liu ZH et al., 2014c [RefID 10411]) was taken forward for BMD analysis (see Chapter 3.2.1). The same endpoint assessed in the studies by Liu ZH et al. (2015) [RefID 4535] and Elsworth et al. (2013) [RefID 1810] was not taken forward because the first is a Tier 3 study and the second is a single-dose Tier 1 study.

The CEP Panel considered that the evidence from the studies available showed a Likely effect of BPA in the exposure period Growth phase/young age for the endpoints 'Number of neurons in hippocampus (CA1 and CA3 areas)' (Zhou YX et al., 2017 [RefID 9083]), 'Dendritic spine density in CA1 pyramidal cells' (Bowman et al., 2014 [RefID 687]; Bowman et al., 2015 [RefID 688]; Chen Z et al., 2018 [RefID 11734]), and 'Dendritic spine density in pyramidal cells in medial part of PFC' (Bowman et al., 2014

[RefID 687]; Bowman et al., 2015 [RefID 688]). The endpoints 'Number of neurons in hippocampal CA1 area' (Zhou YX et al., 2017 [RefID 9083]), 'Number of neurons in hippocampal CA3 area' (Zhou YX et al., 2017b [RefID 9083]) and 'Dendritic spine density in CA1 pyramidal cells' (Chen Z et al., 2018 [RefID 11734]) were taken forward for BMD analysis (see Chapter 3.2.1). Both endpoints 'Dendritic spine density in CA1 pyramidal cells' and 'Dendritic spine density in pyramidal cells in medial part of PFC (Bowman et al., 2014 [RefID 687]; Bowman et al., 2015 [RefID 688]) were not taken forward for BMD analysis because these two Tier 1 studies are both single-dose studies.

The CEP Panel assigned a likelihood level of Inadequate evidence to the effects of BPA on neuromorphology in the exposure period 'adult exposure (after puberty)'. Therefore, none of the endpoints was taken forward for BMD analysis.

The overall likelihood across all exposure periods, i.e. the highest likelihood given in the cluster Neuromorphology, was Likely.

Nervous system functionality

As for the cluster Neuromorphology, this cluster includes various endpoints that relate to different types of actions or responses that can be observed in animals or humans. For the sake of clarity, the results of the WoE exercise will be presented by endpoint to demonstrate which brain functioning measurements were found to be affected by BPA exposure of a certain age group and which were not. The specific measurements that were included for the effects of BPA on brain functionality concern various neurotransmitter systems (GABA, GLU, aspartate (ASP), TAU, GLY, monoaminergic systems), acetylcholine esterase (AChE) activity, leptin secretion and corticosterone secretion and regulation. Most of these studies report such measurements in various parts of the brain, cover more than one endpoint and assess such endpoints relative to specific behavioural performances.

The complete cluster Nervous system functionality includes 10 studies, six in rats and four in mice. Animals were exposed during development in four studies (two in rats, two in mice) or during the growth phase/young age in two studies (one in rats and one in mice). Four studies were performed in adult animals (one in mice and three in rats). Two studies in rats exposed the tested animals by indirect germline exposure.

Developmental exposure (pre-natal and/or post-natal until weaning)

Four studies in two animal species were identified for this part, two in mice (Xin et al., 2018 [RefID 13482]; MacKay et al., 2017 [RefID 4767]) and two in rats (Witchey et al., 2019 [RefID 13782]; Fujimoto and Aou, 2018 [RefID 11960]).

The Tier 2 study from Xin et al. (2018) [RefID 13482] investigated the brain levels of various neurotransmitters in the hippocampus of adult mice (21 weeks after birth) born from dams exposed to BPA from preconception (2 weeks before mating) until weaning (PND21) through the diet at two doses (10 and 10000 µg/kg bw per day). Results showed a significant dose-dependent decrease in serotonin (5HT) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) in high-dose BPA-exposed adult male, while females were unaffected. Levels of noradrenaline (NA) were sex-specifically affected by BPA with a significant reduction in noradrenaline in low-dose BPA-exposed females. Dopamine (DA) levels were not affected in either sex at the low dose (10 µg/kg bw per day). A reduced level of the dopamine metabolite homovanillic acid (HVA) was also observed in low-dose BPA-exposed females. GABA, noradrenaline, dopamine and its metabolite and serotonin are endpoints which were judged as ALAN whereas the others (ASP, GLU and GLN) were judged as Not Likely.

The Tier 1 electrophysiological study from Fujimoto and Aou (2018) [RefID 11960] investigated the extracellular responses of neurons in the medial amygdala to the presentation of three plant odours and three predator odourants in adult male rats exposed to BPA during early development (GD0–PND14, 15 µg/kg bw per day through drinking water). Results showed a greater activity of amygdala odour-responsive neurons to two odourants (fox odour and whisky lactone) in BPA-exposed rats compared with the control animals. Females were not tested.

This study was considered to be Inadequate evidence for the BPA brain risk assessment for the endpoint of excitatory and inhibitory responsiveness of recorded medial amygdala neurons to neurochemical signals.

A Tier 1 study (Witchey et al., 2019 [RefID 13782]) revealed significant sexually dimorphic differences in the density of oxytocin receptors (OTR) between control male and female rats aged 28 days after birth in three hypothalamic regions [Bed Nucleus of Striatal Terminalis (BNST), Paraventricular Nucleus (PVN) and Ventromedial Hypothalamus (VMH)]. In rats of the same age (PND28) exposed to BPA from GD6 to PND21 at 2.5, 25 or 2500 µg/kg bw per day, OTR binding was significantly increased compared with controls at 2.5 and 25 µg/kg bw per day in males only in the $BNST_{dl}$, which eliminated the sex-specific difference observed in the control group. No significant main effects of BPA exposure were measured in any other hypothalamic areas ($BNST_{p}$, PVN and VMH). No effects of BPA were found in females. This endpoint was judged as ALAN for concluding on the likelihood of the BPA effects.

The Tier 1 study from MacKay et al. (2017) [RefID 4767] revealed a delayed serum leptin surge from PND8 in control pups to PND10–12 in BPA-exposed rats. Exposure to BPA was performed in dams from GD0 to GD12 and in the offspring up to PND21 at a dose of 3 µg/kg bw per day. In addition, results showed a decrease in leptin sensitivity reflected by a lack of reduction in body weight in adult (PND130) BPA early exposed females and males after two days of leptin administration. Females were more strongly affected compared with the males.

This evidence was considered to be Inadequate for concluding on the likelihood of the BPA effectson the endpoint leptin (sensitivity and blood concentration).

The CEP Panel assigned a likelihood level of ALAN to the neurofunctional effects of BPA in the developmental exposure period, therefore none of the endpoints was taken forward for BMD analysis. However, the ALAN endpoints were considered in the uncertainty analysis (see Appendix D).

Developmental and adult exposure (pre-natal and/or post-natal in pups until adulthood)

No studies were available for this exposure period.

Growth phase/young age

Two studies were identified during this exposure period. One was performed in mice (Luo et al., 2013 [RefID 11330]), while the other one was done in rats (Bowman et al., 2015 [RefID 688]).

The Tier 1 study from Luo et al. (2013) [RefID 11330] reported a significant decrease in hippocampal AChE activity in young adult male rats immediately after the end of the period of exposure to BPA (PND30 to PND70; 10000 µg/kg bw per day) whereas the enzyme activity remained unchanged in the other brain regions studied (PFC, hypothalamus, cerebellum). Females were not tested. The evidence was considered to be Inadequate to conclude on the likelihood of the BPA effect on the endpoint 'AChE activity'.

The second study is also a Tier 1 study (Bowman et al., 2015 [RefID 688]) which reported a slight non-significant increase in corticosterone blood levels in male (+24%) and female (+16%) adult rats (PND91) exposed to BPA from PND42 to PND49 (40 µg/kg bw per day, s.c. equivalent to an oral dose of 1428 µg/kg bw per day) in a model of restraint-induced stress (1 hour, 21°C). The evidence was considered to be Inadequate to conclude on the likelihood of the effect.

The CEP Panel assigned a likelihood level of Inadequate evidence to the neurofunctional effects of BPA in the growth phase/young age exposure period, so, none of the endpoints was taken forward for BMD analysis.

Adult exposure (after puberty)

Four studies in two animal species were identified for this exposure period, one in mice (Khan et al., 2018 [RefID 12311]) and three in rats (Fan et al., 2013 [RefID 1907]; Khadrawy et al., 2016 [RefID 3462]; Fan et al., 2018 [RefID 11915]).

The study from Khan et al. (2018) [RefID 12311], which was allocated to Tier 2, reported a concomitant 30% decrease in AChE activity and significant increase (+40%) in monoamine oxidase (MAO) activity in the brain homogenate of adult male mice exposed to BPA at a dose of 10000 µg/kg per day (PND63–

PND91). Only males were tested. The evidence was considered Inadequate for the endpoint MAO and as Likely for the other endpoint, AChE activity.

A Tier 1 study (Fan et al., 2013 [RefID 1907]) reported a significant reduction of AChE activity measured in hippocampus of adult male rats at 144 days after birth and exposed to BPA 50 µg/kg per day for 10 weeks. Females were not considered for this study. The evidence for this endpoint was judged as Likely.

The Tier 2 study from Khadrawy et al. (2016) [RefID 3462] intend to assess the effects of BPA on AChE activity and excitatory (GLN, GLU and aspartate (ASP)) and inhibitory (GABA, GLY and TAU) amino acid neurotransmitter levels in the cortex and hippocampus of adult male rats. Females were not tested. Two protocols of exposure to BPA were used: (1) BPA administered at two levels of doses, 10000 and 25000 µg/kg bw per day, for 6 weeks, and (2) BPA administered at only one level of dose, 10000 µg/kg bw per day, with two exposure times, 6 or 10 weeks. The results related to the AChE activity measured in the cortex and hippocampus revealed a significant dose-dependent and duration-dependent increase in the enzyme activity in both regions. Significant increases were observed in the cortex whatever the treatment regimen used. In the hippocampus, AChE activity was significantly increased only at the two dose levels administered for 6 weeks.

There were concomitant increases in hippocampal levels of both excitatory (GLN, GLU and ASP) and inhibitory (GABA, GLY and TAU) amino acid neurotransmitters with the two levels of doses given during 6 weeks. In the cortex, the level of the GLU and ASP metabolic precursor GLN was reduced regardless of the dose or duration used for exposure. Cortical GLU and ASP levels were increased in a significant way only in animals exposed to 10000 µg/kg bw per day for 10 weeks, or to 25000 µg/kg bw per day for 6 weeks. Cortical GABA and GLY were significantly decreased in the same exposure groups. Finally, TAU was decreased in a significant way only in rats exposed to 25000 µg/kg bw per day of BPA during 6 weeks. The endpoint AChE activity was judged as Likely while the other ones (excitatory and inhibitory amino acid neurotransmitters) were judged as ALAN.

A Tier 1 study performed in adult male rats (Fan et al., 2018 [RefID 11915]) dosed 0 or 50 µg/kg bw per day for 21 weeks through the diet) reported a significant increase in corticosterone blood levels 30 minutes after being tested in the open field compared with the basal level measured before the open field. The highest variation was observed in BPA-exposed rats compared with controls. Females were not included in this study. The evidence on serum corticosterone was judged Inadequate to conclude on the likelihood of the effect.

The CEP Panel assigned a likelihood level of Likely to the Neurofunctional adverse effect of BPA in the adult exposure period. The likelihood level for this cluster is Likely for the endpoint 'AChE activity' based on Khadrawy et al. (2016) [RefID 3462], therefore this endpoint was taken forward for BMD analysis.

Other endpoints related to this period of exposure, namely 'excitatory or inhibitory neurotransmitters (GABA, ASP, GLU, GLN, GLY and TAU)' (Khadrawy et al., 2016 [RefID 3462]), 'MAO activity' (Khan et al., 2018 [RefID 12311]) and 'blood corticosterone production' (Fan et al., 2018 [RefID 11915]) were not considered for BMD analysis due to a likelihood level of ALAN based ona Tier 2 study (Khadrawy et al., 2016 [RefID 3462]) or considered of Inadequate Evidence since only two single-dose Tier 1 or Tier 2 studies (Fan et al., 2018 [RefID 11915] and Khan et al., 2018 [RefID 12311], respectively) were available.

The Likely and ALAN endpoints were considered in the uncertainty analysis (see Appendix D).

Indirect (germline) exposure

Two Tier 1 studies both performed in rats were identified (Fan et al., 2018 [RefID 11915]; Fan et al., 2013 [RefID 1907]) regarding this period of exposure.

The Tier 1 study from Fan et al. (2018) [RefID 11915] reported a significant increase (+25%) in corticosterone blood levels measured in adult female rats (PND56) derived from male parents exposed to BPA 50 µg/kg bw per day for 21 weeks before mating. Blood was examined 30 minutes after the FST which was the last one of a series of three consecutive behavioural tests including the Open Field, the EPM and the Forced Swim Test. No effect was observed in males.

This evidence was considered to be Inadequate to conclude on the likelihood of the effect The second Tier 1 study (Fan et al., 2013 [RefID 1907]) reported a lack of significant changes in the hippocampal

AChE activity measured in adult F1 male and female rats (PND56) issued from non-exposed F0 females being mated with F0 males exposed to BPA (50 µg/kg bw per day) for 10 weeks before mating.

This evidence was considered to be Inadequate to conclude on the likelihood of the effect. The CEP Panel assigned a likelihood level of Inadequate Evidence to the neurofunctional effects of BPA in the indirect (germline) exposure period, so, none of the endpoints was taken forward for BMD analysis.

Overall cluster selection of the endpoints/studies for BMD analysis for Nervous system functionality

The CEP Panel considered that the evidence from the studies available showed a Likely effect of BPA in the adult exposure (after puberty) period for the endpoint 'AChE activity' (Fan et al., 2013 [RefID 1907]; Khadrawy et al., 2016 [RefID 3462]; Khan et al., 2018 [RefID 12311]). However, only the study Khadrawy et al., 2016 [RefID 3462] assessing this endpoint was taken forward for BMD analysis and not the other two because these were all single-dose studies/experiments. Other endpoints related to this period of exposure, namely 'excitatory or inhibitory neurotransmitters (GABA, ASP, GLU, GLN, GLY and TAU)' (Khadrawy et al., 2016 [RefID 3462]), 'MAO activity' (Khan et al., 2018 [RefID 12311]) and 'blood corticosterone production' (Fan et al., 2018 [RefID 11915]) were not considered for BMD analysis due to a likelihood level of ALAN based on a Tier 2 study (Khadrawy et al., 2016 [RefID 3462]) or considered of Inadequate evidence since only two single-dose Tier 1 or Tier 2 studies (Fan et al., 2018 [RefID 11915]; Khan et al., 2018 and [RefID 12311], respectively) were available.

The overall likelihood across all exposure periods, i.e. the highest likelihood given in the cluster Nervous system functionality, was Likely.

Behaviour

The cluster Behaviour comprises a number of different endpoints that relate to different types of actions or responses that can be observed in animals or humans. For the sake of clarity, the results of the WoE exercise will be presented by endpoint to demonstrate which behavioural measurements were susceptible to BPA at different exposure periods. The specific measurements that were included for the effects of BPA on behaviour belong to the endpoints of anxiety-related and emotional behaviour, learning and memory, locomotor activity and exploration, social behaviour and preference behaviour. Note that most studies cover more than one endpoint and some cover different stage of the animal life.

Overall, the cluster Behaviour comprises 17 studies performed in rats (of which two studies have indirect germline exposure through the male parent before conception, 10 studies relate to exposed animals during development until weaning, two studies relate to growth phase/young age and five studies relate to exposure in adulthood) and 13 studies performed in mice. Among these, two studies relate to indirect exposure of the male or the female parent before conception, seven studies relate to exposure during development until weaning, two to exposure in growth phase/young age and five to exposure in adulthood.

In addition, there were three studies performed in monkeys and one study in prairie voles.

Developmental exposure (pre-natal and/or post-natal until weaning)

For effects of BPA in the developmental exposure period, 20 studies in total (in rats, mice, monkeys or prairie voles) were identified. From the rat studies, five were allocated to Tier 1 (Ferguson SA et al., 2014 [RefID 1998]; Fujimoto et al. 2013 [RefID 2102]; Fujimoto et al., 2015 [RefID 2104]; Hicks et al., 2016 [RefID 2704]; Johnson et al., 2016 [RefID 3241]), three to Tier 2 (Hass et al., 2016 [RefID 2610]; Wang C et al., 2016 [RefID 7576]; Wang C et al., 2014 [RefID 7579]), and two to Tier 3 (Rebuli et al., 2015 [RefID 6127]; Sadowski et al., 2014a [RefID 6361]). One mouse study was allocated to Tier 1 (Luo et al., 2014 [RefID 4660]), six to Tier 2 (Kumar and Thakur, 2017 [RefID 3737]; Nagao et al., 2014 [RefID 5295]; Naule et al., 2014 [RefID 5335]; Picot et al., 2014 [RefID 5830]; Sobolewski et al., 2014 [RefID 6802]; Xin et al., 2018 [RefID 13482]) and one to Tier 3 (Kundakovic et al., 2013 [RefID 3755]). The study in cynomolgus monkeys (*Macaca fascicularis*) (Negishi et al., 2014 [RefID 5351]) was in Tier 2 and the study in prairie voles (*Microtus ochrogaster*) (Sullivan et al., 2014 [RefID 6954]) was allocated to Tier 3.

- Anxiety/emotionality

For the endpoint anxiety/emotionality, five studies in rats, four studies in mice and one study in prairie voles were included. In most cases, the exposure started at implantation of the conceptus (2 studies in rats, 1 study in mice) or before or directly after mating of the parent animals (3 studies in mice, 1 study in rats). One study in rats examined animals exposed during the fetal period, whereas another study in rats and the study in prairie voles restricted exposure to the post-natal period.

No changes of anxiety or emotionality parameters were found in adult male or female rats in a Tier 2 study by Hass et al. (2016) [RefID 2610] with pre-natal and post-natal exposure covering a large dose range (25, 250, 5000, 50000 µg/kg bw per day). The test was performed in an EPM. The finding is supported by a Tier 3 study by Rebuli et al. (2015) [RefID 6127]. These authors examined the behaviour of juvenile and adult rat offspring after pri-and post-natal exposure to 2.5, 25 and 250 µg/kg bw per day in the EPM and the Open Field and tested adult offspring also in the Zero Maze. None of the tests gave evidence for changes in anxiety-related behaviour following developmental BPA exposure. In contrast, Hicks et al. (2016) [RefID 2704] found evidence for increased anxiety in the Open Field in a single-dose study with exposure through the drinking water throughout pregnancy and lactation (80–195 µg/kg bw per day). Young adult male and female offspring from the BPA group spent less time in the centre area, away from the walls. Fujimoto et al. (2013) [RefID 2102] did not find effects on anxiety either in the EPM in young adult rats of both sexes in a single-dose study (24 µg/kg per day) with exposure during the first pre-natal week. However, they observed an influence on depression-like behaviour in the Forced Swim Test in which males and females of the BPA group displayed a decrease in latency to immobility and males showed a prolonged duration of immobility. Another indication for effects of developmental BPA exposure on anxiety-related parameters comes from a single-dose study (15 µg/kg bw per day) by Fujimoto et al. (2015) [RefID 2104]. The authors reported an increase in the avoidance response to fox odour by adult males and females after exposure to BPA during the second half of the gestation period. Overall, the data in rats indicate effects of BPA only in the more stressful tests that examine the endpoint of anxiety/emotionality.

A single-dose Tier 2 study with mice detected increased anxiety in pre-natally and post-natally exposed males in the Open Field and the EPM at a dose of 50 µg/kg bw per day (Kumar and Thakur, 2017 [RefID 3737]). The males were tested at the age of 8 weeks and the BPA group showed a lower inclination to visit the unprotected areas in both tests. Females were not examined in this study, but data from the single-dose study of Luo et al. (2014) [RefID 4660] conducted with a much higher dose (10000 µg/kg bw per day) indicate a comparable effect in female mice shortly after the end of exposure at weaning in the Open Field and in the EZM. The finding in females is supported by a Tier 3 study with pre-natal exposure. Females tested at the age of 8 weeks spent less time in the central area of the Open Field at the dose levels of 20 and 200 µg/kg bw per day (Kundakovic et al., 2013 [RefID 3755]). However, male mice in this study exhibited a change in the opposite direction at all doses tested (2, 20 and 200 µg/kg bw per day). They spent more time in the unprotected area, a behaviour that resembles the normal pattern of control females.

Xin et al. (2018) [RefID 13482] did not observe increased anxiety in adult male mouse offspring from dams treated with 10 or 10000 µg/kg bw per day from before pregnancy until weaning when testing them in the EZM. However, they discovered an increase in depression-like behaviour (time spent immobile) in the Forced Swim Test which was similar in extent at both dose levels. Females did not show an increase in depression-like behaviour in this test.

Effects of BPA exposure appear Likely based on effects in Open Field, Elevated Maze and Forced Swim Test in Tier 1 and Tier 2 mouse studies and on findings in the Forced Swim Test and a predator odour avoidance test in rats.

- Learning and memory

The endpoint learning and memory was examined in a single-dose study in mice (Tier 2) and five studies in rats with three or more dose groups and a control. Sobolewski et al. (2014) [RefID 6802] exposed pregnant/lactating mice from implantation until weaning to a dose of 50 µg/kg bw per day. Male and female offspring were tested as young adults in a novel object exploration and recognition test that examined response to novelty and short-term memory of familiar objects. In the BPA group both sexes showed a significant decrease in their initial exploration time spent with a novel object and the males also exhibited a decrease in overall exploration time. Females compensated the decreased duration of the exploration bouts with an increase in the number of approaches to the object. No effects

were seen on short-term memory in this test. During fixed interval (60 s) reinforcement sessions, BPA-group males, but not females, exhibited significant reductions in response rates which could indicate an attention deficit or a lack of motivation to respond for food rewards.

In rats, Hass et al. (2016) [RefID 2610] did not identify learning deficits in a MWM in 4–6-month-old male or female offspring after developmental exposure (GD7 to PND22) to doses between 25 and 50000 µg/kg bw per day in their Tier 2 study.

In a Tier 3 study, Sadowski et al. (2014a) [RefID 6361] did not find changes in working or reference memory in BPA-exposed offspring tested as adults in a 17-arm radial maze. The rats had been exposed from GD0 until PND9 to doses between 4, 40 or 400 µg/kg bw per day. However, with male rats tested after weaning and exposed only during pre-natal development (GD9 to GD20), Wang C et al. (2014) [RefID 7579] found an increase in 8-arm radial maze working memory errors compared with the control group. The effect was seen at all doses (50, 500, 5000 and 50000 µg/kg bw per day), especially during the first half of the 14-day trial period. A similar, albeit smaller, effect was seen for reference memory errors in this Tier 2 study. An increased error rate (incidence of sniffing incorrect holes) was also observed in a Tier 1 study by Johnson et al. (2016) [RefID 3241] in male and female rats exposed from GD6 to PND21 to a dose of 2500 µg/kg per day and tested as adults in a Barnes maze. In addition, the female rats exhibited an increased latency to locate the escape box in this test. No effects on Barnes maze performance were seen at lower doses (2.5 and 25 µg/kg per day).

In another Tier 2 study, a test for object recognition in weanling male rats exposed from GD9 to GD20 resulted in a decrease of the object recognition index as a measure of short-term memory (1.5 hours) at 500, 5000 and 50000 µg/kg bw per day. Long-term memory (24 hours) was also impaired but only at 5000 and 50000 µg/kg bw per day. No effect on either measure was seen at the dose of 50 µg/kg bw per day (Wang C et al., 2016 [RefID 7576]). This is compatible with the negative result in mice after developmental exposure to this low dose only (Sobolewski et al., 2014 [RefID 6802]).

Based on findings of memory impairment, impaired novel object exploration/recognition and increased error rate an effect of BPA on these endpoints was judged to be Likely.

- Locomotor activity/exploration

Regarding the endpoint locomotor activity/exploration the motor activity was studied either in the home cage of the animals or in an apparatus like the open field that allowed spontaneous movement and exploration in most cases. Five studies in mice with either pre-natal (Nagao et al., 2014 [RefID 5295]; Kundakovic et al., 2013 [RefID 3755]) or pre-natal and post-natal exposure (Luo et al., 2014 [RefID 4660]; Sobolewski et al., 2014 [RefID 6802]; Xin et al., 2018 [RefID 13482]) were available. Four of these studies did not identify an effect of BPA on this endpoint at doses between 2 and 10000 µg/kg bw per day. Only the Tier 3 study of Kundakovic et al. (2013) [RefID 3755] described a sexually dimorphic effect on distance travelled in the Open Field for males at all doses tested (2, 20 and 200 µg/kg bw per day) and for females at the two highest doses. Similarly, out of eight studies in rats, only the study of Wang C et al. (2014) [RefID 7579], found a slight reduction between 10 and 30% in locomotor activity over a wide dose range (50, 500, 5000 or 50000 µg/kg bw per day) in males after pre-natal exposure. No clear dose dependency was observed. Females were not included in this Tier 2 study. In a different test setting, the same group (Wang C et al., 2016 [RefID 7576]) detected no change in the time period the males spent exploring. Other investigators did not report changes in motor activity of rats after either pre-natal (Fujimoto et al., 2015 [RefID 2104]), pre-natal and post-natal (Rebuli et al., 2015 [RefID 6127]; Hicks et al., 2016 [RefID 2704]; Ferguson SA et al., 2014 [RefID 1998]; Hass et al., 2016 [RefID 2610]) or post-natal exposure (Fujimoto et al., 2013 [RefID 2102]), although the study by Hass et al. covered a similar dose range (25, 250, 5000, 50000 µg/kg bw per day) as the study by Wang C et al. (2014) [RefID 7579]. The only other study in which an effect was observed was a Tier 3 study in prairie voles (Sullivan et al., 2014 [RefID 6954]) that identified a reduction of activity after exposure during the second PNW to the dose of 50000 µg/kg bw per day in the female sex only. No effects occurred at lower doses or in males.

Overall, the available data suggest that an effect of BPA on this endpoint is Not Likely.

- Social interaction

The effects of developmental exposure of BPA on various aspects of social behaviour were examined in rats (pre-natal and post-natal), mice (pre-natal and post-natal), prairie voles (post-natal) and cynomolgus monkeys (pre-natal). No effects on sexual behaviour were seen in intact female rats exposed to doses of 2.5 or 25 µg/kg per day from implantation to weaning in a Tier 1 study (Ferguson SA et al., 2014 [RefID 1998]) and in male mice exposed to doses of 50 or 5000 µg/kg bw per day from the fetal period until weaning in a Tier 2 study (Picot et al., 2014 [RefID 5830]). BPA also did not change non-sexual social interactions in male and female rat offspring in a single-dose Tier 1 study with exposure through the drinking water amounting to 80–195 µg/kg bw per day (Hicks et al., 2016 [RefID 2704]) or in a Tier 2 study with male mice exposed to 10 or 10000 µg/kg bw per day (Xin et al., 2018 [RefID 13482]). Only the Tier 3 study by Kundakovic et al. (2013) [RefID 3755] reported a decrease in chasing behaviour directed to cage mates in male mice at the highest dose tested (200 µg/kg bw per day).

A Tier 2 study in cynomolgus monkeys reported a decrease in social interaction in males after pre-natal exposure to BPA after subcutaneous exposure equivalent to an oral dose of about 1000 µg/kg bw per day (Negishi et al., 2014 [RefID 5351]). In addition, the exposure to BPA abolished the known difference in discriminant scores that is present between control males and females.

In a Tier 3 study with a monogamous rodent species, the prairie vole, Sullivan et al. (2014) [RefID 6954] observed BPA effects on social interaction after exposure on PND8 to PND14. Females at a dose of 50000 µg/kg bw per day spent more time investigating a same sex stimulus animal, whereas males at 50 and 50000 µg/kg bw per day spent less time. Consequently, the normal sex difference of the response was lost or inverted. In the same study, female prairie voles seemed to show a decrease in formation of opposite sex partner preference bonding at all doses tested (5, 50 or 50000 µg/kg bw per day) based on a low number of females that spent time with a strange male in addition to their partner. This parameter was not affected in males.

There were no effects on social behaviour in Tier 1 and Tier 2 studies in rats and mice; weak effects were observed in a small single-dose level Tier 2 study in monkeys and in a Tier 3 study in female prairie voles. Overall, an effect of BPA on this endpoint was judged as Not Likely.

- Preference behaviour

A Tier 1 (Ferguson SA et al., 2014 [RefID 1998]) and a Tier 2 study (Hass et al., 2016 [RefID 2610]) examined preference for sweet solution in male and female rats exposed during *in utero* development until weaning. No effect of BPA was detected in either study at doses equal to or lower than 5000 µg/kg bw per day. The dose of 50000 µg/kg bw per day used in the study of Hass et al. decreased the intake of saccharin solution by approximately 25% in females only, which may indicate masculinisation or de-feminisation of this behaviour at high doses.

BPA exposure to doses of 2.5 or 25 µg/kg bw per day did not change the preference for sodium chloride solution in males and females (Ferguson SA et al., 2014 [RefID 1998]).

The available data indicate that changes in preference behaviour are Not Likely.

Overall, the CEP Panel assigned a likelihood level of Likely to the behavioural effects of BPA in the developmental exposure period (pre-natal and/or post-natal until weaning). Since the likelihood level for this cluster is Likely for the endpoints anxiety/emotionality (Xin et al., 2018 [RefID 13482]) and learning and memory (Johnson et al., 2016 [RefID 3241]; Wang C et al., 2016 [RefID 7576]; Wang C et al., 2014 [RefID 7579]), these were taken forward for BMD analysis (see Chapter 3.2.1) and uncertainty analysis (see Appendix D). The endpoints anxiety/emotionality investigated in the studies by Kumar and Thakur (2017) [RefID 3737] and Luo et al. (2014) [RefID 4660] were not taken forward for BMD analysis because both are single-dose studies.

<u>Developmental and adult exposure (pre-natal and post-natal in pups until adulthood)</u>

No studies were available for this exposure period.

<u>Growth phase/young age exposure</u>

For this exposure period, two studies in rats (Bowman et al., 2015 [RefID 688]; Chen Z et al., 2018 [RefID 11734]), two studies in mice (Zhou YX et al., 2017 [RefID 9083]; Lou et al., 2013 [RefID 11330])

and one study in vervet monkeys (*Chlorocebus sabaeus*) (Elsworth et al., 2013 [RefID 1810]) were identified. All studies were allocated to Tier 1.

- Anxiety/emotionality

The endpoint anxiety/emotionality was studied in rats and mice. Chen Z et al. (2018) [RefID 11734] tested young adult rats that had been dosed orally with 40, 400 and 4000 µg/kg bw per day from weaning until PND49. They observed a statistically significant increase of latency to first entry into the centre area of an open field in males at 4000 µg/kg bw per day, as well as slight, non-significant increases in both lower dose groups. No effects were seen in females. Lou et al. (2013) [RefID 11330] examined adult male mice that had been given 10000 µg/kg bw per day from PND30 until PND70 in a dark/light test and the EPM and found evidence for increased anxiety at this dose with both tests.

Based on these findings, effects of BPA on anxiety/emotionality were judged as Likely for males.

- Learning and memory

For the endpoint of learning and memory, studies in rats, mice and monkeys were available. In the study of Chen Z et al. (2018) [RefID 11734] male rats displayed reduced memory for platform location in the MWM in the mid and the high-dose group (400 and 4000 µg/kg bw per day). Memory was unaffected in females. In male mice treated with doses of 0.5, 50 and 5000 µg/kg bw per day from 4–12 weeks of age, Zhou YX et al. (2017) [RefID 9083] found a decrease in learning performance in the high-dose group. These animals required an increased number of trials to qualify to the learning standard (90% correct response) in a Y maze test.

The other studies for this endpoint were conducted with subcutaneous exposure. In a study with rats employing only a single dose (40 µg/kg bw per day s.c., equivalent to 1428 µg/kg bw per day orally) Bowman et al. (2015) [RefID 688] observed a decrease in overall exploration in males and in females, but no effect on spatial memory performance. In addition, males but not females spent less time with novel objects than the control group. No effect was seen on the working memory of pre-pubertal male and female monkeys exposed to plasma levels of about 15 ng/ml (equivalent to 4500 µg/kg bw per day orally) for 30 days in the study by Elsworth et al. (2013) [RefID 1810].

The available data indicate a Likely effect of BPA on learning/memory for males but not for females.

- Locomotor activity/exploration

No or no consistent effects on the other two endpoints examined for this exposure period, preference behaviour and locomotor behaviour, were found by Bowman et al. (2015) [RefID 688] in their single-dose study with rats. Since this single-dose study was the only study available for these endpoints, there was Inadequate evidence to judge the likelihood of these effects.

Overall, the CEP Panel assigned a likelihood level of Likely to the behavioural effects of BPA in the exposure period Growth phase/young age. Since the likelihood level for this cluster is Likely for the endpoints anxiety/emotionality and learning and memory (in Chen Z et al., 2018 [RefID 11734] and Zhou YX et al., 2017 [RefID 9083], respectively) in males, these were taken forward for BMD analysis (see Chapter 3.2.1) and uncertainty analysis (see Appendix D).

Adult exposure (after puberty)

In this exposure group, 10 studies from three species were identified, four in rats, five in mice and one in vervet monkeys (*Chlorocebus sabaeus*). Three of the rat studies were allocated to Tier 1 (Fan et al., 2018 [RefID 11915]; Fan et al., 2013 [RefID 1907]; Nuñez et al., 2018 [RefID 11199]), one to Tier 2 (Nojima et al., 2013 [RefID 5435]). The database for mice consisted of one Tier 1 study (Xu XH et al., 2015 [RefID 8232]), three Tier 2 studies (Picot et al., 2014 [RefID 5830]; Khan et al., 2018 [RefID 12311]; Xin et al., 2018 [RefID 13482]) and one Tier 3 study with subcutaneous exposure (Liang et al., 2018 [RefID 12508]) which was considered supportive for the endpoints anxiety/emotionality and locomotor activity/exploration identified in Tier 1 studies. The single study in monkeys (Elsworth et al., 2015 [RefID 1809]) was allocated to Tier 1.

- Anxiety/emotionality

For the endpoint anxiety/emotionality, one study in rats and two studies in mice identified an increase of the level of anxiety in male animals. Fan et al. (2018) [RefID 11915] observed that male rats treated

with the single dose of 50 µg/kg bw per day for 23 weeks spent about 40% less time in the centre area of an open field, whereas Xu XH et al. (2015) [RefID 8232] and Liang et al. (2018) [RefID 12508] found that male mice were not responsive in this type of test in their studies. However, they displayed dose-dependent increases in anxiety or emotionality in two other tests, EPM and the FST. In the study by Xu XH et al. (2015) [RefID 8232], the effect was noted from the lowest dose tested (40 µg/kg bw per day) in the FST and for all doses in the EPM except the lowest one. Female mice showed either no or opposite effects (decreased anxiety/emotionality), consistent with possible sexual dimorphic effects of BPA in the brain. An effect of BPA on the endpoint anxiety/emotionality was judged as Likely.

- Learning and memory

With respect to learning and memory, the data available from single-dose studies in three species (rats, mice and vervet monkeys) indicate impaired cognition in males. Fan et al. (2013) [RefID 1907] observed that male rats given 50 µg/kg bw per day for 10 weeks in a small amount of food (5 g) exhibited increased swimming distance and escape latency during the learning phase for the location of the platform in the MWM test . In the probe trial that tested how well platform location had been memorised, they spent less time in the target quadrant and thus displayed a reduced capacity to remember. In the study of Khan et al. (2018) [RefID 12311], male mice showed a reduced attraction to novel objects after treatment with 10000 µg/kg bw per day for 5 weeks. This can be interpreted as a lack of discrimination between objects of different familiarity or, alternatively, as a fear of novelty. A study conducted with young adult male vervet monkeys (Elsworth et al., 2015 [RefID 1809]), pretrained in a two-choice spatial delayed response test, found a decrease in median per cent correct responses from 90% to about 83% 1 week after the start of exposure to 5555 µg/kg bw per day. The effect was reversible and full recovery was achieved 2 weeks after the end of the exposure. Considering the above evidence, the effects of BPA on the endpoint learning/memory are judged as Likely.

- Locomotor activity/exploration

No effects of BPA on locomotor activity in the open field test (OFT) was seen in a Tier 1 study in male rats with a single-dose level of 50 µg/kg bw per day (Fan et al., 2018 [RefID 11915]), a Tier 3 study in male mice with oral equivalent dose levels of 8888, 88880, 888800 µg/kg bw per day (Liang et al., 2018 [RefID 12508]), and a Tier 1 study in male and female mice with dose levels of 40, 400, 4000 and 40000 µg/kg bw per day (Xu XH et al., 2015 [RefID 8232]). In the latter study, the animals were also tested in the EPM and a mirrored maze that examine entry into specific parts of the maze as an indicator for anxiety and may give some information on locomotor activity when total activity is considered. In both sexes, no effects were seen in the mirrored maze which is more similar to the OFT. BPA treatment also did not affect overall locomotion of female mice in the EPM, whereas males showed reduced overall activity at the two highest doses. However, it is considered that the lower activity of males resulted from the dose-related increase in anxiety and not from a direct impact on locomotor behaviour.

Nojima et al. (2013) [RefID 5435] conducted a single-dose study (Tier 2) with intraperitoneal exposure by minipump that measured spontaneous motor activity of male rats in their home cage. At a dose equivalent to 918 µg/kg bw per day orally they noted a difference in activity to the control group that was most pronounced shortly before and after the transition from dark to light phase. The deviation became statistically significant for the light phase only on days 11–12 after implantation of the minipump. However, the circadian activity pattern was not changed, and the BPA group showed slightly higher overall activity throughout the test period. As no pre-treatment data are reported in the study it is not entirely clear if this is a substance effect or if the rats chosen for the BPA group already showed a higher home cage activity before the experiment started.

Overall, there is sufficient evidence to conclude that effects of BPA on the endpoint locomotor activity are Not Likely.

- Sensory-motor coordination

Khan et al. (2018) [RefID 12311] found sensory-motor coordination deficits in male mice exposed to 10000 µg/kg bw per day. The animals showed a decreased performance on the rotarod and in a grip strength test. Since only one Tier 2 single-dose study was available the evidence was considered Inadequate.

- Social interaction

Two Tier 2 studies in mice examined the effects of BPA on adult social behaviour. Xin et al. (2018) [RefID 13482] studied maternal behaviour in females on lactation day 1 after exposure from before mating and throughout pregnancy. They found no effects on nest building, pup retrieval or time spent in the nest at doses of 10 or 10000 µg/kg bw per day. In the study of Picot et al. (2014) [RefID 5830], sexual behaviour was affected in male mice exposed from week 8 to week 12 of age at a dose of 50 µg/kg bw per day but not at 5000 µg/kg bw per day. The males in the low dose required longer latencies to accomplish their first mount, intromission, thrust and ejaculation; in addition, the number of mounts with intromission and thrusts was also reduced. Therefore, it is judged as Likely that BPA affects male sexual behaviour.

- Preference behaviour

Nuñez et al. (2018) [RefID 11199] examined the preference for sodium salt intake in male and ovariectomised (OVX) female rats during seven days of subcutaneous exposure to BPA in oral equivalent doses of 357, 1785, 3570 and 17850 µg/kg bw per day. The males decreased both their spontaneous water intake and the consumption of NaCl solution at all doses except in the lowest dose group and showed a decreased preference for saline after fluid deprivation at 357, 3570 and 17850 µg/kg bw per day. No differences in preference were noted between the groups. For OVX females, decreased water intake was only observed at the highest dose, but they showed reductions in spontaneous salt intake and reduced preference for salt after fluid deprivation at the same doses as the males. The treated females showed a reduced preference for 2.7% NaCl at all doses when compared with the control group, but the effect was only statistically significant at 3570 µg/kg bw per day. The findings may indicate an effect of BPA on body fluid regulation that leads to differences in intake behaviour. The BPA effects on this endpoint is judged as Likely.

Overall, the CEP Panel assigned a likelihood level of Likely to the behavioural effects of BPA exposure in adult males and females based on increased anxiety/depression-like behaviour in male mice (Xu XH et al., 2015 [RefID 8232] and Liang et al., 2018 [RefID 12508]) and decreased anxiety in female mice (Xu XH et al., 2015 [RefID 8232]); impaired male sexual behaviour in mice (Picot et al., 2014 [RefID 5830]) and changes in salt preference in rats (Nuñez et al., 2018 [RefID 11199]). Therefore, these endpoints were taken forward for BMD analysis (see Chapter 3.2.1) and for uncertainty analysis (see Appendix D). The endpoints anxiety/depression-like behaviour in male rats (Fan et al., 2018 [RefID 11915]) and impaired learning and/or memory in male rats (Fan et al., 2013 [RefID 1907]), mice (Khan et al., 2018 [RefID 12311]) and monkeys (Elsworth et al., 2015 [RefID 1809]) were not taken forward for BMD analysis because the studies are single-dose studies.

Indirect (germline) exposure

Behavioural endpoints after exposure through the germline were examined in the offspring of rats and mice that had been treated with BPA before mating of the animals began. For effects through the female germline one study in mice (Xin et al., 2018 [RefID 13482]) allocated to Tier 2 with a low and a high-dose group (10 and 10000 µg/kg bw per day) was available, in which the female parents had been exposed throughout their own development until weaning. This study did not identify any changes in the two endpoints that were examined in their male offspring (anxiety/emotionality, locomotor activity) at any dose. Female offspring was not tested.

Two Tier 1 studies in rats (Fan et al., 2018 [RefID 11915]; Fan et al., 2013 [RefID 1907]) and one Tier 1 study in mice (Luo et al., 2017 [RefID 4661]) dealt with possible consequences of the exposure of the male parent and compared only a single-dose group of BPA of either 50 µg/kg bw per day (in rats) or 10000 µg/kg bw per day (in mice) to a control group.

- Anxiety/emotionality

The endpoint of anxiety/emotionality was examined by three different procedures in young adult rats offspring (Fan et al., 2018 [RefID 11915]) and identified increased anxiety in females with all three tests (Forced Swim Test, Open Field and Elevated Maze). In the males, this finding was only obvious in the Forced Swim Test, which can be considered to impose a higher level of stress on the animals compared with the other testing procedures, open field and elevated maze. In the mouse study (Luo et al., 2017 [RefID 4661]), these latter tests indicated increased anxiety in juvenile and young adult

male offspring, respectively. Female mice were not tested. Effects on this endpoint were judged as Likely.

- Learning and memory

Spatial learning of male and female offspring was affected in the MWM in a rat study (Fan et al., 2013 [RefID 1907]). The animals needed more time and longer swimming distances to locate the escape platform during the acquisition process. However, memory of the learned positions appeared affected only in females, not in males. As only a single-dose study was available, this evidence was considered Inadequate.

- Locomotor activity/exploration

For the endpoint locomotor activity/exploration one study in rats (Fan et al., 2018 [RefID 11915]) and one study in mice (Luo et al., 2017 [RefID 4661]) were available. In young adult rats, no effects were observed on the distance travelled in the open field in either sex. Juvenile male mice showed reduced overall locomotor activity in the same test. No data were obtained for female mice. In addition, a study with male mice (Xin et al., 2018 [RefID 13482]) did not find any changes of exploratory/locomotor activity in a hole board test. An effect of BPA on locomotor behaviour was considered Not Likely.

- Social interaction

Social interaction was studied in male mice only (Luo et al., 2017 [RefID 4661]) and revealed a decrease in exploration time of same sex strangers and a reduced preference for social contact. As only a single-dose study was available, this evidence was considered Inadequate.

Overall, The CEP Panel assigned a likelihood level of Likely to the behavioural effects of BPA exposure through the male germline based on consistent findings in two species (rats and mice) for the endpoint anxiety/emotionality (in Fan et al., 2018 [RefID 11915] and Luo et al., 2017 [RefID 4661]). However, both of these studies were conducted with only a single dose of BPA and none of them was taken forward for BMD analysis.

Overall cluster selection of the endpoints/studies for BMD analysis for Behaviour

Overall, the CEP Panel assigned a likelihood level of Likely to effects of BPA on behaviour in the exposure periods developmental until weaning, growth phase/young age, adult age and indirect (germline) exposure periods.

The CEP Panel considered that the evidence from the studies available showed a Likely effect of BPA in the exposure period developmental (pre-natal and/or post-natal development until weaning) for the endpoints anxiety/emotionality (Xin et al., 2018 [RefID 13482]) and learning and memory (Johnson et al., 2016 [RefID 3241]; Wang C et al., 2016 [RefID 7576]; Wang C et al., 2014 [RefID 7579]). Therefore, these endpoints were taken forward for BMD analysis (see Chapter 3.2.1).

The CEP Panel considered that the evidence from the studies available showed a Likely effect of BPA in the exposure period growth phase/young age for the endpoints anxiety/emotionality (Chen Z et al., 2018 [RefID 11734]; Luo et al., 2013 [RefID 11330]) and learning and memory (Chen Z et al., 2018 [RefID 11734]; Zhou YX et al., 2017 [RefID 9083]) in males.

The CEP Panel considered that the evidence from the studies available showed a Likely effect of BPA in the exposure period adult age for the endpoints anxiety/emotionality (Xu XH et al., 2015 [RefID 8232]), sensory-motor coordination (Khan et al., 2018 [RefID 12311]), and salt preference (Nuñez et al., 2018 [RefID 11199]). Therefore, these endpoints were taken forward for BMD analysis (see Chapter 3.2.1).

The CEP Panel considered that the evidence from the studies available showed a Likely effect of BPA in the exposure period of the male germline for the endpoint anxiety/emotionality (Fan et al., 2018 [RefID 11915]; Luo et al., 2017 [RefID 4661]). These endpoints were not taken forward for BMD analysis because single-dose studies.

The overall likelihood across all exposure periods, i.e. the highest likelihood given in the cluster effects on behaviour, was Likely.

3.1.5.3. Integration of likelihoods from human and animal studies

Table 12 presents the overall likelihood per cluster for the human and animal stream separately, as well as the integration of the likelihoods from the human and animal studies for Neurotoxicity and developmental neurotoxicity.

Table 12: Integration of the human and animal studies for Neurotoxicity and developmental neurotoxicity.

Human stream		Animal stream		Integrated likelihood
Cluster: Neurodevelopment (behaviour after developmental exposure)		**Cluster: Behaviour**		
Exposure during Pregnancy	Not Likely	Developmental (pre-natal and/or post-natal until weaning)	Likely	
Exposure during Childhood	Not Likely	Growth phase/young age	Likely	
		Adult exposure (after puberty)	Likely	
		Indirect (germline) exposure	Likely	
Overall likelihood:	*Not Likely*	*Overall likelihood:*	*Likely*	*Likely*
Cluster: Neuromorphology		**Cluster: Neuromorphology**		
Not applicable		Developmental (pre-natal and/or post-natal until weaning)	Likely	
		Growth phase/young age	Likely	
		Adult exposure (after puberty)	Inadequate evidence	
		Overall likelihood:	*Likely*	*Likely*
Cluster: Nervous system functionality		**Cluster: Nervous system functionality**		
Not applicable		Developmental (pre-natal and/or post-natal until weaning)	ALAN	
		Growth phase/young age	Inadequate evidence	
		Adult exposure (after puberty)	Likely	
		Indirect (germline) exposure	Inadequate evidence	
		Overall likelihood:	*Likely*	*Likely*

3.1.5.4. *In vitro* and Mechanistic studies

BPA effects are reported over a huge range of effective concentrations/doses (low nM and µg/kg per day to high µM and >1 mg/kg). This always needs to be considered; there may be qualitative as well as quantitative differences in mechanisms, depending on dose. In addition, apparent inconsistencies between studies may result from heterogeneity in study design (experimental models, route and window of exposure, dose of BPA, age at time of assessment, testing procedure, etc.).

It is clear that many of the reported effects of BPA on Neurotoxicity and developmental neurotoxicity endpoints are downstream pleiotropic effects (e.g. altered expression of many genes and proteins; ERK1/2 signalling, Wnt/β-catenin, Tmprss2, Foxa1, NGF, SOX2, Pax6, Grin2b, JNK, CREB and p53-mitochondrial apoptosis pathways, Gnrh1, Calbindin-D28, Kisspeptin (Kiss1), GNRH, DNA methylation (Dnmt, MECP2), hypotaurine, NMDA, GABA, oxytocin, serotonin and dopamine signalling, hypothalamic–pituitary axes (HPA, HPT and HPG), BDNF-NTRK2 neurotrophin system, etc.)

Details of upstream mechanisms, which are more likely to be BPA specific, are unclear, but receptor interactions are an obvious candidate mechanism. In particular, ER-dependent pathways have been implicated in a number of studies (Mhaouty-Kodja et al., 2018).

Mhaouty-Kodja et al. (2018) concluded that effects of BPA on learning and memory appear to be associated with the disruption of oestrogen-dependent pathways and of cerebral glutamate-NMDAR pathway (downstream targets leading to gene transcription of ERK, CREB, Brain-derived neurotrophic factor BDNF and synaptic proteins involved in synaptic plasticity).

It is unfortunately difficult to generalise data from studies reporting receptor-dependency of BPA effects, because steroid receptor properties and interactions are dynamic; substance effects can vary by tissue, life-stage and/or dose. In fact, much of the complexity of steroid receptor mechanisms has been revealed by research with BPA.

Oxidative stress generation is an additional candidate mechanism for BPA effects on AChE activity and other endpoints. There is some evidence that BPA-induced oxidative stress is involved in brain functional effects at both low and high doses, but the data are not consistent. In humans, Condolot et al. (2016) [RefID 3646] sought to identify correlations between BPA levels and oxidant-antioxidant parameters in autistic and non-autistic children. No significant correlations were observed.

Neuromorphology

There was no human evidence available for the cluster Neuromorphology. Thus, the overall likelihood of effects of BPA for this cluster was scored Likely, based on the animal evidence.

Low-dose BPA-related reduction of dendritic spine density and/or cell number during development and/or growth phase (hippocampal CA1 and CA3) was judged as Likely.

BPA-induced mitochondria-related oxidative stress is one possible MoA.

Agarwal et al. (2016) [RefID 52] concluded that pre-natal and post-pubertal low dose (40 μg/kg bw per day) of BPA impaired rat hippocampus mitochondrial fusion/fission dynamics and autophagy-mediated mitochondrial turnover, leading to increased oxidative stress, mitochondrial fragmentation, and apoptosis in hippocampal neural stem cells (NSCs), and inhibited hippocampal derived NSC proliferation and differentiation.

A possible mechanism for BPA effects on mitochondria is interaction with calcium channels.

Michaela et al. (2014) [RefID 5081] reported that nanomolar concentrations of BPA inhibited calcium current through T-type calcium channels (TCCs) in HEK cells. They suggested that this high-affinity low-efficacy inhibition may be caused by direct binding of BPA to TCCs in their resting state.

Chen et al. (2020) reported that TCC blockade (in C2C12 myoblasts) induced mitochondria-related apoptosis and reduced mitochondrial transmembrane potential (MMP), induced mito-ROS generation, and enhanced expression of mitochondrial apoptosis proteins.

Jiang et al. (2015) [RefID 3189] reported BPA-related decreased activities of mitochondrial respiratory complexes and abnormalities in mitochondrial morphology in rat cardiac myofibrils, including decreased mitochondrial volume density, reduced cristae density and increased vacuoles, after low-dose BPA (50 μg/kg bw per day) for 48 weeks. They considered it possible that the changes in mitochondrial function were a primary event to observed BPA-induced myocardial hypertrophy.

Mitochondria are the major source of cellular ROS, and there is evidence that neural stem cell activity is modulated by ROS (Hou et al., 2012; Adusumilli et al., 2021). BPA-induced ROS could be a possible MoA for the reported alterations in hippocampal cell numbers.

Overall, there is some evidence for a connection between BPA-induced mitochondria-related oxidative stress, possibly related to calcium channel blockade, and effects on neuromorphology.

Nervous system functionality

There was very limited human evidence available for the cluster Nervous system functionality, not fitting for the WoE. Thus, the overall likelihood of effects of BPA for this cluster was scored Likely, based on the animal evidence.

More specifically, Alavian-Ghavanini et al. (2018) [RefID 11471] investigated initially if low-dose developmental BPA exposure affects DNA methylation and expression of Grin2b (glutamate ionotropic receptor NMDA type subunit 2B) in brains of adult rats. They reported that developmental exposure to BPA changes the DNA methylation level in the Grin2b promoter, results in altered gene expression levels in female, but not in male, rats 1 year after the exposure had ceased. Extending their investigation to humans, the authors report that pre-natal BPA exposure was associated with increased methylation levels in girls. Attempting an indirect link they also report that low APGAR scores, a predictor for increased risk for neurodevelopmental diseases, were associated with higher Grin2b methylation levels in girls than boys.

Kundakovic et al. (2015) [RefID 3756] showed that pre-natal exposure to BPA induces lasting DNA methylation changes in the transcriptionally relevant region of the BDNF gene in the hippocampus and blood of BALB/c mice. Extending their work in humans, they examined BDNF IV DNA methylation in cord blood samples from the CCCEH cohort where high maternal BPA exposure had been associated with adverse behavioural effects in different sex groups. High pregnancy BPA levels (based on maternal spot urine) were associated with altered DNA methylation of two CpG sites in the human cord blood with an observed trend for a sex-specific effect of high BPA on CpG1A methylation and a significant sex-specific effect of high BPA exposure on CpG1B methylation levels.

Yang CW et al. (2014) [RefID 8324] identified candidate genes of neuronal development by implementing a gene ontology analysis and formed a reconstructed neuronal sub-network. Subsequently, their gene expressions were determined in 20 umbilical cord blood samples dichotomised into high and low BPA level tiers. Two neuronal genes, sex determining region Y-box 2 (Sox2) and paired box 6 (Pax6), had preferentially downregulated expression in response to BPA exposure. Fetal cord blood samples had the obviously attenuated gene expression of Sox2 and Pax6 in high BPA group referred to low BPA group. Visualised gene network of Cytoscape analysis showed that Sox2 and Pax6 which were contributed to neural precursor cell proliferation and neuronal differentiation might be downregulated through sonic hedgehog (Shh), vascular endothelial growth factor A (VEGFA) and Notch signalling. These results indicated that trans-placental BPA exposure downregulated gene expression of Sox2 and Pax6 potentially underlying the adverse effect on childhood neuronal development.

In animals, changes in neurotransmitters (GABA, noradrenaline NA, dopamine DA, 5-hydroxytryptamine 5HT serotonin) and OR were judged ALAN during developmental (pre-natal and/or post-natal until weaning) exposure.

At high doses (≥10000 µg/kg bw per day), BPA increased hippocampal neurotransmitters and also increased markers of oxidative stress and lipid peroxidation (Khadrawy et al., 2016 [RefID 3462]). It is therefore possible that these neurotransmitter changes were downstream to (i.e. a result of) BPA-induced oxidative stress.

At low dose (10 µg/kg bw per day), mouse hippocampal DA was increased in males only, and NA was decreased in females only, without changes in GABA and GLN (Xin et al., 2018 [RefID 13482]).

Low-dose BPA (≤2500 µg/kg bw per day) increased male OR density, and thus eliminated sex differences, in various sexually dimorphic brain nuclei ($BNST_{dl}$, VMH, paraventricular hypothalamic nucleus PVN (Witchey et al., 2019 [RefID 13782]).

Given that these low-dose effects are gender-specific, it is possible that the MoA involves interactions with oestrogen (ER) and androgen (AR) receptors.

Altered AChE activity after adult exposure to BPA was judged Likely.

Low dose BPA (50 µg/kg bw per day) decreased AChE activity (−36%) in the rat hippocampus and impaired spatial memory (Fan et al., 2013 [RefID 1907]). Given that BPA is reported by others to induce oxidative stress at low doses, this reduced AChE activity could be caused by oxidative stress, cf. Schallreuter et al. (2004).

At higher doses (20000–50000 µg/kg bw per day), BPA increased cortical AChE activity (by about 20–50%) (Khadrawy et al., 2016 [RefID 3462]). Hippocampal AChE activity was similarly significantly increased after 10000 µg/kg for 6 weeks and 25000 µg/kg for 6 weeks, but not after 25000 µg/kg bw per day for 6 weeks, i.e. this was not a consistent effect.

A possible mechanism for the increased brain AChE activity at high doses is altered membrane fluidity leading to increased extracellular enzyme exposure, as proposed in the study by Macczak et al. (2017) [RefID 4760] for erythrocyte AChE activity ('AChE is located in erythrocyte membrane on phosphatidylinositol, and thus changes in membrane fluidity may lead to stronger exposure of this enzyme outside of the cell, which results in an increase of its activity.'). Such a mechanism implies cell damage, but brain histopathology, reported by others, was not measured in Macczak et al. (2017) [RefID 4760].

By contrast, Khan et al. (2018) [RefID 12311] reported reduced AChE activity (about −35%) in whole brain of adult mice dosed orally with BPA 10000 µg/kg bw per day for 30 days. Oxidative stress biomarkers in brain homogenates were also significantly increased (and neurobehavioural and cognitive performance was reduced). These study data support the hypothesis that oxidative stress is a causative upstream event leading to the observed decrease in AChE activity.

The reported relationship between both high-dose and low-dose BPA exposure and brain AChE activity is inconsistent, thus, it is not possible to propose a single mechanism, although there is some evidence suggesting that BPA-induced oxidative stress may be involved.

Behaviour

After the integration of the human and animal evidence, the overall likelihood of effects of BPA for the cluster Effects on behaviour was scored Likely.

Changes in behavioural endpoints are expressed downstream of functional and/or structural changes in the brain and are considered the apical indication that a living system has exhausted its innate ability to compensate for changes induced in the underlying processes.

Effects of BPA on learning and memory performances were judged as Likely in both sexes for two periods of exposure, i.e. the developmental (pre-natal and/or post-natal until weaning) and the adult period (after puberty), whereas it was limited to the males for BPA exposure in the growth phase and/or the young age. Neurofunctional clusters interrelated with learning and memory performances were judged as Likely for the hippocampal and cortical AChE activity for an exposure occurring at the adult stage (after puberty), and ALAN for neurotransmitter systems in various parts of the brain following a developmental exposure (pre-natal and/or post-natal until weaning). Concomitant neuromorphological changes including dendritic morphology and spine density in hippocampus and cortex were also judged as Likely for the two earliest periods of exposure (development until weaning and growth phase and/or young age) and as ALAN for exposure limited to the adult stage (after weaning).

Effects of BPA on anxiety and depression-like behaviour were judged as Likely in all exposure periods: during pre-natal and/or post-natal exposure until weaning (in mice); during the growth phase and young age (in male rats and mice); in adult animals; and also, after indirect exposure through the germline of the male parent. Possible mechanisms include changes in corticosteroid regulation and in Wnt/β-catenin signalling.

Corticosterone regulation

Sex-specific functional alterations of the hypothalamic–pituitary–adrenal (HPA) axis and concomitant changes in anxiety-like behaviour have been described after developmental exposure to BPA. Chen F et al. (2014, 2015) [RefID 1012, RefID 1013] reported a reduction in the HPA axis response to stress for females ('anti-anxiety-like' behaviour), whereas in males they found a hyperactivation. BPA-exposed males, but not females, had higher basal levels of serum corticosterone and adrenocorticotrophic hormone (ACTH), as well as an increase of corticotropin releasing hormone (CRH) mRNA in the hypothalamic PVN.

Pubertal female rats exposed to BPA exposure at 40 µg/kg bw per day during pregnancy and lactation showed increased basal corticosterone and reduced hypothalamic GR levels. A stress challenge elicited more anxiety-like behavioural coping and a weaker corticosterone response compared with control females (Panagiotidou et al., 2014 [RefID 5627]). In female rat offspring exposed to BPA at 40 µg/kg bw per day during pregnancy and lactation, Zhou et al. (2015) [RefID 9062] found a significant increase in both basal (morning) and peak (afternoon) corticosterone release compared with controls and increased basal and peak plasma ACTH at the same time points. In the hippocampus, mRNA expression

for GR, mGlu2 and mGlu3 receptors as well as proteins levels of mGlu2/3 receptors were decreased, suggesting that both increased corticosterone levels and decreased signalling through hippocampal mGlu2/3 contribute to the anxiety and depression-like behaviour observed after BPA exposure.

The increased corticosterone production observed in BPA-exposed animals seems to involve impaired feedback from the hippocampus to the HPA axis which leads to increased levels in CRH and ACTH. The CRH signalling pathway was identified among the top scoring pathways in the transcriptome of the amygdala in male and female neonatal rats after pre-natal BPA exposure at 25 µg/kg bw per day (Arambula et al., 2018 [RefID 11050]). An activation of Cyp11A1 (P450scc) in the adrenal was shown by Medwid et al. (2016) [RefID 4996] in mice at 5000 µg/kg bw per day and by Lan et al. (2015) [RefID 3825] in mouse Y1 adrenal cortex cells in the BPA concentration range 50–1000 nM, and in rats injected subcutaneously with BPA (0.5 µg/kg bw per day for 3 days, equivalent to oral 17.85 µg/kg bw per day). In addition to this increase of adrenal steroid synthesis, an upregulation of corticosterone production could also occur in the neurons themselves; hippocampal neurons contain the complete pathway of corticosteroid synthesis and normally produce low levels of corticosterone (approximately 7 nM) that are sufficient to modulate synaptic plasticity (Hojo et al., 2011). Thus, a cell-autonomous overproduction could augment the adverse effects of circulating corticosteroids on synaptic plasticity and affective behaviours.

A possible mechanism for the effects of BPA on both corticosterone and anxiety is via ER-mediated altered hippocampal expression of FKBP5 (FKBP51), a GR binding protein that negatively regulates GR, which is upregulated by stress, and is connected to neuronal synaptic plasticity (Qiu et al., 2019). Increased Fkbp5 promoter methylation (decreased protein expression) has been reported to be associated with an anxiety phenotype and increased corticosterone levels in mice after pre-natal trauma (Plank et al., 2021). Kitraki et al. (2015) [RefID 3589] found an increase in DNA methylation of this gene at a BPA dose of 40 µg/kg bw per day in male rats, which probably resulted from a reduction of ERβ binding to a site in intron 5 of the gene.

Similar to BPA, increased corticosterone levels have been associated with a reduction in dendritic spine density in hippocampus and PFC and an increase in anxiety/depression-like behaviour (Wang G et al., 2013a).

In conclusion, it appears that the effects on the HPA axis at the molecular level, the morphological decrease of dendritic spine density in hippocampus and PFC, and the apical change in affective behaviour (anxiety/depression-like behaviour) are causally connected and delineate a MoA for BPA neurotoxicity. In the dose range between 2 and 40 µg/kg bw per day, BPA exposure increased serum corticosterone by 10–60%. It decreased dendritic spine density or dendritic spine synapses in layer II/III mPFC pyramidal cells and hippocampus for all exposure periods (lowest effective dose reported was 40 µg/kg bw per day) and increased anxiety/depression-like behaviour (lowest effective dose reported at 10–20 µg/kg bw per day). A MoA involving increased corticosterone production could be envisaged as follows:

- Binding of BPA to ERβ changes specificity for target genes (including Fkbp5 in hippocampus) imply a decreased Fkbp5 expression, which would lead to an increase in CRH and ACTH. This activates Cyp11A1 (P450scc) in mitochondria of adrenal cells (and possibly in hippocampal neurons), causing an increase of corticosterone production through the c-Jun JNK) signalling pathway, an increase of serum corticosterone and a corticosterone-dependent decrease in dendritic spine density associated with increased anxiety/depression.

Altered Wnt/β-catenin pathway activity is a further possible MoA. Arambula et al. (2018) [RefID 11050] identified Wnt/β-catenin signalling (four genes) as one of the top pathways affected by BPA in neonate amygdala at 25 µg/kg bw per day in males. BPA was shown to downregulate this pathway and to increase β-catenin degradation in rat brain and in cultured hippocampal neurons in studies by Liu ZH et al. (2014, 2015) [RefID 10411, RefID 4535] and by Tiwari et al. (2015, 2016) [RefID 7220, RefID 7221]. BPA exposure upregulated GSK3β which phosphorylates β-catenin and marks it for degradation in the absence of ligand. As a consequence, nuclear translocation of β-catenin was decreased in the hippocampus and the subventricular zone (SVZ). Proliferation and neuronal differentiation of hippocampus-derived neural stem cell as well as hippocampal and subventricular zone neurogenesis were impaired (Tiwari et al., 2015 [RefID 7220]). BPA-induced decreases in dendritic spine density were noted in the dentate gyrus and the CA1 area of the hippocampus by Liu ZH et al. (2015) [RefID

4535]. This finding was replicated in cultured CA1 neurons with BPA concentrations of 10 nM or greater. Importantly, this *in vitro* effect was abolished by addition of a Wnt ligand (Wnt7a) to the culture, suggesting that Wnt is causally involved in the effect.

Wnt signalling together with BDNF cooperatively regulates dendritic spine formation; Wnt signalling inhibition in cultured cortical neurons disrupts dendritic spine development (Hiester et al., 2013). BDNF is a direct target of Wnt signalling, being induced through Wnt-dependent TCF/LEF transcription factors (Yi et al., 2012) and then can exert a positive feedback on Wnt signalling through induction of Wnt2 which is sufficient to promote cortical dendrite growth and dendritic spine formation *in vitro*. BPA diminished the expression of LEF-1 and TCF mRNAs and proteins in the hippocampus of rats (Tiwari et al., 2015 [RefID 7220]).

Activation of Wnt-dependent β-catenin signalling decreased the expression of steroidogenic genes (including Cyp11a1) in an adrenocortical cell line and caused a reduction in the release of corticosterone (Walczak et al., 2014). The inhibitory effect of BPA on the Wnt pathway may contribute to the upregulation of corticosterone production.

In addition to the canonical (β-catenin dependent) pathway, non-canonical Wnt pathways appear to be involved in BPA effects on neurons. Liu ZH et al. (2014) [RefID 10411] reported a dose-dependent decrease of the canonical ligand Wnt7a while the non-canonical ligand Wnt5a increased in hippocampal dentate gyrus homogenates. Wnt5a has been shown to modulate mitochondrial dynamics in cultured hippocampal neurons and to promote fission of mitochondria through recruitment of the fission regulator Drp1 from the cytosol to the mitochondria. The change in mitochondrial morphology was associated with significant increases in cytosolic and mitochondrial Ca^{2+} levels (Godoy et al., 2014). Agarwal et al. (2016) [RefID 52] showed that BPA increased mitochondrial fragmentation in the hippocampus (including dentate gyrus and CA regions) of the rat brain at a dose level of 40 µg/kg bw per day and in hippocampal NSC-derived neuron cultures at a concentration of 100 µM.

In conclusion, these studies support the involvement of canonical and non-canonical Wnt pathways in the effects of BPA on functional and structural parameters in the brain and their downstream consequences for behavioural endpoints.

3.1.5.5. Conclusion on hazard identification for Neurotoxicity and developmental neurotoxicity of BPA

In the EFSA opinion 2015 (EFSA CEF Panel, 2015), a likelihood level of ALAN was assigned to neurological, neurodevelopmental and neuroendocrine effects of BPA in a WoE approach. This was based on the results from prospective epidemiological studies examining children exposed to BPA during the pre-natal period. The studies provided evidence for an association with sex-dependent behavioural problems but not sufficient proof for a causal link, due to inconsistent findings across studies. Animal studies, while indicating a possible impairment of brain functions and behavioural parameters such as anxiety-like behaviour, learning and memory, social behaviour and sensory-motor function, presented methodological shortcomings as well as inconsistent results from different studies. Therefore, the CEP Panel decided not to take these effects forward to derive the toxicological RP but used them in the analysis of uncertainty for hazard characterisation and risk characterisation.

In the current assessment, epidemiological evidence derived from newly available longitudinal studies examining children with exposure during pregnancy or post-natally did not suggest any endpoints related to neurodevelopment as critical for risk assessment. The children were followed for various time periods, up to the age of 10 years. Although some statistically significant associations were observed, none of them occurred in more than one study and subsequent research failed to replicate the results.

The available cross-sectional studies produced some evidence for associations of BPA exposure and various neurodevelopmental endpoints in children but are not considered robust enough on their own to support an adverse association.

With respect to the animal studies, the results of the present evaluation extend the previous database and indicate possible effects of BPA during development and in adults mainly on anxiety and depression-related behaviours, learning and memory, as well as on dendritic spine density and AChE activity in the hippocampus and the PFC. Loss of spines in these brain regions has also been observed in association with impaired cognitive ability and mood disturbances in a number of other animal models. Thus, it can

be assumed that the reduction of dendritic spine density forms the structural basis for the behavioural changes induced by BPA. The mechanisms that underly the effect on spine density are less clear. Spine formation may be affected by various signals, including neurosteroids, neurotransmitters and their receptors, synaptic plasticity-promoting proteins, signalling pathways and oxidative stress, most of which have also been identified in studies that examined possible MoAs for BPA. This includes for example reductions in the expression of ERs, overproduction of corticosterone, downregulation of NMDA receptors, changes in PSD-95 expression and interference with Wnt/β-catenin signalling.

Overall, the CEP Panel considers that the different effects observed on brain structure, neurochemistry and functional outcome, e.g. behaviour, can be integrated into a convincing picture that indicates the existence of a neurotoxic hazard of BPA in developing, growing and adult animals.

Using a WoE approach, the CEP Panel assigned a likelihood level of Likely to the effect of BPA on anxiety/emotionally, learning and memory, salt preference, dendritic spine density and AChE activity. Therefore, these endpoints were brought forward for BMD analysis (see Chapter 3.2.1).

3.1.6. Reproductive and developmental toxicity

3.1.6.1. Epidemiological studies

For the HOC Reproductive and developmental toxicity, a total of 47 studies was appraised by the CEP Panel. The details of the appraisals (internal validity) are reported in Annex B.

Identification of the clusters to be considered for WoE

On the basis on the approach described in Chapter 2.3.2 'Definition of Health Outcome Categories and Clusters', the following Clusters (C) and Exposure periods (Exp) were brought forward to WoE analysis:

- C: Fetal and post-natal growth
  - Exp: Adulthood
- C: Prematurity
  - Exp: Pregnancy
- C: Pre-eclampsia
  - Exp: Adulthood
- C: Male fertility
  - Exp: Adulthood
- C: Female fertility
  - Exp: Adulthood

WoE of the relevant clusters

The main information extracted from the studies included in relevant clusters in the HOC Reproductive and developmental toxicity are summarised in Annex C. The outcome of the weight of the evidence is described in the text below and presented in a tabulated format in Annex D.

Cluster Fetal and post-natal growth

Exposure during pregnancy

A total of 13 studies reported results on indices of fetal growth (Burstyn et al., 2013 [RefID 762]; Philippat et al., 2014 [RefID 5821]; Smarr et al., 2015 [RefID 6784]; Veiga-Lopez et al., 2015 [RefID 7422]; Birks et al., 2016 [RefID 591]; Casas et al., 2016 [RefID 879]; Ferguson et al., 2016b [RefID 1995]; Huang YF et al., 2017a [RefID 2925]; Pinney et al., 2017 [RefID 5838]; Woods et al., 2017 [RefID 8003]; Lee YM et al., 2018 [RefID 11150]; Lester et al., 2018 [RefID 12446]; Mustieles et al.,

2018b [RefID 12784]). Of these two studies examined associations with measures of growth *in utero* based on ultrasound measures (Philippat et al., 2014 [RefID 5821]; Lee YM et al., 2018 [RefID 11150]) and two studies examined associations between maternal pregnancy BPA concentrations with measures of post-natal growth up to 3 (Philippat et al., 2014 [RefID 5821]) and 6 years of age (Lee YM et al., 2018 [RefID 11150]). Overall, no consistent associations with BW, birth length, head circumference or other indices measured *in utero* or at birth were observed. In most cases the effect estimates were centred around the NULL with wide confidence intervals but significant associations were observed in a few studies. As an example, a study by Lee YM et al. (2018) [RefID 11150] reported a significant increase in BW with higher maternal exposure while a significant decrease in femoral length *in utero* was observed. Another study by Mustieles et al. (2018b) [RefID 12784] examined associations with BW among 346 subjects who were having their fertility status examined. In that study a significant inverse association between maternal pre-pregnancy BPA concentration and BW was observed [~79 g decrease in BW (95%CI: −153, −5) for ~3-fold increase in BPA exposure]. For maternal samples collected during pregnancy this association was in the same direction but non-significant [−38 g (95%CI: −101, 25)]. Although interesting, this inverse association for maternal pre-pregnancy concentration would need to be replicated in another study before any robust conclusions can be drawn.

In terms of possible high exposures, Birks et al. (2016) [RefID 591] used individual data from 13 European birth cohorts to identify pregnant women who had been occupationally exposed (based on self-report) to BPA during pregnancy. Of 133,957 individuals a total of 59 women with occupational exposure were identified. Mean birth eight among these exposed women was not significantly different from those unexposed.

The few studies that examined more clinically relevant birth outcomes such as small for gestational age (i.e. babies with LBW (below the 10th percentile), when controlling for gestational age) and LBW did not find any association with maternal BPA exposure (Burstyn et al., 2013 [RefID 762]; Lester et al., 2018 [RefID 12446]). However, only the case–control study by Burstyn et al. (2013) [RefID 762] had sufficient statistical power to evaluate this outcome with some accuracy.

Concerning post-natal growth, the study by Lee YM et al. (2018) [RefID 11150] also reported some significant associations between maternal concentrations of BPA in pregnancy and weight and weight for length from age 3 to 6 years of age. In contrast, no association with weight or length up to 3 years of age was observed in the study by Philippat et al. (2014) [RefID 5821].

Overall conclusions

On the basis of the above, the CEP Panel concluded that an association between maternal BPA exposure and impaired pre-natal and post-natal growth is Not Likely.

Cluster Prematurity

Exposure during pregnancy

A total of seven studies examined the association between maternal BPA concentrations and length of gestation or preterm delivery (Weinberger et al., 2014 [RefID 7909]; Cantonwine et al., 2015 [RefID 823]; Smarr et al., 2015 [RefID 6784]; Veiga-Lopez et al., 2015 [RefID 7422]; Birks et al., 2016 [RefID 591]; Casas et al., 2016 [RefID 879]; Pinney et al., 2017 [RefID 5838]).

In a cohort of 72 pregnant women Weinberger et al. (2014) [RefID 7909] reported a significant inverse association between total BPA concentration in urine and length of gestation (~1 day shorter gestation for each interquartile increase in exposure). In contrast, a relatively large case–control study sampling 130 preterm cases and 352 random controls sampled from a larger birth cohort (n = 2246) did not find any association between maternal urinary BPA exposure (samples collected minimum three times during pregnancy) and preterm delivery (Cantonwine et al., 2015 [RefID 823]). No association was consistently observed in other studies looking at length of gestation.

In studies examining associations with length of gestation no associations were observed (Smarr et al., 2015 [RefID 6784]; Veiga-Lopez et al., 2015 [RefID 7422]; Casas et al., 2016 [RefID 879]; Pinney et al., 2017 [RefID 5838]).

In terms of possible high exposures Birks et al. (2016) [RefID 591] reported a slightly longer gestation (~4 days) among 59 that were likely to have been occupationally exposed to BPA (based on self-report) compared with unexposed women (n = 116,358).

Overall conclusions

On the basis of these studies, the CEP Panel concluded an association between BPA exposure and shorter duration of gestation or increased risk of preterm delivery is Not Likely.

Cluster Pre-eclampsia

Exposure during adulthood

Two case–control studies, Ye et al. (2017) [RefID 8483] n = 173 and Cantonwine et al. (2016) [RefID 824], n = 481 for a total number of cases of 123, assessed the association between BPA exposure measured in spot urine (n = 1) or serum (n = 1) samples in pregnancy and endpoints related to pre-eclampsia (onset and/or severity). Their detailed description and risk of bias assessment related to these studies are provided in Annexes C and D. One study was conducted in USA (Cantonwine et al., 2016 [RefID 824]) and one study was conducted in China (Ye et al., 2017 [RefID 8483]) with similar endpoint definitions. In the latter, BPA exposure (continuous and per tertile) was statistically significantly associated with pre-eclampsia, pre-eclampsia onset and pre-eclampsia severity.

Overall conclusions

On the basis of the above, the CEP Panel concluded that the evidence for a positive association between BPA exposure and pre-eclampsia is ALAN**.**

Cluster Male fertility

Exposure during adulthood

A total of five studies (Buck Louis et al., 2014 [RefID 4599]; Bae et al., 2015 [RefID 347]; Dodge et al., 2015 [RefID 1648]; Goldstone et al., 2015 [RefID 2304]; Buck Louis et al., 2018 [RefID 12602]) in three different cohorts examined the relationship between urinary concentrations of BPA and fertility in males and females.

Among couples (n = 218) seeking fertility treatment Dodge et al. (2015) [RefID 1648] found no association between urinary BPA concentrations in men and fertilisation or live birth following *in vitro* fertilisation or insemination. Similarly, Buck Louis et al. (2014) [RefID 4599] examined associations between urinary concentrations in both male and female couples (n = 501) with fecundability. No association was observed for urinary BPA concentrations in males and females.

In a later study of 339 males form the same cohort (Buck Louis et al., 2018 [RefID 12602]) no association between BPA concentrations in seminal plasma and fecundity was also observed.

These findings are in line with a study by Goldstone et al. (2015) [RefID 2304] where no association was observed between urinary BPA concentrations and semen quality (total count, concentration or morphology) in 418 males.

Finally, Bae et al. (2015) [RefID 347] reported association between paternal BPA exposure and fewer male births (lower male to female sex ratio). As no other studies have reported on this outcome and in the light of the fact that none of the other studies found consistent associations with live birth rate, fecundability or other fertility outcomes a chance finding seems plausible.

Overall conclusions

On the basis of the above, the CEP Panel concluded that an association between exposure to BPA measured in spot urine and reduced fertility is considered Not Likely.

Cluster Female fertility

Exposure during adulthood

A total of 11 studies examined the association between exposure to BPA during adult life with fertility in females (Souter et al., 2013 [RefID 6856]; Buck Louis et al., 2014 [RefID 4599]; Lathi et al., 2014 [RefID 3864]; Bae et al., 2015 [RefID 347]; Minguez-Alarcon et al., 2015 [RefID 5118]; Chavarro et al., 2016 [RefID 988]; Jukic et al., 2016 [RefID 3276]; Minguez-Alarcon et al., 2016 [RefID 5117]; Chin et al., 2018 [RefID 11745]; Pollack et al., 2018 [RefID 12909]; Wang B et al., 2018 [RefID 13333]).

These included associations between BPA exposures during pregnancy with fecundability and miscarriage and offspring sex ratio in the more general population (Buck Louis et al., 2014 [RefID 4599]; Lathi et al., 2014 [RefID 3864]; Bae et al., 2015 [RefID 347]; Jukic et al., 2016 [RefID 3276]; Chin et al., 2018 [RefID 11745]; Wang B et al., 2018 [RefID 13333]) or associations with fertility outcomes in a more selective group of women seeking fertility treatment (Souter et al., 2013 [RefID 6856]; Minguez-Alarcon et al., 2015 [RefID 5118]; Minguez-Alarcon et al., 2016 [RefID 5117]).

For the studies recruiting the more general population associations with fecundability were inconsistent with three studies reporting no association (Buck Louis et al., 2014 [RefID 4599]; Jukic et al., 2016 [RefID 3276]; Chin et al., 2018 [RefID 11745]). One study reported associations between maternal BPA exposure and decreased fecundability (Wang B et al., 2018 [RefID 13333]). A study by Lathi et al. (2014) [RefID 3864] also reported a significant positive association between BPA and aneuploid pregnancy and risk of miscarriage.

In a group of selected women seeking fertility treatment, Chavarro et al. (2016) [RefID 988] reported a significant decrease in live births among strata of women who did not consume soy (n = 100), while no such association were observed among majority of study participants that did consume soy (n = 247). As women who seek treatment may change their dietary habits before treatment it is possible that women who did not consume soy in a group of selected women may have underlying different fertility compared with those who reported to consume soy. Similarly, in a group of selected women (Minguez-Alarcon et al., 2016 [RefID 5117]) reported decreased probability of implantation and clinical pregnancies with higher BPA exposures.

Overall conclusions

Overall, the results from these studies conducted in both selected and more unselected group of women are inconsistent and an association between BPA exposure in adult life and reduced fertility was judged as ALAN.

Cluster Pubertal/endocrine

Exposure during pregnancy

Watkins et al. (2017b) [RefID 7876] examined association between pregnancy exposure to BPA and pubertal development in 109 male offspring aged 8 to 14 years. In a separate publication based on the same cohort associations with pubertal development were also examined in 120–129 female offspring aged 8 to 13 years (Watkins et al., 2014 [RefID 7877]; Watkins et al., 2017a [RefID 7875]). No associations with markers of pubertal development were observed in either studies.

Similarly, no association between pregnancy exposure to BPA and markers of puberty was observed in 112 boys in a study by Ferguson KK et al. (2014) [RefID 1996]. However, a study by Berger et al. (2018) [RefID 11600] examining the relation between maternal BPA exposure and pubertal development in 179 females and 159 male offspring at age 9 to 13 years found associations with later and earlier pubertal development in female and male offspring, respectively.

Finally, three studies reported associations with anogenital distance (Barrett et al., 2017 [RefID 434]; Arbuckle et al., 2018 [RefID 11520]; Sun et al., 2018 [RefID 13199]), an outcome that is often used as predictor for later reproductive disorders. Overall, despite a few significant findings observed the overall findings from these three studies were inconsistent.

On the basis of these studies, the CEP Panel concluded that an association between BPA exposure during pregnancy and pubertal development is considered as ALAN.

Exposure during childhood

A total of five studies examined prospectively associations between exposure to BPA measured in urine during childhood and pubertal development (Lee et al., 2013 [RefID 3911]; Wolff et al., 2015 [RefID 7982]; Kasper-Sonnenberg et al., 2017 [RefID 3401]; Wolff et al., 2017 [RefID 9215]; Binder et al., 2018 [RefID 11612]). However, two of these studies were conducted in the same study population but with different length of follow-up (Wolff et al., 2015 [RefID 7982]; Wolff et al., 2017 [RefID 9215]).

Overall, no association between childhood exposures to BPA and pubertal development were observed in these studies.

On the basis of these studies, the CEP Panel concluded that an association between BPA exposure during childhood and pubertal development is considered Not Likely.

Overall conclusions

On the basis of these studies, the CEP Panel concluded that an association between BPA exposure and pubertal development is considered ALAN.

Cross-sectional studies

Fetal and post-natal growth

Several cross-sectional studies using maternal blood or urine drawn at end of pregnancy or cord blood examined the associations with length of gestation and measures of fetal growth. Several studies found no significant associations with these outcome measures (Tang et al., 2013 [RefID 7111]; Ding et al., 2017 [RefID 1610]; Huang YF et al., 2018 [RefID 12183]; Mammadov et al., 2018 [RefID 11173]; Wan et al., 2018 [RefID 13328]). One reported a positive association with length at birth (Xu XJ et al., 2015 [RefID 8238]) and one study reported an inverse association (Youssef et al., 2016 [RefID 9542]). One study reported higher placental BPA concentration in LBW infants (<2,500g) compared with controls (Huo et al., 2015 [RefID 2951]). Since BPA has very short elimination half-life it is difficult to explain how placental concentration at time of birth might reflect concentrations during previous months when the fetus was growing making it likely that the observed association is coincidental rather than causal. Overall, the results from these cross-sectional studies were in line with those from prospective studies showing relatively consistent NULL associations.

Prematurity

One cross-sectional study found no association between maternal urine (n = 567) concentration at time of delivery and length of gestation (Tang et al., 2013 [RefID 7111]), while another small (n = 80) study reported an inverse association (Youssef et al., 2016 [RefID 9542]). Overall, no meaningful conclusions on possible association with length of gestation can be drawn from these two studies.

Pre-eclampsia

One cross-sectional study investigated the relationship between BPA exposure (maternal and fetal serum, placenta) and pre-eclampsia (Leclerc et al., 2014 [RefID 3888]) and found no statistical significance. Only in placental tissue, concentrations of BPA were higher in preeclamptic women compared with normotensive pregnant women (p-value = 0.04).

Male fertility

A total of 15 studies (see external report) (Knez et al., 2014 [RefID 3608]; Lassen et al., 2014 [RefID 3859]; Komarowska et al., 2015 [RefID 3642]; La Rocca et al., 2015 [RefID 3791]; Liu XQ et al., 2015 [RefID 4492]; Vitku et al., 2015 [RefID 7499]; Zhuang et al., 2015 [RefID 9129]; Vitku et al., 2016 [RefID 7498]; Liang H et al.,2017 [RefID 4236]; Wang ZL et al., 2017 [RefID 7850]; Adoamnei et al., 2018 [RefID 11046]; Joensen et al., 2018 [RefID 12254]; Mustieles et al., 2018a [RefID 11188]; Omran et al., 2018 [RefID 12844]; Radwan et al., 2018 [RefID 12945]) examined cross-sectionally the association between BPA and male reproduction. Of those four studies focused on reproductive hormones only (Liu XQ et al., 2015 [RefID 4492]; Zhuang et al., 2015 [RefID 9129]; Liang H et al., 2017 [RefID 4236]; Mustieles et al., 2018a [RefID 11188]), while others also focused on more direct measures of male fertility (e.g. semen quality, n = 9) (Knez et al., 2014 [RefID 3608]; Lassen et al., 2014 [RefID 3859]; La Rocca et al., 2015 [RefID 3791]; Vitku et al., 2015 [RefID 7499]; Vitku et al., 2016 [RefID 7498]; Adoamnei et al., 2018 [RefID 11046]; Joensen et al., 2018 [RefID 12254]; Omran et al., 2018 [RefID 12844]; Radwan et al., 2018 [RefID 12945])or pubertal development (n = 2) (Wang

ZL et al., 2017 [RefID 7850]; Mustieles et al., 2018a [RefID 11188]) or birth malformation (cryptorchidism, n = 1) (Komarowska et al., 2015 [RefID 3642]). Among the studies examining associations between BPA exposure (in blood or urine) and semen quality, four studies reported lower sperm concentrations with higher serum or urinary BPA exposure (Knez et al., 2014 [RefID 3608]; Vitku et al., 2015 [RefID 7499]; Adoamnei et al., 2018 [RefID 11046]; Omran et al., 2018 [RefID 12844]); while other studies reported inverse association with other parameters (e.g. sperm motility but not sperm concentrations) of semen quality (Lassen et al., 2014 [RefID 3859]; La Rocca et al., 2015 [RefID 3791]; Joensen et al., 2018 [RefID 12254]; Radwan et al., 2018 [RefID 12945]). One study also quantified BPA in seminal fluid where an inverse association with sperm count was also observed (Vitku et al., 2016 [RefID 7498]). Significant associations between serum or urinary BPA concentrations and reproductive hormones were also reported in several studies. Although, the associations were not entirely consistent in terms of directionality, findings from some individual studies could be interpreted as being adverse for male reproductive function.

The results from these cross-sectional analyses are in stark contrast to the few prospective cohorts on male fertility described above where no association for semen quality, fecundability and live birth rate were observed. The cross-sectional inverse association observed for reduced semen quality is also difficult to interpret in terms of the temporal separation between the exposure and outcome. A single measure of urinary or blood BPA reflects only few hours of past exposures while the process of spermatogenesis takes around 3 months. With only a single measure of BPA cross-sectionally it seems biologically implausible that such short time exposure quantified at that point in time could affect sperm quality. An alternative explanation for these cross-sectional findings is that those with reduced fertility may be differently exposed to BPA through difference in lifestyle or behaviour. Alternatively, these associations may also reflect biological differences in rate of excretion of BPA. Studies relaying on repeated measures of BPA would to some extent be able to address these limitations. Similarly, the cross-sectional association between urinary BPA concentration in 1–4-year-old boys and cryptorchidism (Komarowska et al., 2015 [RefID 3642]) is hard to explain biologically as this malformation has origin during pre-natal but not post-natal development.

In summary, in the absence of support from other lines of evidence, including prospective studies or results from animal experiments, the associations reported in these studies are most likely to be coincidental rather than causal.

Female fertility

Few cross-sectional associations were reported on reproductive hormones in adult women. No consistent findings were reported in these studies. Seven studies examined cross-sectionally using case–control design association between BPA concentration and polycystic ovary syndrome (PCOS). Higher BPA concentrations were observed in four of these studies (Akin et al., 2015 [RefID 93]; Vahedi et al., 2016 [RefID 7370]; Rashidi et al., 2017 [RefID 9297]; Konieczna et al., 2018 [RefID 12363]), while no association was observed in three studies (Vagi et al., 2014 [RefID 7369]; Yang QY et al., 2015 [RefID 8385]; Gu et al., 2018 [RefID 12041]). Two studies reported higher BPA controls among women diagnosed with endometriosis compared with controls (Upson et al., 2014 [RefID 7361]; Simonelli et al., 2017 [RefID 6742]). Similarly higher BPA concentrations were reported in women with diminished ovarian reserves (Cao et al., 2018 [RefID 11666]) and women diagnosed as infertile (La Rocca et al., 2014 [RefID 3792]) compared with controls. In addition, three cross-sectional studies found higher BPA concentration among women with uterine leiomyoma compared with controls (Jeong et al., 2013 [RefID 11125]; Shen et al., 2013 [RefID 6644]; Shen et al., 2016 [RefID 6640]). The directionality of these findings showing higher BPA concentrations among women diagnosed with PCOS, endometriosis, women with no or reduced fertility and uterine leiomyoma is difficult to evaluate. The measured BPA concentrations reflect exposure during the previous few hours and as such, in the absence of further evidence, are unlikely to reflect past exposure that may have led to development of the underlying disease condition. Differences in lifestyle factors that may affect exposure to BPA or rate of uptake or excretion among cases may equally explain the observed findings, particularly in the absence of similar findings from prospective studies.

Pubertal/endocrine

A study of 665 girls 9–18 years of age reported positive association between BPA concentrations in urine and age at menarche (Miao et al., 2017 [RefID 5074]), while another study recruiting 987

adolescent girls from the US National Health and Nutrition Examination Survey (NHANES) found no such association (McGuinn et al., 2015 [RefID 4984]). One small (n = 88) study reported higher BPA concentration in girls with more advanced puberty (Supornsilchai et al., 2016 [RefID 7015]). Another study also found higher BPA concentrations in girls with isolated breast development before the age of 8 compared with controls (Durmaz et al., 2018 [RefID 11867]). Limited conclusions can be drawn from these studies. Also, a few cross-sectional associations were reported on reproductive hormones in adolescent girls. No consistent findings were reported in these studies.

3.1.6.2. Animal studies

For the HOC Reproductive and developmental toxicity a total of 153 studies were appraised by the CEP Panel. The details of the appraisals (internal and external validity) are reported in Annex E.

The endpoints for each study identified as relevant in this opinion are reported in Annex F.

Identification of clusters of relevant endpoints

Endpoints for which statistically significant changes were reported were extracted from the available literature in accordance with the protocol and grouped into three clusters:

- Developmental toxicity
- Female reproductive toxicity
- Male reproductive toxicity.

These clusters thus include endpoints identified as relevant in the 2015 opinion and considered in the uncertainty analysis (EFSA CEF Panel, 2015, Section 4.3.2), i.e. endometrial hyperplasia, ovarian cysts and anogenital distance (AGD), the first two of which were also identified as relevant in the newly compiled studies. Because it was previously identified as relevant, AGD was included and considered relevant in the new assessment, together with the others. For more details see Annex A, Section 2.5.

MoAs of developmental toxicants are generally distinctly different from those of male and female reproductive toxicants. Developmental toxicants can have multiple pleiotropic effects.

WoE of the clusters of relevant endpoints

The main information extracted from the studies addressing relevant endpoints in the HOC Reproductive and developmental Toxicity are summarised in Annex G. The outcome of the weight of the evidence is described in the text below and presented in a tabulated format in Annex H.

Developmental toxicity

Within the cluster developmental toxicity, nine studies were on mice, of which seven studies had exposure during development until weaning, two had exposure during development until adulthood, one had exposure during the growth phase. Of the 13 studies on rats, 10 studies had exposure during the development until weaning, four had exposure during development until adulthood and one was exposed as adults. Some studies assessed multiple exposure periods.

The specific endpoints that were included for effects of BPA on the developmental toxicity cluster were blastocyte outgrowth incidence, embryo development, age/day of first oestrus, AGD, mammary gland histology, mammary gland weight, bone development and body weight of F1/F2/F3 generation, as well as body weight of F0 dams.

The assessment of body weight for each exposure period is described in detail in the Metabolic effects category (Chapter 3.1.4.2).

<u>Developmental exposure (pre-natal and/or post-natal until weaning)</u>

AGD: One Tier 2 study in rats (Spörndly-Nees et al., 2018 [RefID 13164]) was available. There was a not statistically significant increase of AGD at nominal 0.5 (achieved 0.4) µg/kg bw per day after 12 months only but no changes on PND35, and at nominal 50 (achieved 40) µg/kg bw per day at both time points. The study assessed males only. As only one Tier 2 study showed an unexpected increase without a clear dose–response, the endpoint is rated as Not Likely.

Age at first oestrus: One Tier 1 study in mice (Tucker et al., 2018 [RefID 13275]) was available. No effect was seen at 500, 5000 and 50000 µg/kg bw per day, twice daily, from GD10.5–17.5. A change in the age at first oestrus was judged Not Likely after developmental exposure.

Bone development: Two Tier 1 studies in rats (Lejonklou et al., 2016 [RefID 3974]; Lind et al., 2017 [RefID 4350]) and one Tier 1 study in mice were available (van Esterik, 2014 [RefID 7393]). In Lejonklou et al. (2016) [RefID 3974] in Wistar rats, femur cortical thickness was increased in males, while in females femur length was increased. In Lind et al. (2017) [RefID 4350] in F344 rats; femur length was decreased in males; in females no effects were observed. In van Esterik (2014) [RefID 7393] no effect on femur length was observed in males or females in C57BL/6J mice. Femoral diaphyseal cortex thickness (F1 males, 25 µg/kg bw per day) and the femoral length (F1 females, 25 and 5,000 µg/kg bw per day) were increased in Wistar rats (Lejonklou et al., 2016 [RefID 3974]) whereas the femur length (F1 males) was decreased dose dependently, at 0.5 and 50 µg/kg bw per day in F344 rats (Lind et al., 2017 [RefID 4350]). No effect on femur length was described after exposure of mice to 3, 10, 30, 100, 300, 1000 or 3000 µg/kg bw per day) 2 weeks before mating until PND21 (van Esterik, 2014 [RefID 7393]). Both Tier 1 studies in rats show inconsistent effects according to doses, directions, sex, and strains and mice are without effect. The CEP Panel considered effects on bone development ALAN.

Mammary gland weight: For effects on mammary gland weight in this exposure period only one Tier 1 study in rats was identified (Montévil et al., 2020 [RefID 13788]).

The mammary gland weight in female rats was determined at PND90 (Montévil et al., 2020 [RefID 13788]). Based on the authors' description it appears that a data-driven approach was used for identifying a NMDR by defining a step function around the doses of 25 and 250 µg/kg bw per day. However, when using more conventional statistical methods, e.g. modelling the data in PROAST (Hill and Exponential models) or using spline and polynomial fit (without overfitting the data) no dose–response was identified by the CEP Panel. The CEP Panel considered that alternative interpretations of the data may be plausible, e.g. a significant difference between the dose group with 25 µg/kg bw per day and the controls – in the absence of dose–response – may be likely explained by random fluctuations and variability in the data. The CEP Panel assigned a likelihood level of Not Likely to the mammary gland weight effects of BPA in the developmental exposure period.

Mammary gland histology:

Males: Out of six rat and one mouse studies, three rat studies (all Tier 1) (Kass et al., 2015 [RefID 3402]; Mandrup et al., 2016 [RefID 4831]; NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) assessed the mammary gland histology of male pups. There were no neoplastic changes but changes in mammary gland growth observed in Kass et al. (2015) [RefID 3402] and Mandrup et al. (2016) [RefID 4831]. In Kass et al. (2015) [RefID 3402] male pups from dams exposed to BPA by s.c. injection of 25 and 250 µg/kg bw per day (converted to the equivalent oral doses 892.5 and 8925 µg/kg bw per day) from GD9 until GD23, showed an increased ductal growth at PND5 (transient effect) and a delay in ductal growth when assessed on PND30, both at the higher dose. A parallel experiment applying 64 µg/kg bw per day (oral dose from drinking water) (GD9–PND21) showed a reduced number of terminal end buds (TEBs) at PND15 and 30. The other oral study (Mandrup et al., 2016 [RefID 4831]) showed a decrease in male and increase in female-like mammary gland structures in male rat pups assessed PND100 when doses of 5,000 and 50000 µg/kg bw per day were applied from GD7 until birth. Assessment of the same dose groups at PND22 did not show any changes. However, lower doses in this study (25 and 250 µg/kg bw per day), which were also assessed at PND22, showed an increase in mammary gland growth at 25 µg/kg bw per day. No effects were reported on mammary glands in males in the NTP Clarity Report (2018)/Camacho et al. (2019) [RefID 11370]. The CEP Panel judged the male mammary gland effects as ALAN.

Females: Non-neoplastic findings on female rat mammary gland were observed in six Tier 1 (Kass et al., 2015 [RefID 3402]; Grassi et al., 2016 [RefID 2387]; NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]; Montévil et al., 2020 [RefID 13788]; Tucker et al., 2018 [RefID 13275]; Mandrup et al., 2016 [RefID 4831]) studies and one Tier 3 study (Leung et al., 2017 [RefID 3990]). Delayed ductal growth was observed at PND30 after s.c. application of 250 µg/kg bw per day (equal to 8925 µg/kg bw per day, GD7–23, Kass et al., 2015 [RefID 3402]). In another study, increased branching score and number of terminal ducts (TDs) and TEB+TDs are induced by 25 µg/kg bw per day (but not

at 250 µg/kg bw per day) on PND21 after exposure from GD10 until GD21 (Grassi et al., 2016 [RefID 2387]). This finding is supported by a Tier 3 study (Leung et al., 2017 [RefID 3990]) showing also increased numbers of TEBs at 2.5 and 25 µg/kg bw per day. A comprehensive Tier 1 study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370], doses applied: 2.5, 25, 250, 2500 and 25000 µg/kg bw per day from GD6 to PND0 and from PND1 to PND21 (stop-dose group of that study)) showed a decreased incidence of ductal dilatation in F1 females at 1 year (trend, significant at 250 and 25000 µg/kg bw per day) and significant in all doses at 2 years. Furthermore, Montévil et al. (2020) [RefID 13788] (Tier 1), who assessed samples from the CLARITY study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]), reported several effects at single doses in female F1 pups mammary glands: there were increases of gland density, Fractal dimension 3D, Dim 3 (third dimension from Principle Component Analysis) and angles of branches between beginning and end with a breaking point between 25 and 250 µg/kg bw per day, of thickness of the epithelium and of variation of ductal thickness at 25 µg/kg bw per day and an increased average branch length at 250 µg/kg bw per day. In addition, there were decreases of the gland depth, of the proportion of the small and very small branches, of the maximum branch length and topological asymmetry, of the lateral branching at 250 µg/kg bw per day and of the aspect ratio at 2.5 and 250 µg/kg bw per day. All effects with indications of a dose–response for which individual data were available (gland density, Dimension 3D and angles of branches between beginning and end, thickness of epithelium, variation of ductal thickness, aspect ratio) were statistically re-analysed by the CEP Panel. This re-analysis revealed that a formal dose–response could not be identified by fitting flexible biologically based functions or polynomials that are commonly used to describe biological systems except for ductal thickness and aspect ratio, for which a potential non-monotonic dose–response was identified statistically.

Within another Tier 1 study (Tucker et al., 2018 [RefID 13275]) pups exposed twice daily to 500, 5000 and 50000 µg/kg bw per day from GD10.5 to 17.5 showed increased branching density at low dose, whereas increased length of TEBs, mammary gland development score and number of TEBs are increased at mid dose. All effects occurred only at PND20 and not at later time points assessed (PND28, 35, 56). These non-neoplastic findings are thus not consistent over studies with different study designs. Pre-neoplastic findings in adult females (PND400) were seen in one Tier 1 study, Mandrup et al. (2016) [RefID 4831], showing an increased intraductal hyperplasia at 250 µg/kg bw per day after treatment by gavage from GD7 until birth. One Tier1 study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) revealed a neoplastic effect, an increase in adenoma and adenocarcinoma in the lowest dose group (2.5 µg/kg bw per day, exposure period: GD6–PND21, assessed at terminal sacrifice after 2 years. Four other Tier 1 studies with shorter timepoints for measuring or higher doses showed no pre-neoplastic effects. One neoplastic lesion was reported, i.e. stromal polyps, in the NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]. For this endpoint, a decrease (no adverse effect) was seen in this Tier 1 study at the highest dose after 2 years. The CEP Panel concludes, based on the inconsistent findings of non-neoplastic effects as well as the unconfirmed neoplastic or pre-neoplastic effects, that effects on the female mammary gland are ALAN.

Overall, the CEP Panel assigned a likelihood level of ALAN to the developmental toxicity effects of BPA in the developmental exposure period based on bone development, mammary gland histology and body weight. Therefore, none of the endpoints are taken forward for BMD analysis. However, the ALAN endpoints were considered in the uncertainty analysis (see Appendix D).

Developmental and adult (pre-natal and post-natal in pups until adulthood)

AGD: As only one single-dose study (Tier 3, Patel et al., 2013 [RefID 5697]) assessed the AGD after exposure from GD11.5 until the assessment at 4 months of age, there is Inadequate evidence for this endpoint.

Embryo development: The only study available is a Tier 3 study (Dobrzynska et al., 2018 [RefID 11837]), thus there is Inadequate evidence for this endpoint.

Bone development: As only one single-dose study (Tier 2, Auger et al., 2013 [RefID 301]) is available, there is Inadequate evidence for this endpoint.

Mammary gland histology: Two Tier 1 studies (Montévil et al., 2020 [RefID 13788]; NTP Clarity Report 2018/Camacho et al., 2019 [RefID 11370]) assessed the mammary glands of female rats at doses of 2.5, 25, 250, 2500 and 25000 µg/kg bw per day by continuous dosing from GD6 to PND90 or

6months. In Montévil et al. (2020) [RefID 13788] an additional pilot study dosing 2.5, 25, 260 and 2700 µg/kg bw per day from GD6 to terminal measurement at PND90 is reported. Non-neoplastic effects were only observed at single doses without any dose–response, apart for mammary gland scores, where the data were in line with the definition by the CEP Panel of indications for a NMDR. For Montévil et al. (2020) [RefID 13788] the CEP Panel re-evaluated the dose–response for gland density by fitting flexible biologically based functions or polynomials that are commonly used to describe biological systems and concluded that there is no dose–response.

Among the non-neoplastic effects identified in this exposure group several changes were only reported for one dose group in female rats, i.e. an increase in lobular alveolar budding at 250 µg/kg bw per day at PND90 (Montévil et al., 2020 [RefID 13788]), changes in ductal dilatation (increased at 1 year ; decreased at 2 years; adversity unclear) and a decrease in lobular hyperplasia, both at 25 µg/kg bw per day (NTP Clarity Report2018/Camacho et al.2019 [RefID 11370]). The BPA-induced decreases are different from results with E2 treatment which resulted in clear increases in duct dilatation and lobular hyperplasia as well in adenocarcinomas. An increase in alveolar dilatation was only reported in males at the lowest dose after 2 years (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) while the effect was not significant in females in both studies. Therefore, no dose–response could be established for non-neoplastic effects. Whereas for neoplastic effects, NTP Clarity Report (2018)/Camacho et al. (2019) [RefID 11370] showed an increase in atypical foci and adenocarcinomas at 2.5 µg/kg bw per day. In addition, there were non-significant increases in atypical foci in the 25 and 250 µg/kg bw per day dose groups (9% and 8%, respectively, vs 0% in controls) at 1 year (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]). One neoplastic lesion was also reported in NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370], i.e. stromal polyps. For this endpoint, however, a significant dose trend towards increased incidence at the higher doses at 1 year was observed, while a negative trend (no adverse effect) was observed at 2 years in this Tier 1 study. The CEP Panel therefore considered this result biologically implausible.

Based on the above results, histological effects on mammary gland induced by BPA were judged as ALAN by the CEP Panel.

Overall, the CEP Panel assigned a likelihood level of ALAN to the developmental toxicity effects of BPA in the developmental and adult exposure period based on effects on body weight and mammary gland histology. Therefore, none of the endpoints was taken forward for BMD analysis. However, the ALAN endpoints were considered in the uncertainty analysis (see Appendix D).

Growth phase/young age

Age at first oestrus: Based on one Tier 1 study (Li et al., 2016 [RefID 4128]) in mice, the likelihood of changes in age at first oestrus was rated ALAN by the CEP Panel. The mice showed a decreased age at first oestrus at 60 and an increased age at first oestrus at 600 µg/kg bw per day with a micropipette application three times per day after 5 weeks of exposure starting at PND22.

The CEP Panel assigned a likelihood of ALAN to the cluster of developmental toxicity of BPA in the growth phase/young age exposure period. Therefore, none of the endpoints was taken forward for BMD analysis. However, both body weight and age at first oestrus were considered in the uncertainty analysis (see Appendix D).

Adult exposure (after puberty)

Blastocyst outgrowth and F1 embryo development: There is Inadequate Evidence for these endpoints as both are only assessed in one single dose Tier 1 Study (Martinez et al., 2015 [RefID 4902]).

The CEP Panel assigned a likelihood level of Not Likely to the developmental toxicity effects of BPA in the adult exposure period based on body weight. Therefore, none of the endpoints were taken forward for BMD analysis.

Indirect (germline) exposure

Bone development: As only one single-dose study (Tier 2, Auger et al., 2013 [RefID 301]) is available, there is Inadequate Evidence for this endpoint to conclude on the likelihood.

The CEP Panel assigned a likelihood level of Not Likely to the developmental toxicity effects of BPA in the indirect germline exposure period based on body weight. Therefore, this endpoint was not taken forward for BMD analysis.

Overall cluster selection for endpoints/studies for BMD for developmental toxicity

Overall, the CEP Panel assigned a likelihood level of ALAN, to the developmental toxicity effects of BPA in the exposure periods developmental, developmental and adult and growth phase/young age, and of Not Likely in the adult and indirect (germline) exposure.

The overall likelihood across all exposure periods, i.e. the highest likelihood given in the cluster developmental toxicity was ALAN.

The CEP Panel considered that the evidence from the studies available showed ALAN effects of BPA for the endpoints bone development, mammary gland histology, body weight (developmental exposure), body weight and mammary gland histology (developmental and adult exposure) as well as body weight and age at first oestrus (growth phase/young age). These endpoints were therefore not brought forward for BMD analysis.

Female reproductive toxicity

Within the cluster female reproductive toxicity, 17 studies were on mice, of which seven studies had exposure during development until weaning, one had exposure during development until adulthood, one had exposure during the growth phase, seven were exposed as adults and four had indirect germline exposure (some studies tested multiple exposure periods). Of the 16 studies on rats, 11 studies had exposure during development until weaning, three had exposure during development until adulthood, four were exposed as adults. There was one study in hamsters which had exposure during development until weaning. In addition, three studies were on sheep, two had exposure during the development until weaning and one as adults.

The specific endpoints that were included for effects of BPA on female reproductive toxicity cluster were plasma/serum thyroid hormones, testosterone, oestrus cyclicity, age at first oestrus, fertilisation rate and implantation incidences, ovary weight and histology, uterus weight and histology.

Developmental exposure (pre-natal and/or post-natal until weaning)

Plasma/serum thyroid hormones: For this exposure period the following studies were identified. **T_3:** one Tier 1 rat study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]), **T_4:** two Tier 1 rat studies (Bansal and Zoeller, 2019 [RefID 13783]; NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]), one Tier 1 sheep study (Guignard et al., 2017 [RefID 2451]) and one Tier 3 mouse study (Bodin et al., 2014 [RefID 623]), and **T_3/TT_4:** one Tier 1 sheep study (Guignard et al., 2017 [RefID 2451]).

No effect was seen on **T_3** in the Tier 1 rat study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]). Rats were dosed with 2.5, 25, 250, 2500 or 25000 µg/kg bw per day; F0 dams from GD6 to PND0 and F1 pups from PND1 to PND21 at interim sacrifice (1 year).

No effect was seen on **T_4** in the Tier 1 rat study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]). Rats were dosed with 2.5, 25, 250, 2500 or 25000 µg/kg bw per day; F0 dams from GD6 to PND0 and F1 pups from PND1 to PND21. On PND15, no effect was observed in the Tier 1 rat study (Bansal and Zoeller, 2019 [RefID 13783]) in which females were dosed from GD6–PND15 with 2.5, 25, 250, 2500 or 25000 µg/kg bw per day. No change in T4 was observed in fetuses (GD28–132/134) in the sheep Tier 1 study (Guignard et al., 2017 [RefID 2451]) after s.c. dosing with 5, 50 or 5000 µg/kg bw per day (using respectively 125 and 37 as dose conversion factors to oral dose in sheep, the equivalent oral doses are 625, 6250 or 625000 µg/kg bw per day and 185, 1850 or 185000 µg/kg bw per day).

No change was observed on the serum ratio **T_3/TT_4** in the sheep Tier 1 study (Guignard et al., 2017 [RefID 2451]).

During this exposure period the likelihood of changes in thyroid hormones (T_3, T_4, rT_3/TT_4) were judged as Not Likely by the CEP Panel as no effects were observed in Tier 1 and Tier 2 studies in rats and in a Tier 1 study in sheep.

Plasma/serum testosterone: For this exposure period, only three Tier 3 studies in rats (Castro et al., 2018 [RefID 11674]; Leung et al., 2017 [RefID 3990]; Johnson et al., 2016 [RefID 3241]) and two Tier 3 studies (Mahalingam et al., 2017 [RefID 4779]; Tucker et al., 2018 [RefID 13275]) in mice were identified for this endpoint. The CEP Panel noted that as only Tier 3 studies were available, the likelihood for this endpoint could not be determined because of Inadequate evidence.

Oestrus cyclicity: For this exposure period, four Tier 1 studies (Hass et al., 2016 [RefID 2610], Ferguson SA et al., 2014 [RefID 1998]; NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]; Franssen et al., 2016 [RefID 2073]) and one Tier 2 study (Santamaria et al., 2016 [RefID 6448]) in rats, one Tier 1 study (Tucker et al., 2018 [RefID 13275]) and one Tier 2 study (Acevedo et al., 2018 [RefID 11434]) in mice and one Tier 2 study (Veiga-Lopez et al., 2014 [RefID 7421]) in sheep were identified. Furthermore, a Tier 3 rat study (Leung et al., 2017 [RefID 3990]) and a Tier 3 mouse study (Wang W et al., 2014 [RefID 7759]) were identified.

In the Tier 1 rat study (Hass et al., 2016 [RefID 2610]), no change in oestrus cyclicity was observed when measured in F1 females at 12 months; female rats were treated at oral doses of 25, 250 µg, 5000 µg or 50000 µg/kg bw per day GD7–PND22. In the Tier 1 rat study (Ferguson SA et al., 2014 [RefID 1998]) no change in oestrus cyclicity was observed (PND82–109); dams were treated daily by gavage from GD6–21 and pups PND1–21 at doses of 2.5 or 25 µg/kg bw per day. In the Tier 1 rat study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) no change in oestrus cycle was found after 1 year; dams were treated daily at doses of 2.5, 25, 250, 2500 or 25000 µg/kg bw per day from GD6 to PND0 and F1 pups from PND1 to PND21. In the Tier 1 rat study (Franssen et al., 2016 [RefID 2073]) no change in oestrus cycle was identified when female pups received a subcutaneous injection of 0.025 µg, 25 µg or 5,000 µg/kg per day from PND1–5 or PND1–15 (equivalent to oral doses of 0.8925, 892.5 or 178500 µg/kg bw per day); measured daily from PND25 to PND80.

In a Tier 1 mouse study (Tucker et al., 2018 [RefID 13275]) no change in oestrus cyclicity (PND63–84) was observed in F1 females after exposure by gavage at doses of 500, 5000 or 50000 µg/kg bw per day, twice daily from GD10.5–GD17.5 of F0 dams. In a Tier 2 mouse study (Acevedo et al., 2018 [RefID 11434]) only a decrease in oestrus cyclicity by 6 months at the lowest dose (0.025 µg/kg bw per day) in F1 females was seen. In this study F0 dams were exposed subcutaneously from GD8 to PND16 through osmotic mini pumps to 0.025 µg, 0.250 µg or 25 µg/kg bw per day (equivalent to oral doses 5.5, 55, 5550 µg/kg bw per day) and oestrus cycle was examined at 5.5 and 8.5 months of age (F1) for 2 weeks.

In the Tier 2 study in sheep (Veiga-Lopez et al., 2014 [RefID 7421]) a change in oestrus cyclicity (decrease in follicular count trajectories) in all dose groups was observed. Pregnant sheep were exposed from GD30–GD90 by subcutaneous injections 50, 500 or 5000 µg/kg bw per day (using respectively 125 and 37 as dose conversion factors to oral dose in sheep, the equivalent oral doses are 6250, 62500 or 625000 µg/kg bw per day and 1850, 18500 or 185000 µg/kg bw per day). Time of examination for oestrus cyclicity was not described but considered at least after 8 weeks (weaning) and when the F1 females weighed more than 40 kg.

Apart from the decreased oestrus cyclicity in only the lowest dose and one timepoint in the Tier 2 mouse study (Acevedo et al., 2018 [RefID 11434]) and a decrease in oestrus cyclicity (decrease in follicular count trajectories) in the sheep study (Veiga-Lopez et al., 2014 [RefID 7421]), no change in oestrus cycle was observed in the other five Tier 1 studies in rats and mice. Therefore, the CEP Panel considered that the likelihood of a change in oestrus cyclicity is Not Likely.

Ovary weight: For this exposure period one Tier 1 study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) and one Tier 2 study (Santamaria et al., 2016 [RefID 6448]) in rats and one Tier 3 mouse study (Patel et al., 2013 [RefID 5697]) were identified.

In the Tier 1 study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]), rats were dosed with 2.5, 25, 250, 2500 or 25000 µg/kg bw per day; F0 dams from GD6 to PND0 and F1 pups from PND1 to PND21. A decrease was observed in the high-dose group with a trend in the other dose groups. Ovary weight was decreased in the F1 females at 7 weeks of age. In a Tier 2 rat study (Santamaria et al., 2016 [RefID 6448]) an approximately equal decrease was observed on ovary weight in F1 animals of both BPA-treated dose groups at PND90; F0 dams and F1 female offspring were given 0.5 µg and 50 µg/kg bw per day (via drinking water) from GD9–PND21.

The CEP Panel considered the likelihood of the decrease of ovary weight as Likely as there was a trend in the Tier 1 study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) supported by one Tier 2 study with effects at lower doses without dose–response (Santamaria et al., 2016 [RefID 6448]).

Ovary histology (follicle count, cellular hypertrophy, follicular cysts): For this exposure period one Tier 1 study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]), one Tier 2 study (Santamaria et al., 2016 [RefID 6448]) and one Tier 3 study (Patel et al., 2017 [RefID 5708]) in rats and three Tier 3 studies in mice (Mahalingam et al., 2017 [RefID 4779]; Wang W et al., 2014 [RefID 7759]; Berger et al., 2016 [RefID 524]) were identified.

In the Tier 1 study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]), rats were dosed with 2.5, 25, 250, 2500 or 25000 µg/kg bw per day; F0 dams from GD6 to PND0 and F1 pups from PND1 to PND21. No change was observed in cell hypertrophy. The incidence in follicular cysts was increased in the highest dose group and in all dose groups there was an increased trend in incidence of follicular cysts.

In the Tier 2 (Santamaria et al., 2016 [RefID 6448]) rats a decrease in the number of growing follicles was observed in the BPA-treated groups on PND90; the decrease was comparable in both groups (oral (via drinking water) 0.5 µg and 50 µg BPA/kg bw per day; GD9–PND21).

The CEP Panel considered for the exposure period developmental exposure (pre-natal and/or post-natal until weaning) the likelihood of histological changes in the ovary as Likely.

Uterus weight: For this exposure period one Tier 1 study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) in rats, one Tier 1 study in hamsters (Radko et al., 2015 [RefID 6046]) and one Tier 3 study in mice (Patel et al., 2013 [RefID 5697]) were identified.

In the Tier 1 rat study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]), animals were dosed with 2.5, 25, 250, 2500 and 25000 µg/kg bw per day; F0 dams from GD6 to PND0 and F1 pups from PND1 to PND21. No change in uterus weight was observed. In the Tier 1 hamster study uterus weight (wet and dry) on PND21 was statistically significantly increased at 160000 µg/kg bw per day; female hamster pups were dosed with three daily oral doses (PND18–PND20) of 8000; 40000 and 160000 µg/kg bw per day.

The effect on uterus weight was considered as ALAN as no effect was observed in the Tier 1 study in rats and an increase in weaning hamsters (uterotropic assay) was only seen at the highest dose (160000 µg/kg bw per day).

Uterus histology (cystic endometrial hyperplasia, uterine dilation, squamous metaplasia, apoptosis in the luminal epithelial cells in the endometrium, endometrial hyperplasia, luminal epithelial anomalies and glands with cellular anomalies): Two Tier 1 studies in rats (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]; Vigezzi et al., 2015 [RefID 7472]) were identified for this exposure period.

In the Tier 1 rat study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]), animals were dosed with 2.5, 25, 250, 2500 or 25000 µg/kg bw per day; F0 dams from GD6 to PND0 and F1 pups from PND1 to PND21. In this study a statistically significant increase was observed in cystic endometrial hyperplasia at the interim sacrifice (1 year) in the highest dose group and at terminal sacrifice (2 years) in the two highest dose groups (2500 or 25000 µg/kg bw per day group). At interim sacrifice uterine dilation and squamous metaplasia were increased in the 250 µg/kg bw per day and in the 25,000 µg/kg bw per day dose groups, respectively. There was a non-significant increase observed in the incidence of apoptosis in the luminal epithelial cells in the endometrium in the high-dose group. No change was observed in endometrial hyperplasia in any of the dose groups in this study.

In the other Tier 1 rat study (Vigezzi et al., 2015 [RefID 7472]) dams were administered with 0.5 or 50 µg/kg bw per day in drinking water from GD9–PND21; F1 females were necropsied on PND90 and 360. No changes in squamous metaplasia and luminal epithelial anomalies were observed. At necropsy on PND360 an increase in glands with cellular anomalies was observed in the 50 µg/kg bw per day; on PND90 no effects were seen in the 50 µg/kg bw per day group or at both times of necropsy in the 0.5 µg/kg bw per day.

The CEP Panel considered the likelihood during this exposure period to be Likely for the endpoint uterus histology based on effects seen at histological examination of the uterus in two rat Tier 1 studies (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]; Vigezzi et al., 2015 [RefID 7472]).

During developmental exposure (pre-natal and/or post-natal until weaning), the CEP Panel assigned a likelihood level of Likely to the cluster female reproductive toxicity of BPA. Since the likelihood level is Likely for the endpoint ovary weight in Tier 1 rat study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]), for the endpoint ovary histology (follicle count and follicle cysts) in one rat study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370] (Tier1)) and the endpoint uterus histology in two Tier 1 rat studies (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]; Vigezzi et al., 2015 [RefID 7472]), these were taken forward for BMD analysis (see Chapter 3.2.1) and uncertainty analysis (see Appendix D).

Developmental and adult exposure (pre-natal and post-natal in pups until adulthood)

Plasma/serum thyroid hormones: For this exposure period one Tier 1 rat study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) was identified. No effect was seen on **T_3 or T_4** in this study. Rats were dosed with 2.5, 25, 250, 2500 or 25000 µg/kg bw per day; F0 dams from GD6 to PND0 and F1 pups from PND1 to 1 year (continuous-dose group).

Based on this Tier 1 rat study, the CEP Panel considered an effect on thyroid hormones (T_3, T_4) as Not Likely.

Oestrus cyclicity: For this exposure period one Tier 1 rat study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) was identified. No effect was seen on oestrus cyclicity in this study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]). Rats were dosed with 2.5, 25, 250, 2500 or 25000 µg/kg bw per day; F0 dams from GD6 to PND0 and F1 pups from PND1 to interim (1 year) and terminal sacrifice (2 years) (continuous-dose group).

Based on this Tier 1 rat study, the CEP Panel judged an effect on oestrus cyclicity as Not Likely.

Ovary weight: for this exposure period one Tier 1 rat study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) and one Tier 3 mouse study (Patel et al., 2013 [RefID 5697]) were identified. No effect was seen on ovary weight in the Tier 1 rat study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]). Rats were dosed with 2.5, 25, 250, 2500 or 25000 µg/kg bw per day; F0 dams from GD6 to PND0 and F1 pups from PND1 to interim sacrifice (1 year) (continuous-dose group).

Based on this Tier 1 rat study, the CEP Panel judged an effect on ovary weight as Not Likely.

Ovary histology (interstitial cell hypertrophy and follicle cysts): For this exposure period one Tier 1 rat study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) was identified. In this Tier 1 rat study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) a statistically significant increase was observed in interstitial cell hypertrophy in the 2,500 and 25,000 µg/kg bw per day groups at interim sacrifice. Rats were dosed with 2.5, 25, 250, 2500 or 25000 µg/kg bw per day; F0 dams from GD6 to PND0 and F1 pups from PND1 to 1 or 2 years (continuous-dose group). In the same study no change in follicular cysts was observed. The CEP Panel judged the effect on interstitial cell hypertrophy at a dose level of 2500 and 25000 µg/kg bw per day as Likely.

Uterus weight: For this exposure period two Tier 1 rat studies (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]; Leung et al., 2020 [RefID 13789]) and one Tier 3 mouse study (Patel et al., 2013 [RefID 5697]) were identified. No effect was seen on uterus weight in the Tier 1 rat study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]). Rats were dosed with 2.5, 25, 250, 2500 or 25000 µg/kg bw per day; F0 dams from GD6 to PND0 and F1 pups from PND1 to interim sacrifice (1 year) (continuous-dose group). In addition, no effects were observed on uterus weight in the other Tier 1 study (Leung et al., 2020 [RefID 13789]); dams were exposed from GD6 to PND0 and pups from PND1 to 1 year (continuous-dose group) to 2.5, 25, 250, 2500 or 25000 µg/kg bw per day; measurements were performed at PND90, 6 months and 1 year.

Based on these two Tier 1 rat studies, the CEP Panel judged an effect on uterus weight as Not Likely.

Uterus histology: (squamous metaplasia, apoptosis, uterine dilation, endometrial hyperplasia, squamous metaplasia): For this exposure period two Tier 1 rat studies (Leung et al., 2020 [RefID 13789]; NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) were identified. In the Tier 1

rat study (Leung et al., 2020 [RefID 13789]) no effects were observed on squamous metaplasia and apoptosis; dams were exposed from GD6 to PND0 and pups from PND1 to 1 year (continuous-dose group) to 2.5, 25, 250, 2500 and 25000 µg/kg bw per day; measurements were performed at PND90, 6 months and 1 year. In the Tier 1 rat study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) animals were dosed with 2.5, 25, 250, 2500 or 25000 µg/kg bw per day; F0 dams from GD6 to PND0 and F1 pups from PND1 to 1 or 2 years (continuous-dose group). In this Tier 1 study the following effects (statistically significant) were observed at sacrifice at interim sacrifice (1 year): increased uterine dilation (250 µg/kg bw per day), increased endometrial hyperplasia (2.5 or 250 µg/kg bw per day), apoptosis and squamous metaplasia (25,000 µg/kg bw per day) and a decreased cystic endometrial hyperplasia (2.5 µg/kg bw per day). No other statistically significant effects were observed at interim or terminal sacrifice in this study.

During the exposure period developmental until adult, no effect was seen in squamous metaplasia and apoptosis in one Tier 1 rat study (Leung et al., 2020 [RefID 13789]). In another Tier 1 rat study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]), in which the exposure time and dose range (2.5, 25, 250, 2500 and 25000 µg/kg bw per day) was the same, only an effect was observed at the highest dose. The other histological effects were only seen at low concentrations (uterine dilation at 250 µg/kg bw per day and cystic endometrial hyperplasia (2.5 µg/kg bw per day)). Therefore, the CEP Panel considered the likelihood for this endpoint to be ALAN.

Number of implantation sites: Within a Tier 1 rat study (Boudalia et al., 2014 [RefID 670]) dams were exposed from GD1 to LD21 by micropipette with 5 µg/kg bw. The examination of the dams at LD/PND21 revealed no change in the number of implantation sites. As this study is a single-dose study the CEP Panel considered the available study data of Inadequate evidence for any further conclusions:

During developmental and adult exposure (pre-natal and/or post-natal in pups until adulthood), the CEP Panel assigned a likelihood level of Likely to the cluster female reproductive toxicity of BPA. As the likelihood level is Likely for the endpoint ovary histology (interstitial cell hypertrophy in the Tier 1 study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]); this study was taken forward for BMD analysis (see Chapter 3.2.1). Moreover, the Likely and ALAN endpoints were considered for uncertainty analysis (see Appendix D)

Growth phase/young age exposure

Oestrus cyclicity: For this exposure period one Tier 1 mouse study (Li et al., 2016 [RefID 4128]), in which the oestrus cyclicity was studied, was identified. Mice were dosed from PND22 for 5 weeks (3 times per day) orally (via micropipette) with 0, 60 and 600 µg/kg bw per day of BPA. In the high-dose group, the oestrous cyclicity was decreased (the females spent less time in pro-estrus and oestrus and more time in met and diestrus). The CEP Panel judged this endpoint as ALAN.

Implantation rate: The implantation incidence was decreased in a dose-dependent manner in a Tier 1 mouse study (Li et al., 2016 [RefID 4128]), in which mice were fed split doses of BPA from PND22 for 5 weeks (0, 60 and 600 µg/kg bw per day). The CEP Panel judged this endpoint as Likely.

During the growth phase/young age, the CEP Panel assigned a likelihood level of Likely to the cluster of female reproductive toxicity based on one Tier 1 mouse study (Li et al., 2016 [RefID 4128]) in which the implantation incidence was decreased in a dose-dependent manner. The study (Li et al., 2016 [RefID 4128]) was taken forward for BMD analysis (see Chapter 3.2.1). Moreover, the Likely and ALAN endpoints were considered for uncertainty analysis (see Appendix D)

Adult exposure (after puberty)

Plasma/serum thyroid hormones: For this exposure period one Tier 1 sheep study (Guignard et al., 2017 [RefID 2451]) and one Tier 2 rat study (Zhang J et al., 2017 [RefID 8770]) were identified.

The female sheep in the Tier 1 study (Guignard et al., 2017 [RefID 2451]) were injected s.c. with 5, 50 or 5000 µg/kg bw per day (equivalent to oral doses of 185/625; 1850/6250; 185000/625000 µg/kg bw per day depending on the conversion factor of 37 or 125). In this study no effect was observed on **TT_4**. A decrease was observed in **TT_3** at 50 µg/kg bw per day and in **FT_4** at 50 or 5000 µg/kg bw per day. Furthermore, an increase in **reverse T_3/T_4** was observed at a s.c. dose of at 50 µg/kg bw per day (equivalent to oral dose of 1850/6250 µg/kg bw per day).

In the Tier 2 rat study (Zhang J et al., 2017 [RefID 8770]) the female rats were dosed with 250 and 1000 µg/kg bw per day from 6 weeks of age for 64 weeks. In this study a statistically significant increase was seen on **FT_4** at 1000 µg/kg bw per day and no changes in **FT_3**.

The effects on **T_3** were judged as Not Likely as at approximately the same dose no effects were measured in rats (Zhang J et al., 2017 [RefID 8770]), but an effect without dose relationship was seen in sheep (Guignard et al., 2017 [RefID 2451]); the effect in sheep is considered to be a variation.

The effects on **T_4** were judged as Not Likely as no dose–response was seen in sheep (Guignard et al., 2017 [RefID 2451]) for **FT_4** and an effect in opposite direction (increase) in rats (Zhang J et al., 2017 [RefID 8770]) at a similar dose was observed.

In the sheep study (Guignard et al., 2017 [RefID 2451]), the increase on reverse **T_3/T_4** was only seen at 50 µg/kg bw per day, the mid dose. Therefore, this effect was judged as Not Likely.

During this exposure period the likelihood of changes in **thyroid hormones (T_3, T_4, FT_4 rT_3/TT_4)** was considered as Not Likely by the CEP Panel as no consistent effects were observed in a Tier 2 study in rats and in a Tier 1 study in sheep.

Plasma/serum testosterone: For this exposure period one Tier 3 rat study (Rashid et al., 2018 [RefID 12963]) and two Tier 3 mouse studies (Hu et al., 2018 [RefID 11119]; Xu XH et al., 2015 [RefID 8232]) were identified. Therefore, data were Inadequate to judge the likelihood of an effect on testosterone levels.

Fertilisation rate: one Tier 1 mouse study (Moore-Ambriz et al., 2015 [RefID 5196]), in which fertilisation rate was determined, was identified for this exposure period. In adult female mice exposed from the day of the first oestrus until the completion of three oestrous cycles orally (via pipette) at a dose of 50 µg/kg bw per day a decreased fertilisation rate was observed. As only one single-dose Tier 1 mouse study was available data were Inadequate to judge the likelihood of an effect on fertilisation rate.

Implantation rate: As only one single-dose Tier 1 study (Boudalia et al., 2014 [RefID 670]) and two Tier 3 studies (Yuan et al., 2018 [RefID 13593]; Dobrzynska et al., 2018 [RefID 11837]) are available, there is Inadequate evidence to conclude on a likelihood of an effect.

Oestrus cyclicity: For this exposure period one Tier 1 study (Moore-Ambriz et al., 2015 [RefID 5196]) and one Tier 3 study (Cao et al., 2018 [RefID 11666]) in mice and one Tier 2 rat study (Zaid et al., 2014 [RefID 10261]) were identified.

No effect on oestrous cyclicity was observed in adult female mice (Tier 1 study, Moore-Ambriz et al., 2015 [RefID 5196]) exposed orally (via pipette) from the day of the first oestrus until the completion of three oestrous cycles at a dose of 50 µg/kg bw per day. In the Tier 2 rat study (Zaid et al., 2014 [RefID 10261]) the number of females which were in persistent diestrous was increased after daily dosing of 10000 µg/kg bw per day (single-dose level) from PND28 for 6 weeks.

The effect on oestrous cyclicity was judged ALAN. No effect was seen in the lower dose (50 µg/kg bw per day) Tier 1 mouse study, but an effect at higher dose level, 10000 µg/kg bw per day was observed in the Tier 2 rat study; both studies were single-dose studies.

Ovary weight: One Tier 2 rat study (Zaid et al., 2014 [RefID 10261]) was identified for this exposure period. No change in absolute ovary weight was observed after daily dosing female rats from PND28 for 6 weeks with 10000 µg/kg bw per day (only dose tested). Therefore, data were Inadequate to judge the likelihood of an effect on ovary weight.

Ovary histology: (follicles count, premature activation of primordial follicles, large antral-like and atretic cystic-like follicles): For this exposure period one Tier 1 study (Moore-Ambriz et al., 2015 [RefID 5196]) and one Tier 2 study (Hu et al., 2018 [RefID 11119]) in mice and one Tier 2 rat study (Zaid et al., 2014 [RefID 10261]) were identified.

No effect on follicle count was observed in adult female mice (Tier 1 study Moore-Ambriz et al., 2015 [RefID 5196]) exposed orally (via pipette) from the day of the first oestrus until the completion of three oestrous cycles at a dose of 50 µg/kg bw per day. In the Tier 2 mouse study (Hu et al., 2018 [RefID 11119]) in all dose groups, a dose-related decrease was observed in the number of primordial follicles

and the premature activation of primordial follicles; in this study adult female mice were dosed orally for 28 days with 1 µg, 10 µg, 100 µg, 1000 µg and 10000 µg/kg bw per day. In the Tier 2 rat study (Zaid et al., 2014 [RefID 10261]) female rats were dosed orally from PND28 for 6 weeks with 10000 µg/kg bw per day (only dose tested). In this study, the numbers of atretic follicles, atretic cystic-like and large antral-like follicles were increased in the BPA-treated group when compared with the controls.

The effects on ovary histology were judged as Likely. As the Tier 2 rat study (Zaid et al., 2014 [RefID 10261]) is a single-dose study, only the Tier 2 mouse study (Hu et al., 2018 [RefID 11119]) was taken forward for BMD analysis (see Chapter 3.2.1).

Uterus histology: (gland nests density, gland nests): For this exposure period a Tier 1 study in CD1 mice (Kendziorski and Belcher, 2015 [RefID 3453]) and a Tier 3 study in C57Bl/6J mice (Kendziorski and Belcher, 2015 [RefID 3453]) were identified. In the Tier 1 study, CD-1 mice were exposed for 12–15 weeks via the diet to doses equivalent to 4, 40, 400, 4000 and 40000 µg/kg bw per day. Gland nests density and the number of gland nests was increased in the high-dose group. The design of the Tier 3 study in C57Bl/6J mice was identical. In this study no effect on the number of gland nests was observed and the gland nests density was increased in the 4, 4000 and 40000 µg/kg bw per day group.

The likelihood of the effects on uterus histology was considered as ALAN as gland nest number and density showed inconsistent effects; in the Tier 1 study in CD-1 mice an increase in gland nest number and density was observed only at high dose, in the Tier 3 study with a low number of C57BI/6J mice, varying effect were seen.

The CEP Panel assigned a likelihood level of Likely to the female reproductive toxicity cluster in the exposure period adulthood. The likelihood for the endpoint ovary histology is Likely. In a Tier 2 mouse study (Hu et al., 2018 [RefID 11119]), a dose-related decrease in the number of primordial follicles and the premature activation of primordial follicles was observed. This study is taken forward for BMD analysis (see Chapter 3.2.1). In addition, an increase in follicle abnormalities was reported in a single-dose rat Tier 2 study (Zaid et al., 2014 [RefID 10261]). As this study tested only one dose, this study will not be taken forward for BMD analysis. The Likely and ALAN endpoints were also considered for uncertainty analysis (see Appendix D).

Indirect (germline) exposure

For this exposure period five studies were assessed: three Tier 3 mouse studies (Ziv-Gal et al., 2015 [RefID 9143]; Berger et al., 2016 [RefID 524]; Mahalingam et al., 2017 [RefID 4779]), in which the F2 and F3 generation were studied and two Tier 3 mouse studies (Dobrzynska et al., 2015 [RefID 1644]; Mahalingam et al., 2017 [RefID 4779]) in which the F2 generation were studied. In the study by Ziv-Gal et al. (2015) [RefID 9143] the age at first oestrus, in the study by Dobrzynska et al. (2015) [RefID 1644] the embryo implantation incidence and in the study by Berger et al. (2016) [RefID 524] and Mahalingam et al. (2017) [RefID 4779] the follicle count (ovary histology) were reported.

The CEP Panel noted that since for indirect (germline) exposure only Tier 3 studies were available, the likelihood for this endpoint could not be determined because of Inadequate evidence.

Overall cluster selection for endpoints/studies for BMD for female reproductive toxicity

Overall, the CEP Panel assigned a likelihood level of Likely, to the female reproductive toxicity cluster in the exposure periods developmental (pre-natal and/or post-natal until weaning), developmental and adult (pre-natal and/or post-natal until adulthood) and growth phase/young age, and of Inadequate Evidence in the adult and indirect (germline) exposure periods.

The overall likelihood across all exposure periods, i.e. the highest likelihood given in the cluster female reproductive toxicity was Likely.

The CEP Panel considered that the evidence from the studies available showed a Likely effect for ovary weight (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]), for uterus histology (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370] and Vigezzi et al., 2015 [RefID 7472]) and ovary histology (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) during the developmental exposure period, for ovary histology (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) during developmental and adult exposure and for ovary histology (Hu et al., 2018 [RefID 11119]) during

adult exposure and for decreased implantation incidence during the growth phase (Li et al., 2016 [RefID 4128]). Therefore, these endpoints were taken forward for BMD analysis (see Chapter 3.2.1).

Male reproductive toxicity

Within the cluster male reproductive toxicity, 15 studies were on mice, of which six studies had exposure during the development until weaning, two had exposure during development until adulthood, seven were exposed as adults and two had germline exposure (some studies tested multiple exposure periods). Of the 26 studies on rats, 14 studies had exposure during development until weaning, four had exposure during development until adulthood, five had exposure during the growth phase, five were exposed as adults. In addition, one study was on sheep, which had exposure during the development until weaning and one study in monkeys during adult exposure period.

The specific endpoints that were included for effects of BPA on the male reproductive toxicity cluster were plasma/serum thyroid hormones, testosterone, epididymis weight and histology, prostate histology, seminal vesicle weight, sperm count/morphology/motility/viability, testis weight and histology.

Developmental exposure (pre-natal and/or post-natal until weaning)

Plasma/serum thyroid hormones: For this exposure period the following studies were identified. **T3:** one Tier 1 rat study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]), **T_4:** two Tier 1 rat studies (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]; Bansal and Zoeller, 2019 [RefID 13783]) and one Tier 1 sheep study (Guignard et al., 2017 [RefID 2451]) and **T_3/TT_4:** one Tier 1 sheep study (Guignard et al., 2017 [RefID 2451]).

No effect was seen on **T3** in the Tier 1 rat study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]). Rats were dosed with 2.5, 25, 250, 2500 or 25000 µg/kg bw per day; F0 dams from GD6 to PND0 and F1 pups from PND1 to PND21 at interim sacrifice (1 year).

Apart from a decrease at the highest dose level (25000 µg/kg bw per day), no effect was seen on **T_4** in the Tier 1 rat study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]). In this study, rats were dosed with 2.5, 25, 250, 2500 or 25000 µg/kg bw per day; F0 dams from GD6 to PND0 and F1 pups from PND1 to PND21 at interim sacrifice. On PND15, no effect was observed in **T_4** in another Tier 1 rat study (Bansal and Zoeller, 2019 [RefID 13783]) in which females were dosed from GD6–PND15 with 2.5, 25, 250, 2500 or 25000 µg/kg bw per day.

No change in **T_4** was observed in fetus (GD113–135) in the sheep Tier 1 study (Guignard et al., 2017 [RefID 2451]) after s.c. dosing with 5, 50 or 5000 µg/kg bw per day (equivalent to oral doses of 185–625; 1850–6250; 185000–625000 µg/kg bw per day). In addition, no change was seen in this study in the ratio **T_3/T_4**.

During this developmental exposure (pre-natal and/or post-natal until weaning) period changes in thyroid hormones (T_3, T_4, rT_3/TT_4) were judged as Not Likely by the CEP Panel.

Plasma/serum testosterone: for this endpoint four Tier 3 studies in rats (Quan et al., 2017 [RefID 6025]; Wang C et al., 2014 [RefID 7579]; Castro et al., 2018 [RefID 11674]; Johnson et al., 2016 [RefID 3241]) and one Tier 3 mouse study (Shi et al., 2018 [RefID 13099]) were identified. Therefore, data were considered Inadequate to judge the likelihood of an effect of BPA on testosterone levels.

Epididymis weight: for this endpoint one Tier 1 (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]), one Tier 2 study (Spörndly-Nees et al., 2018 [RefID 13164]), one Tier 3 rat study (Tarapore et al., 2017 [RefID 7128]) and one Tier 2 mouse study (Meng Y et al., 2018 [RefID 12707]) were identified.

No effect was seen on epididymis weight in the Tier 1 rat study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]). Rats were dosed with 2.5, 25, 250, 2500 or 25000 µg/kg bw per day; F0 dams from GD6 to PND0 and F1 pups from PND1 to PND21 at interim sacrifice (at 1 year).

No effect was seen on epididymis weight in the other Tier 2 rat study (Spörndly-Nees et al., 2018 [RefID 13164]): in this study animals were exposed via the drinking water to nominal doses of 5 or 50 µg/kg

per day (achieved doses 4 or 40 µg/kg per day) from GD3.5–PND22 and necropsied at PND35 or 12 months.

No effect was seen on epididymis weight in the Tier 2 mouse study (Meng Y et al., 2018 [RefID 12707]): in this study animals were exposed via the drinking water to doses equivalent to 18 or 180 µg/kg per day from GD6–PND21 and necropsied at PND50.

As there is no effect in any Tier 1 or Tier 2 study, the likelihood for this endpoint was considered as Not Likely.

Epididymis histology (non-neoplastic, inflammatory changes, inflammation): for this endpoint one Tier 1 rat study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) and one Tier 2 rat study (Spörndly-Nees et al., 2018 [RefID 13164]) were identified.

No effect was seen on epididymis histology (non-neoplastic, inflammatory changes) in the Tier 1 rat study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]). Rats were dosed with 2.5, 25, 250, 2500 or 25000 µg/kg bw per day; F0 dams from GD6 to PND0 and F1 pups from PND1 to PND21 at interim sacrifice (at 1 year).

An increase in inflammatory changes was seen at 40 µg/kg bw per day in the other Tier 2 rat study (Spörndly-Nees et al., 2018 [RefID 13164]): in this study animals were exposed via the drinking water to doses of 4 or 40 µg/kg per day from GD3.5–PND22 and necropsied at PND35 or 12 months.

No effects were seen for the endpoint epididymis histology (non-neoplastic, inflammatory changes, inflammation) in one Tier 1 study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) and an effect only at the highest dose was seen in a Tier 2 rat study (Spörndly-Nees et al., 2018 [RefID 13164]). Therefore, the likelihood assigned to this endpoint is Not Likely.

Prostate histology: for this endpoint four Tier 1 (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]; Bernardo et al., 2015 [RefID 533]; Prins et al., 2018 [RefID 13779]; Brandt et al., 2014 [RefID 700]), one Tier 2 (Hass et al., 2016 [RefID 2610]) and one Tier 3 (Prins et al., 2017 [RefID 5930]) studies in rats were identified. In these studies several histological effects were examined.

No effect was seen on prostate histology (non-neoplastic proliferative lesions, hyperplasia of the ventral prostate, epithelium hyperplasia and inflammatory changes, inflammation, dorsal/lateral prostate histology, suppurative inflammation) in the Tier 1 rat study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]). Rats were dosed with 2.5, 25, 250, 2500 or 25000 µg/kg bw per day; F0 dams from GD6 to PND0 and F1 pups from PND1 to PND21 at interim sacrifice (at 1 year).

At histological examination, an increase (same effect size) in the incidence of inflammatory changes, pre-neoplastic lesions (atypical hyperplasia), non-neoplastic proliferative lesions (reactive hyperplasia), in the prostate was seen in both doses tested in a Tier 1 rat study (Bernardo et al., 2015 [RefID 533]). In this study animals were given 25 or 250 µg/kg bw per day from GD10–21 and necropsied at PND180.

No effect was seen on prostate histology (non-neoplastic proliferative lesions, hyperplasia of the ventral prostate and inflammatory changes, inflammation, dorsal/lateral prostate histology, suppurative inflammation) in the Tier 1 rat study (Prins et al., 2018 [RefID 13779]). Rats were dosed with 2.5, 25, 250, 2500 or 25000 µg/kg bw per day; F0 dams from GD6 to PND0 and F1 pups from PND1 to PND21 and necropsied at 1 year.

In a Tier 1 study (Brandt et al., 2014 [RefID 700]) rats were administered 25 and 250 µg/kg bw per day from GD10–21. F1 pups/adults were sacrificed on PND21/180. At histological examination on PND21 an increase in proliferation and hyperplasia/dysplasia of the prostate was observed in the lowest dose and in apoptosis in the highest dose. At both doses an increase (effect the same size) of multifocal inflammation in the ventral prostate on PND180 was observed.

In the Tier 2 rat study (Hass et al., 2016 [RefID 2610]), no change in prostate histology (interstitial inflammation, proliferation or epithelial atypical hyperplasia) was observed when examined in F1 males at 3 or 8 months; F0 dams were treated at oral doses of 25, 250, 5000 or 50000 µg/kg bw per day GD7–PND22.

In two Tier 1 rat studies (Bernardo et al., 2015 [RefID 533]; Brandt et al., 2014 [RefID 700]) from the same laboratory, inflammatory effects and reactive hyperplasia in the prostate were reported at doses

of 25 and 250 µg/kg bw per day GD10–GD21. This effect was not confirmed in two other Tier 1 rat studies (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]; Prins et al., 2018 [RefID 13779]) at doses of 2.5, 25, 250, 2500 or 25000 µg/kg bw per day from GD6–PND21 or in a Tier 2 rat study (Hass et al., 2016 [RefID 2610]) at doses of 25, 250, 5000 or 50000 µg/kg bw per day GD7–PND22. Therefore, the likelihood for this endpoint was considered to be ALAN.

Seminal vesicle weight: for this endpoint one Tier 1 (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) and one Tier 2 study (Spörndly-Nees et al., 2018 [RefID 13164]) in rats and one Tier 3 mouse study (Patel et al., 2013 [RefID 5697]) were identified.

No effect was seen on seminal vesicle weight in the Tier 1 rat study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]). Rats were dosed with 2.5, 25, 250, 2500 or 25000 µg/kg bw per day; F0 dams from GD6 to PND0 and F1 pups from PND1 to PND21 at interim sacrifice (at 1 year).

No effect was seen on seminal vesicle weight in the other Tier 2 rat study (Spörndly-Nees et al., 2018 [RefID 13164]): in this study animals were exposed via the drinking water to doses of 4 or 40 µg/kg per day from GD3.5–PND22 and necropsied at PND35 or 12 months.

No effect was observed on seminal vesicle weight in one Tier 1 (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) and one Tier 2 rat study (Spörndly-Nees et al., 2018 [RefID 13164]). Therefore, the likelihood assigned to this endpoint is Not Likely.

Sperm count: for this endpoint one Tier 1 (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) and one Tier 3 study (Hass et al., 2016 [RefID 2610]) in rats and two Tier 3 studies in mice (Rahman et al., 2017 [RefID 6060]; Shi et al., 2018 [RefID 13099]) were identified.

No effect was seen on epidydimal sperm count and count of testicular sperm heads in the Tier 1 rat study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]). Rats were dosed with 2.5, 25, 250, 2500 or 25000 µg/kg bw per day; F0 dams from GD6 to PND0 and F1 pups from PND1 to PND21 at interim sacrifice (at 1 year).

No effect was observed on epidydimal sperm count and count of testicular sperm heads in one Tier 1 rat study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) at dose levels of 2.5 to 25000 µg/kg bw per day GD6–PND22. Therefore, the likelihood assigned to this endpoint is Not Likely.

Sperm morphology: for this endpoint one Tier 1 study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) and one Tier 2 study (Spörndly-Nees et al., 2018 [RefID 13164]) in rats and one Tier 3 mouse study (Kalb et al., 2016 [RefID 3312]) were identified.

No effect was identified on sperm morphology in the Tier 1 rat study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]). Rats were dosed with 2.5, 25, 250, 2500 or 25000 µg/kg bw per day; F0 dams from GD6 to PND0 and F1 pups from PND1 to PND21 (at 1 year interim sacrifice).

No effect was seen on sperm morphology in the Tier 2 rat study (Spörndly-Nees et al., 2018 [RefID 13164]): in this study animals were exposed via the drinking water to doses of 4 or 40 µg/kg per day from GD3.5–PND22 and necropsied at 12 months.

No effect was seen on sperm morphology in one Tier 1 rat study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) at dose levels of 2.5 to 25000 µg/kg bw per day GD6–PND22 and in a Tier 2 rat study (Spörndly-Nees et al., 2018 [RefID 13164]) at 4 or 40 µg/kg per day from GD3.5–PND22. Therefore, the likelihood assigned to this endpoint is Not Likely.

Sperm motility: for this endpoint one Tier 1 rat study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) and three Tier 3 studies in mice (Shi et al., 2018 [RefID 13099]; Kalb et al., 2016 [RefID 3312]; Rahman et al., 2017 [RefID 6060]) were identified.

No effect was examined on sperm motility in the Tier 1 rat study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]). Rats were dosed with 2.5, 25, 250, 2500 or 25000 µg/kg bw per day; F0 dams from GD6 to PND0 and F1 pups from PND1 to PND21 at interim sacrifice (at 1 year).

No effect was examined on sperm motility in one Tier 1 rat study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]). Therefore, the likelihood assigned to this endpoint is Not Likely.

Sperm viability: for this endpoint one Tier 3 mouse study (Rahman et al., 2017 [RefID 6060]) was identified. Therefore, data were Inadequate to judge the likelihood of an effect of BPA on sperm viability.

Testis weight: for this endpoint two Tier 1 studies (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]; Cao et al., 2015 [RefID 831]), and one Tier 3 study (Tarapore et al., 2017 [RefID 7128]) in rats and two Tier 2 studies (Meng Y et al., 2018 [RefID 12707]; Shi et al., 2018 [RefID 13099]) and one Tier 3 study (Patel et al., 2013 [RefID 5697]) in mice were identified.

No effect was reported on testis weight in the Tier 1 rat study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]). Rats were dosed with 2.5, 25, 250, 2500 or 25000 µg/kg bw per day; F0 dams from GD6 to PND0 and F1 pups from PND1 to PND21 at interim sacrifice (at 1 year).

On PND120, an increase in testis weight was observed in a single-dose Tier 1 rat study (Cao et al., 2015 [RefID 831]); the F0 females were administered via the drinking water (2 mg BPA/L; equivalent to 100 µg/kg bw per day) and co-treated with soy in the diet from GD1–PND21. No change was observed in the BPA-treated group with a soy-free diet.

No effect was seen on testis weight in a Tier 2 mouse study (Meng Y et al., 2018 [RefID 12707]): in this study animals were exposed via the drinking water to doses equivalent to 18 or 180 µg/kg per day from GD6–PND21 and necropsied at PND50.

No effect was seen on testis weight in the other Tier 2 mouse study (Shi et al., 2018 [RefID 13099]): in this study animals were exposed via the drinking water to doses equivalent to 0.5, 20 or 50 µg/kg bw per day from GD11 to birth and necropsied at PND60.

As no effect was observed in the Tier 1 rat study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) and two Tier 2 mouse studies (Meng Y et al., 2018 [RefID 12707]; Shi et al., 2018 [RefID 13099]), the likelihood was considered to be Not Likely for this endpoint.

Testis histology: for this endpoint one Tier 1 rat study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]), two rat Tier 2 studies (Quan et al., 2017 [RefID 6025]; Spörndly-Nees et al., [RefID 13164]), two Tier 2 mouse studies (Shi et al., 2018 [RefID 13099]; Xie et al., 2016 [RefID 8130]) and one Tier 3 mouse study (Rahman et al., 2017 [RefID 6060]) were identified.

At histological examination, increased incidence of testis (and pancreas) polyarteritis was seen in the Tier 1 rat study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) at a dose of 2500 µg/kg bw per day. Rats were dosed with 2.5, 25, 250, 2500 or 25000 µg/kg bw per day; F0 dams from GD6 to PND0 and F1 pups from PND1 to PND21 at interim sacrifice (at 1 year).

In the Tier 2 rat study (Quan et al., 2017 [RefID 6025]) an increase was seen at all dose levels (no dose–response) in the testis (seminiferous tubular changes, cell-specific and/or stage-specific: degeneration, germ cell) at PND50 in the F1 rats. The F0 animals were dosed with 1000; 10000 or 100000 µg/kg bw from GD14–21.

At histological examination at necropsy at 12 months, an increase was seen in inflammatory changes in the testis of the low-dose group (4 µg/kg per day from GD3.5–PND22) in a Tier 2 rat study (Spörndly-Nees et al., [RefID 13164]). No such effects were seen at the other dose tested (40 µg/kg per day) or at both dose levels on other histological effects in the testis (seminiferous tubular changes, non-specific seminiferous epithelial height and seminiferous tubule diameter).

At histological examination of the testis in a Tier 2 mouse study (Shi et al., 2018 [RefID 13099]) at PND60, a decrease of seminiferous tubular changes, cell and/or stage-specific, in stage VII seminiferous epithelial cells and an increase in stage VIII seminiferous epithelial cells was seen in the mid-dose group (20 µg/kg bw per day). On PND12 an increase without dose–response was seen in testicular apoptosis in the mid and high-dose groups. In this study mice were dosed with 0.5, 20 or 50 µg/kg bw per day from GD11 to birth.

In another Tier 2 mouse study (Xie et al., 2016 [RefID 8130]) a dose-related increase was observed at histological examination of the testis (degeneration, germ cell). In this study male mice were s.c. injected 10, 100 or 5000 µg/kg bw per day (equivalent to oral doses of 2222; 22220 or 1111000 µg/kg bw per day).

The Tier 1 rat study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) reported increased incidence of polyarteritis at 2500 µg/kg bw per day only (not at other doses in the range from 2.5–25000 µg/kg bw per day. The Tier 2 rat studies showed effects on inflammatory changes at only one dose (4 µg/kg bw per day) (Spörndly-Nees et al., [RefID 13164]), or effects (apoptosis) without dose–response at 1000–100000 µg/kg bw per day (Quan et al., 2017 [RefID 6025]). In the Tier 2 mouse study (Shi et al., 2018 [RefID 13099]) only the mid dose (20 µg/kg bw per day) showed effects on the testis (decrease in stage VII and decrease in stage VIII seminiferous epithelial cells) and apoptosis in the mid and high dose, 20 or 50 µg/kg bw per day, with no dose–response. In another Tier 2 mouse study (Xie et al., 2016 [RefID 8130]) with s.c. administration dose-related testicular findings (germ cell (apoptosis)) were observed at doses equivalent to oral doses of 2220; 22220 or 1111000 µg/kg bw per day. The likelihood of the histological changes in the testis were considered to be ALAN:

During developmental exposure (pre-natal and/or post-natal until weaning), the CEP Panel assigned a likelihood level of ALAN to the cluster male reproductive toxicity of BPA. Hence, none of these endpoints were taken forward for BMD analysis. However, the Likely and ALAN endpoints were considered in the uncertainty analysis (see Appendix D).

Developmental and adult exposure (pre-natal and post-natal in pups until adulthood)

Plasma/serum thyroid hormones: For this exposure period only one Tier 1 rat study was identified (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]). In this study **T_3** and **T_4** were measured. Rats were dosed with 2.5, 25, 250, 2500 or 25000 µg/kg bw per day; F0 dams from GD6 to PND0 and F1 pups from PND1 to interim sacrifice (at 1 year).

No effect was seen on **T_3** in the Tier 1 rat study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]). According to the authors, **T_4** levels in the serum showed a significant trend, but the nature of the trend was not evident from inspection of the data. The likelihood of an effect on **T_4** was therefore considered as Not Likely (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]).

During this exposure period the likelihood of changes in thyroid hormones (**T_3, T_4**) are considered as Not Likely by the CEP Panel as no effects were observed in one Tier 1 rat study.

Testosterone: For this endpoint one Tier 3 rat study (Gonzalez-Cadavid, 2018 (NTP Grantee study) [RefID 13787) was identified. Therefore, data were Inadequate to judge the likelihood of an effect on serum testosterone.

Epididymis weight: For this exposure period two Tier 1 rat studies (Dere et al., 2018 [RefID 11815]; NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) were identified.

No change in epididymis weight was seen in the Tier 1 rat study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]). Rats were dosed with 2.5, 25, 250, 2500 or 25000 µg/kg bw per day; F0 dams from GD6 to PND0 and F1 pups from PND1 to PND21 to interim sacrifice (at 1 year).

In the other Tier 1 rat study (Dere et al., 2018 [RefID 11815]), rats were dosed with 2.5, 25, 250, 2500 or 25000 µg/kg bw per day; F0 dams from GD6 to PND0 and F1 pups from PND1 to PND90. In this study an extra satellite control and 250000 µg/kg bw per day was added. A decrease in epididymis weight was only seen in the 250000 µg/kg bw per day group when compared with the extra (satellite) control group. This satellite control group showed a higher epididymis weight than the other control group.

The likelihood of an effect on epididymis weight was considered as Not Likely.

Epididymis histology: For this exposure period the following Tier 1 rat study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) was identified; this study had two different times of sacrifice.

In this study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) an increased change in exfoliated germ cells and inflammation was seen at histological examination of the epididymis in the high-dose group (25,000 µg/kg bw per day) at interim sacrifice (1 year); these effects were not observed at terminal sacrifice (2 years). Rats were dosed with 2.5, 25, 250, 2500 or 25000 µg/kg bw per day; F0 dams from GD6 to PND0 and F1 pups from PND1 to PND21 to interim sacrifice (1 year) and terminal sacrifice (2 years).

The likelihood of the changes in epididymis histology (exfoliated germ cells and inflammation) was considered to be Likely, although effects were only seen in the highest dose group (25,000 µg/kg bw per day) at interim sacrifice (1 year) and not at terminal sacrifice (2 years), i.e. the effect was apparently transient.

Prostate histology: For this exposure period the following Tier 1 rat study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) was identified; this study had two different times of sacrifice. In addition, another set of animals of the same study was examined (Prins et al., 2018 [RefID 13779]).

In the Tier one rat study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) animals were dosed with 2.5, 25, 250, 2500 or 25000 µg/kg bw per day; F0 dams from GD6 to PND0 and F1 pups from PND1 to PND21 to interim sacrifice (1 year) or terminal sacrifice (2 years). At interim sacrifice no change in hyperplasia of the epithelium (non-neoplastic, proliferative lesions) was observed and at terminal sacrifice only an increase was observed at 250 µg/kg bw per day. At interim sacrifice an increase without dose–response in inflammatory changes of the prostate was observed at 2.5, 250, 2500 or 25000 µg/kg bw per day; no change was seen at 25 µg/kg bw per day. At terminal sacrifice only an increase in inflammatory changes of the prostate was seen in the lowest dose group.

There was no change in inflammation of the prostate in the Tier 1 rat study (Prins et al., 2018 [RefID 13779]): in this study animals were dose with 2.5, 25, 250, 2500 or 25000 µg/kg bw per day; F0 dams from GD6 to PND0 and F1 pups from PND1 to PND21 to 1 year.

The likelihood of the changes in prostate histology were considered to be Not Likely as effects were not seen in different sets of animals and were only examined at interim sacrifice.

Seminal vesicle weight: For this exposure period one Tier 1 rat study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) and one Tier 3 mouse study (Patel et al., 2013 [RefID 5697]) were identified.

In the Tier 1 rat study animals were dosed with 2.5, 25, 250, 2500 or 25000 µg/kg bw per day; F0 dams from GD6 to PND0 and F1 pups from PND1 to interim sacrifice (1 year). No effects on seminal vesicle weight were observed.

The likelihood of an effect on seminal vesicle weight was considered as Not Likely.

Sperm count: for this endpoint one Tier 1 (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) was identified.

No effect was seen on epidydimal sperm count and count of testicular sperm heads in the Tier 1 rat study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]). Rats were dosed with 2.5, 25, 250, 2500 or 25000 µg/kg bw per day; F0 dams from GD6 to PND0 and F1 pups from PND1 to interim sacrifice (1 year, continuous arm).

The likelihood of an effect on sperm count was considered as Not Likely.

Sperm morphology: for this endpoint one Tier 1 rat study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) and one Tier 3 mouse study (Dobrzynska et al., 2018 [RefID 11837]) were identified.

No effect on sperm morphology was observed in the Tier 1 rat study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]). Rats were dosed with 2.5, 25, 250, 2500 or 25000 µg/kg bw per day; F0 dams from GD6 to PND0 and F1 pups from PND1 to interim sacrifice (1 year).

The likelihood of an effect on sperm morphology was considered as Not Likely.

Sperm motility: for this endpoint one Tier 1 rat study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) and one Tier 3 mouse study (Dobrzynska et al., 2018 [RefID 11837]) were identified.

No effect was seen on sperm motility in the Tier 1 rat study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]). Rats were dosed with 2.5, 25, 250, 2500 or 25000 µg/kg bw per day; F0 dams from GD6 to PND0 and F1 pups from PND1 to interim sacrifice (1 year).

The likelihood of an effect on sperm motility was considered as Not Likely.

Testis weight: For this exposure period two Tier 1 studies (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]; Dere et al., 2018 [RefID 11815]) and one Tier 3 mouse study (Patel et al., 2013 [RefID 5697]) were identified.

No change in testis weight was seen in the Tier 1 rat study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]). Rats were dosed with 2.5, 25, 250, 2500 or 25000 µg/kg bw per day; F0 dams from GD6 to PND0 and F1 pups from PND1 to PND21 to interim sacrifice (1 year).

In the other Tier 1 rat study (Dere et al., 2018 [RefID 11815]), rats were dosed with 2.5, 25, 250, 2500 or 25000 µg/kg bw per day; in this study extra satellite groups, 0 and 250000 µg/kg bw per day were added. F0 dams were exposed from GD6 to PND0 and F1 pups from PND1 to PND90. A decrease in testis weight was only seen in the 250000 µg/kg bw per day group when compared with the extra control group. This extra control group showed a higher testis weight than the other control group.

The likelihood of an effect on testis weight was considered as Not Likely.

Testis histology: For this exposure period two Tier 1 studies (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]; Dere et al., 2018 [RefID 11815]) were identified.

At histological examination in the testis (polyarteritis) of the Tier 1 rat study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) no changes were observed. Rats were dosed with 2.5, 25, 250, 2500 or 25000 µg/kg bw per day; F0 dams from GD6 to PND0 and F1 pups from PND1 to PND21 to interim sacrifice (1 year).

In the other Tier 1 rat study (Dere et al., 2018 [RefID 11815]), rats were dosed with 2.5, 25, 250, 2500, or 25000 µg/kg bw per day; in this study extra satellite groups, 0 and 250000 µg/kg bw per day were added. F0 dams were exposed from GD6 to PND0 and F1 pups from PND1 to PND90. At histological examination of the testis, no changes in apoptosis, sperm production and spermiation were observed.

The likelihood of an effect on testis histology was considered as Not Likely:

During developmental exposure (pre-natal and/or post-natal in pups until adulthood), the CEP Panel assigned a likelihood level of Likely to the cluster male reproductive toxicity of BPA. Since the likelihood level is Likely for the endpoint epididymis histology (exfoliated germ cells and inflammation) in the Tier 1 study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]), this study was taken forward for BMD analysis (see Chapter 3.2.1) and uncertainty analysis (see Appendix D).

Growth phase/young age

Testosterone: For this endpoint three Tier 3 rat studies (Ullah et al., 2018a [RefID 13281]; Ullah et al., 2018b [RefID 13282]; Gurmeet et al., 2014 [RefID 2502]) were identified. In the Tier 3 rat study (Ullah et al., 2018a [RefID 13281]) intratesticular testosterone was measured in addition to serum testosterone. In the other studies only serum/plasma testosterone was measured.

As only Tier 3 studies were identified, data were considered Inadequate to judge the likelihood of an effect on serum testosterone.

Epididymis weight: For this endpoint two Tier 1 rat studies (Ogo et al., 2018 [RefID 11201]; Gurmeet et al., 2014 [RefID 2502]) and one Tier 3 rat study (Ullah et al., 2018b [RefID 13282]) were identified.

No effect on epididymis weight was seen when rats were dosed with 20 or 200 µg/kg bw per day from PND36–66 (Ogo et al., 2018 [RefID 11201]). In another Tier 1 rat study (Gurmeet et al., 2014 [RefID 2502]) no effects were observed on epididymis weight when rats were dosed with 1000, 5000 or 100000 µg/kg bw per day from PND28–70.

The likelihood was considered as Not Likely as no effect was observed on epididymis weight in two Tier 1 rat studies.

Seminal vesicle weight: For this endpoint one Tier 1 rat study (Gurmeet et al., 2014 [RefID 2502]) and one Tier 3 rat study (Ullah et al., 2018b [RefID 13282]) were identified.

No effect on seminal vesicle weight was observed when rats were administered BPA via the drinking water with doses equivalent to oral doses of 1000, 5000 and 100000 µg/kg bw per day from PND28–70 weeks (Gurmeet et al., 2014 [RefID 2502]).

The likelihood was considered as Not Likely as no effect was seen on seminal vesicle weight in one Tier 1 rat study.

Sperm count (testis/epididymis): For this endpoint one Tier 1 rat studies (Ogo et al., 2018 [RefID 11201]) and one Tier 3 rat study (testicular count, Ullah et al., 2018b [RefID 13282]) were identified.

No effect on epididymal sperm count was observed when rats were dosed with 20 or 200 µg/kg bw per day from PND36–66 (Ogo et al., 2018 [RefID 11201]).

The likelihood was considered as Not Likely as no effect was seen on epididymal sperm count in one Tier 1 rat study.

Sperm motility: For this endpoint only one Tier 3 rat study (Ullah et al., 2018b [RefID 13282]) was identified. As only one Tier 3 rat study was identified, data were Inadequate to judge the likelihood of an effect of BPA on sperm motility.

Testis weight: For this endpoint two Tier 1 rat studies (Ullah et al., 2018a [RefID 13281]; Gurmeet et al., 2014 [RefID 2502]), one Tier 2 rat study (Brouard et al., 2016 [RefID 734]) and one Tier 3 rat study (Ullah et al., 2018b [RefID 13282]) were identified.

In the Tier 1 rat study (Ullah et al., 2018a [RefID 13281]) no effect on testis weight was observed. In this study rats were dosed for 4 weeks with 5000, 25000 or 50000 µg/kg bw per day from PND70–80. In another Tier 1 rat study (Gurmeet et al., 2014 [RefID 2502]), no effects were observed on testis weight when rats were dosed with 1000, 5000 or 100000 µg/kg bw per day from PND28–70. In a Tier 2 study (Brouard et al., 2016 [RefID 734]) rats were exposed from PND15–PND30 by s.c. injection with a single dose of 50 µg/kg bw per day (equivalent to an oral dose of 1800 µg/kg bw per day) and testis weight was increased.

The likelihood was considered to be Not Likely as no effects on testis weight were seen in two Tier 1 studies via gavage (dose range 5000–50000 µg/kg bw per day rats of PND70–PND80 for 4 weeks and 1000–100000 µg/kg bw per day from PND23 for 48 weeks) and only an effect was observed in the Tier 2 single-dose rat study via the s.c. route after dosing from PND15–PND30.

Testis histology: For this endpoint two Tier 2 rat studies (Gurmeet et al., 2014 [RefID 2502]; Brouard et al., 2016 [RefID 734]) and two Tier 3 rat studies (Ullah et al., 2018a [RefID 13281]; Ullah et al., 2018b [RefID 13282]) were identified.

In the Tier 2 rat study (Gurmeet et al., 2014 [RefID 2502]), a decrease of the seminiferous tubule diameter was observed in the high-dose group; rats were dosed with 1000, 5000 or 100000 µg/kg bw per day from PND28–PND70. In another Tier 2 study (Brouard et al., 2016 [RefID 734]), rats were exposed from PND15–PND30 by s.c. injection with a single dose of 50 µg/kg bw per day (equivalent to an oral dose of 1800 µg/kg bw per day), and incidences of seminiferous tubules with lumen, with acrosomal vesicles and acrosome reaction were increased in the BPA-treated group.

The likelihood of the effect was considered as Likely as in a Tier 2 rat study (Gurmeet et al., 2014 [RefID 2502]) a decrease in seminiferous tubule diameter at dose level of 100000 µg/kg bw per day was observed. These effects were supported by testicular effects in another Tier 2 rat study (Brouard et al., 2016 [RefID 734]) at a s.c. dose equivalent to an oral dose of 1800 µg/kg bw per day. In this study, incidence of seminiferous tubules with lumen, acrosomal vesicles and acrosome reaction was increased. This single-dose study was not taken forward for BMD analysis:

During the exposure during the growth phase, the CEP Panel assigned a likelihood level of Likely to the cluster male reproductive toxicity of BPA. Since the likelihood level is Likely for the endpoint testis histology (decrease in seminiferous tubule diameter) in the Tier 2 rat study (Gurmeet et al., 2014 [RefID 2502]); this study was taken forward for BMD analysis (see Chapter 3.2.1) and uncertainty analysis (see Appendix D).

Adult exposure (after puberty)

Plasma/serum testosterone: For this endpoint four Tier 3 rat studies (Srivastava and Gupta, 2018 [RefID 13167]; Wu et al., 2016 [RefID 8036]; Huang DY et al., 2018 [RefID 12167]; Rashid et al., 2018 [RefID 12963]), three Tier 3 mouse studies (Xu XH et al., 2015 [RefID 8232]; Chouhan et al., 2015

[RefID 1216]; Gao et al., 2018 [RefID 11972]) and one Tier 3 monkey study (Vijaykumar et al., [RefID 7477]) were identified.

As only Tier 3 studies were identified, data were considered Inadequate to judge the likelihood of an effect on testosterone.

Epididymis weight: For this exposure period only one Tier 3 mouse study (Dobrzynska et al., 2014 [RefID 1645]) was identified in which epididymis weight was measured.

As only one Tier 3 mouse study was identified, data were considered Inadequate to judge the likelihood of an effect on epididymis weight.

Prostate histology: For this exposure period one Tier 2 (Olukole et al., 2018 [RefID 12841]) and two Tier 3 studies (Huang DY et al., 2018 [RefID 12167]; Wu et al., 2016 [RefID 8036]) in rats were identified.

In the Tier 2 study (Olukole et al., 2018 [RefID 12841]), rats were dosed with 10000 µg/kg bw per day for 14 days. The following effects were increased in prostate histology of the single-dose BPA-treated group: degenerative changes, atrophy (atrophic tubules); non-neoplastic proliferative lesion: functional hyperplasia, proliferative lesions, reduced glandular diameter, hyperplasia reactive (accompanied by inflammation); inflammatory changes: inflammation and vascular digestion; atypical hyperplasia.

The likelihood of the effects on prostate histology was judged as Inadequate evidence as the effects on prostate histology were observed in a single-dose Tier 2 rat study (Olukole et al., 2018 [RefID 12841]).

Seminal vesicle weight: For this exposure period one Tier 3 mouse study (Chouhan et al., 2015 [RefID 1216]) was identified.

As only one Tier 3 mouse study was identified, data were considered Inadequate to judge the likelihood of an effect on seminal vesicle weight.

Sperm count: For this exposure period one Tier 1 study (Wang HF et al., 2016 [RefID 7618]), two Tier 2 studies (Park et al., 2018 [RefID 12869]; Yin et al., 2017 [RefID 8510]) and three Tier 3 studies (Dobrzynska et al., 2014 [RefID 1645]; Gao et al., 2018 [RefID 11972]; Chouhan et al., 2015 [RefID 1216]) in mice and one Tier 3 rat study (Srivastava and Gupta, 2018 [RefID 13167]) were identified.

In the Tier 1 study (Wang HF et al., 2016 [RefID 7618]) mice were dosed for 8 weeks with 10, 50 or 250 µg/kg per day; no effect on sperm count was noted. In the Tier 2 mouse study (Yin et al., 2017 [RefID 8510]) no effect was observed on sperm count after 5 weeks dosing with 3000, 30000 or 300000 µg/kg bw per day. In another Tier 2 mouse study (Park et al., 2018 [RefID 12869]) a decrease in sperm count was seen at the only dose tested (10000 µg/kg bw per day) for 12 weeks.

The likelihood of the effect on sperm count was judged as Not Likely as one Tier 1 study (Wang HF et al., 2016 [RefID 7618]) and one Tier 2 study (Yin et al., 2017 [RefID 8510]) in mice were without effects (dose range 10–300000 µg/kg per day) and the decrease on sperm count was seen in a single-dose study (Park et al., 2018 [RefID 12869]) at 10000 µg/kg bw per day.

Sperm motility: For this exposure period one Tier 1 study (Wang HF et al., 2016 [RefID 7618]), one Tier 2 study (Park et al., 2018 [RefID 12869]) and one Tier 3 study (Dobrzynska et al., 2014 [RefID 1645]) in mice were identified.

In the Tier 1 study (Wang HF et al., 2016 [RefID 7618]) mice were dosed for 8 weeks with 10, 50 or 250 µg/kg bw per day; a dose-related decrease on sperm motility was observed. In the Tier 2 mouse study (Park et al., 2018 [RefID 12869]) a decrease in sperm motility was observed at the only dose tested (10000 µg/kg bw per day) for 12 weeks.

The likelihood of the decrease in sperm motility was judged as Likely based on the dose-related decrease in the Tier 1 mouse study (Wang HF et al., 2016 [RefID 7618]) at doses 10, 50 or 250 µg/kg bw per day. This effect was supported by decrease in sperm motility seen in the single dose (10000 µg/kg bw per day) in the Tier 2 study (Park et al., 2018 [RefID 12869]). This single-dose study was not brought forward for BMD analysis.

Sperm morphology: For this exposure period one Tier 2 study (Park et al., 2018 [RefID 12869]) and one Tier 3 study (Dobrzynska et al., 2014 [RefID 1645]) in mice were identified.

In the Tier 2 mouse study (Park et al., 2018 [RefID 12869]) an increase in abnormal sperm was observed at the only dose tested (10000 µg/kg bw per day) for 12 weeks.

The likelihood of the increase of abnormal sperm was judged as Inadequate as the increase was observed in a single dose (10000 µg/kg bw per day) Tier 2 mouse study (Park et al., 2018 [RefID 12869]).

Sperm viability: For this exposure period one Tier 1 mouse (Wang HF et al., 2016 [RefID 7618]) study was identified.

In the Tier 1 study (Wang HF et al., 2016 [RefID 7618]) mice were dosed for 8 weeks with 10, 50 or 250 µg/kg bw per day; a dose-related decrease on sperm motility was observed which was significant at the highest dose level and decreased not statistically significant also in the mid-dose group.

The likelihood of the decrease in sperm viability was judged as Likely based on the dose-related decrease in the Tier 1 mouse study (Wang HF et al., 2016 [RefID 7618]) at doses 10, 50 or 250 µg/kg bw per day.

Sperm acrosome reaction: For this exposure period one Tier 1 mouse study (Wang HF et al., 2016 [RefID 7618]) was identified. In this Tier 1 study (Wang HF et al., 2016 [RefID 7618]) mice were dosed for 8 weeks with 10, 50 or 250 µg/kg bw per day. A dose-related decrease in acrosome reaction was seen at the two highest dose levels.

The likelihood of the decrease in acrosome reaction was considered to be Likely.

Testis weight: For this exposure period one Tier 1 study (Wang HF et al., 2016 [RefID 7618]) and three Tier 3 studies (Dobrzynska et al., 2014 [RefID 1645], Gao et al., 2018 [RefID 11972], Chouhan et al., 2015 [RefID 1216]) in mice were identified. In the Tier 1 study (Wang HF et al., 2016 [RefID 7618]) mice were dosed for 8 weeks with 10, 50 or 250 µg/kg bw per day; no effect on testis weight was noted.

The likelihood for an effect on testis weight was judged as Not Likely as no effect was observed in the Tier 1 mouse study.

Overall, the CEP Panel assigned a likelihood of Likely to the cluster male reproductive toxicity during adult exposure. As the likelihood level for male reproductive toxicity is Likely for the endpoint sperm motility, viability and acrosome reaction in the Tier 1 mouse study (Wang HF et al., 2016 [RefID 7618]) these data were taken forward for BMD analysis (see Chapter 3.2.1) and uncertainty analysis (see Appendix D).

Indirect (germline) exposure

For this exposure period two Tier 3 mouse studies (Dobrzynska et al., 2015 [RefID 1644]; Dobrzynska et al., 2018 [RefID 11837]), in which epididymis weight, sperm count and sperm motility were measured, were identified. In addition, in the Tier 3 mouse study (Dobrzynska et al., 2015 [RefID 1644]) sperm morphology was examined and in the other Tier 3 study (Dobrzynska et al., 2018 [RefID 11837]) testis weight was measured.

The CEP Panel noted that as only Tier 3 studies were available, the evidence was considered Inadequate for this exposure period.

Overall cluster selection for endpoints/studies for BMD analysis for male reproductive toxicity

Overall, the CEP Panel assigned a likelihood level of ALAN to the male reproductive toxicity cluster in the developmental exposure period, of Likely in the developmental and adult, growth phase/young age and adult exposure periods, and of Inadequate evidence in the indirect (germline) exposure period.

The overall likelihood across all exposure periods, i.e. the highest likelihood given in the cluster male reproductive toxicity was Likely.

The CEP Panel considered that the evidence from the studies available showed a Likely effect for epididymis histology (exfoliated germ cells and inflammation) in the Tier 1 rat study (NTP Clarity Report,

2018/Camacho et al., 2019 [RefID 11370]) during the developmental exposure (pre-natal and/or post-natal until adult) period. In addition, with exposure during the growth phase a Likely effect was reported for testis histology (decrease in seminiferous tubule diameter) in a Tier 2 rat study (Gurmeet et al., 2014 [RefID 2502]) and during adult exposure effects on sperm (motility; viability; acrosome reaction) were observed in a Tier 1 mouse study (Wang HF et al., 2016 [RefID 7618]). Therefore, these endpoints were taken forward for BMD analysis (see Chapter 3.2.1).

3.1.6.3 Integration of likelihoods from human and animal studies

Table 13 presents the overall likelihood per cluster for the human and animal stream separately, as well as the integration of the likelihoods from the human and animal studies for reproductive toxicity.

AGD, bone development and breast development are endpoints assessed in two different clusters: in pubertal/endocrine in the human stream and in developmental toxicity in the animal stream. Overall, the animal and human clusters are not comparable. It is however of relevance to analyse the endpoints on which the final conclusion for the likelihood is based, both for animal and human clusters. In case of any similar outcomes the evidence of the respective likelihood would increase. In the human stream the fetal and post-natal growth cluster contains results for body weight and femoral length being comparable to body weight effects and effects on bone development in the developmental cluster for animals. While human studies show Not Likely effects, animal studies result in ALAN effects on body weight and bone development. The further likelihood of effects observed in developmental animal studies is based on ALAN effects on mammary gland development and age of first estrus. Neither of these effects have been described as changed nor as references of a similar likelihood in human studies. The pubertal/endocrine cluster of the human stream bases its ALAN evaluation on changes of the AGD. The assessment of the AGD in animals revealed, however, no effects. Sex hormones are included in the human pubertal/endocrine and in the animal Male and Female reproductive toxicity clusters, both without any influence on the overall conclusion on the respective likelihoods. Thus, finally there is no substantiation of the ALAN likelihood for the animal cluster developmental toxicity nor of the ALAN likelihood for the human cluster pubertal/endocrine. In the animal Male and Female reproductive toxicity clusters, there were Likely effects on sperm, follicles and implantation, but not on fertility. There was no clear overlap between the endpoints assessed in human and animal studies that focused on reproductive toxicity in females. As for effects on mammary gland development, ovary and uterus weight and histology, there are no studies available in human to be compared and integrated with the animal evidence.

Table 13: Integration of the human and animal studies for Reproductive and developmental toxicity.

Human stream	Animal stream	Integrated likelihood
Cluster: Developmental toxicity	**Cluster: Developmental toxicity**	
Not applicable	Developmental exposure (pre-natal and/or post-natal until weaning) ALAN	
	Developmental and adult exposure (pre-natal and/or post-natal in pups until adulthood) ALAN	
	Growth phase/young age exposure ALAN	
	Adult exposure (after puberty) Not Likely	
	Indirect (germline) exposure Not Likely	
	Overall likelihood: ALAN	*ALAN*
Cluster: Fetal and Post-natal Growth	**Cluster: Fetal and Post-natal Growth**	
Exposure during Pregnancy Not Likely	Not applicable	

Overall likelihood:	*Not Likely*			*Not Likely*
Cluster: Pubertal/Endocrine		**Cluster: Pubertal/Endocrine**		
Exposure during Pregnancy	ALAN	Not applicable		
Exposure during Childhood	Not Likely			
Overall likelihood:	*ALAN*			*ALAN*
Cluster: Female fertility		**Cluster: Female reproductive toxicity**		
Exposure during Adulthood	ALAN	Developmental exposure (pre-natal and/or post-natal until weaning)	Likely	
		Developmental and adult exposure (pre-natal and/or post-natal in pups until adulthood)	Likely	
		Growth phase/young age exposure	Likely	
		Adult exposure (after puberty)	Likely	
		Indirect (germline) exposure	Inadequate evidence	
Overall likelihood:	*ALAN*	*Overall likelihood:*	*Likely*	*Likely*
Cluster: Male fertility		**Cluster: Male reproductive toxicity**		
Exposure during Adulthood	Not Likely	Developmental exposure (pre-natal and/or post-natal until weaning)	ALAN	
		Developmental and adult exposure (pre-natal and/or post-natal in pups until adulthood)	Likely	
		Growth phase/young age exposure	Likely	
		Adult exposure (after puberty)	Likely	
		Indirect (germline) exposure	Inadequate evidence	
Overall likelihood:	*Not Likely*	*Overall likelihood:*	*Likely*	*Likely*
Cluster: Prematurity		**Cluster: Prematurity**		
Exposure during Pregnancy	Not Likely	Not applicable		
Overall likelihood:	*Not Likely*			*Not Likely*
Cluster: Pre-eclampsia		**Cluster: Pre-eclampsia**		
Exposure during Adulthood	ALAN	Not applicable		
Overall likelihood:	*ALAN*			*ALAN*

3.1.6.4. *In vitro* and Mechanistic studies

Regarding scoring of likelihood of effects in the WoE in the HoC Reproductive effects, a few cluster/endpoints were scored Likely and several were scored ALAN, either in animals or human studies. In the following, MoA studies for these clusters are considered.

Developmental toxicity

The overall likelihood of effects of BPA in animal studies for the cluster developmental toxicity was scored ALAN.

In humans, no study offered an understanding on putative MoA pathways related to developmental toxicity following exposure to BPA.

In animals, overall, 27 studies considered a MoA related to one of the ALAN effects in animals. For males these effects are body weight (54 studies), mammary gland development (three studies), testis descent (two studies), and bone development (three studies). For the females seven studies assessed

the mammary gland development, three the bone development, one the age of first estrus, and 47 body weight.

Results from *in vitro* experiments were only related to mammary gland development (12 studies).

Body weight changes and their related MoA are described in the Metabolic section (Chapter 3.1.4.4).

The *in vivo* studies on mammary gland effects show evidence that stromal–epithelial interactions may play a crucial role in the BPA-induced developmental changes in the mammary gland. Epigenetic effects of BPA including changes in methylation patterns are frequently reported along with altered expression of genes involved in proliferation/apoptosis and other cellular functions. Results from two breast cancer models (i.e. xenograft mouse model and chemically (DMBA) induced cancer) suggest that BPA may enhance the susceptibility to carcinogenic stimuli. In addition, there is some evidence from *in vitro* studies with breast cancer cells which supports the hypothesis that BPA promotes changes in proliferation and pathological processes which ultimately could contribute to carcinogenesis in the mammary gland. More details are reported in Chapter 3.1.8.4, *In vitro* and Mechanistic studies on Carcinogenicity and mammary gland proliferative effects.

Bone development studies reported both increased male femoral cortical thickness, male femoral diaphyseal cortex thickness and female femur length, and decreased male femur length (Lejonklou et al., 2016 [RefID 3974]; Lind et al., 2017 [RefID 4350]). One of these studies linked the differential expression of bone marrow gene expression to the sex-specific effects observed (Lind et al., 2017 [RefID 4350]). No *in vitro* studies were available on bone development.

Female reproductive toxicity

After the integration of the human and animal evidence, the overall likelihood of effects of BPA for the cluster female reproductive toxicity was scored Likely.

Two studies performed in human populations were deemed relevant to contribute to our understanding on the MoA for female reproductive toxicity (Caserta et al., 2013 [RefID 883]; Cao et al., 2018 [RefID 11666]).

Addressing general aspects of female reproductive toxicity, Caserta et al. (2013) [RefID 883] reported a positive association between BPA and expression of genes involved in hormone response pathways and in steroid biosynthesis (ERα, ERβ, AR, AhR, PXR) in infertile women.

Effects on ovary

BPA appears to affect ovarian follicle development at low doses. BPA-induced oxidative stress leading to altered steroidogenesis is a plausible mechanism.

In humans, Cao et al. (2018) [RefID 11666] found that anti-Müllerian hormone (AMH) and E2 levels in the follicular fluid of patients with decreased ovarian reserve (DOR) were lower than those of non-DOR patients and that the BPA concentration was correlated with the AMH and E2 levels in the follicular fluid.

In animals, Thilagavathi et al. (2017) [RefID 9247] reported that the number of antral follicles in rats was decreased after BPA 10 μg/kg bw per day for 12 weeks but increased after 50 and 100 μg/kg bw per day. Based on their data, they proposed that BPA-induced oxidative stress leads to antioxidant depletion in ovary, which leads to elevated level of TBARS, which leads to overexpression of endothelial nitric oxide (eNOS), which prevents steroidogenic acute regulatory protein (StAR) transport from outer to inner mitochondria membrane, which inhibits CYP11A1, leading to downregulation of aromatase.

Berger et al. (2016) [RefID 524] reported that in mice dosed during gestation with BPA 0.5–50 μg/kg bw per day, germ cell nest breakdown was directly inhibited, probably by altering gene expression for apoptosis, oxidative stress and autophagy. Preantral follicle numbers were decreased, potentially by causing death of these follicles at time points earlier than 3 months. They concluded that *in utero* BPA exposure has different effects on the gene expression of steroidogenic enzymes and these effects depend on the dose of BPA, age and the generation of the mice.

Santamaria et al. (2016) [RefID 6448] reported reduced primordial to primary follicle transition and altered steroidogenesis in adult rats dosed during gestation and lactation with 0.5 or 50 μg/kg bw per day. AR was increased in primordial follicles at 5 μg/kg bw per day and decreased in primary follicles

at 50 μg/kg bw per day. They concluded that BPA could affect ovulation through different mechanisms depending on dose but did not speculate on what was causing the altered steroidogenesis.

Santamaria et al. (2017) [RefID 6449] reported reduced ovulatory response to exogenous gonadotrophins in pre-pubertal female rats dosed during gestation and lactation at 50 μg/kg bw per day. They suggested that different mechanisms might be leading the follicles to atresia and that loss of AR in the later stages of follicular development might be part of a paracrine mechanism that affords protection against premature luteinisation and atresia.

Cao et al. (2018) [RefID 11666] reported that BPA decreased the ovarian reserve in adult mice dosed with 5–500 μg/kg bw per day for 28 days. They hypothesised that BPA may reduce ovarian granulosa cell activity and accelerate its apoptosis, leading to the decreased synthesis of AMH. They did not speculate on upstream mechanisms.

Soleimani Mehranjani and Mansoori (2016) [RefID 5007] reported that adult rats dosed with BPA 60 μg/kg bw per day for 20 days had reduced antral follicles and increased atretic follicles. Concomitant vitamin C ameliorated these adverse effects. They did not propose a MoA, but antagonism of BPA-induced oxidative stress is a plausible possibility.

Ganesan and Keating (2016) [RefID 2141] reported that *in vitro* incubation of perinatal rat ovary with very high dose of BPA (440 μM) reduced primary and secondary follicle numbers after 2 days, followed by a reduction in primordial follicle numbers after four days and induced ovarian DNA damage. They concluded that BPA, via biotransformation, may be converted to a DNA alkylating agent. It is noted that the concentration tested was likely many orders of magnitude higher than achievable *in vivo* levels.

Overall, at lower doses, oxidative stress leading to steroid alterations is a plausible mechanism for reduced follicle development. DNA damage is only reported at a much higher dose/concentration.

Effects on uterus

The possible mechanism of BPA for the effects on uterus in animals (uterus weight and uterus histology, including non-neoplastic lesions, pre-neoplastic lesions, neoplastic lesions, proliferation and apoptosis are described here and are also described in Chapter 3.1.8.4 (MoA on Carcinogenicity).

Uterus weight

The CEP Panel considered the likelihood for an increase in uterus weight ALAN during developmental exposure. No MoA study specially addressed this outcome.

Uterus histology (*non-neoplastic changes*)

No human studies were available addressing these effects, while several non-neoplastic changes were evaluated in animal studies. These changes can be included in the broad categories of hyperplasia and metaplasia.

Some MoA studies have investigated *in vitro* the role of BPA in proliferation of uterine cells derived from tumour cell lines or endometrial biopsies from woman. Wang GH et al. (2013) [RefID 7675] showed that BPA increased human uterine myoma tissue-derived mesenchymal stem cells (hUM-MSCs) proliferation in a dose-dependent manner. The same group (Wang KH et al., 2015 [RefID 7676]) showed that BPA increased growth rate efficiency in a dose-dependent manner in human endometrial carcinoma cells. According to Mannelli et al. (2015) [RefID 4841], BPA demonstrated a proliferative effect at a concentration of 100 nM after 48 h in *in vitro* decidualised endometrial stromal cells (ESCs) from hysterectomy specimens. On the contrary, in another study (Forte et al., 2016 [RefID 2056]) BPA did not affect cell proliferation on human ESCs, derived from endometrial biopsies from woman not affected by endometriosis.

In conclusion, most MoA studies indicate that BPA may increase the proliferative rate of different types of uterine cells.

Uterus histology (pre-neoplastic lesions)

No pre-neoplastic lesions were evaluated in human or animal studies and no MoA study specifically addressed this outcome.

Uterus histology (neoplastic lesions)

No neoplastic lesions were evaluated in human studies. Based on evidence from animal (rats and mice) studies, the overall likelihood of effects of BPA for uterine neoplastic lesions was considered Not Likely.

Uterus histology (proliferation and apoptosis)

Proliferation or apoptosis in uterus were not evaluated in human studies. In animals, apoptosis was evaluated with standard histology in one study in rats (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) that reported an increase (trend) at the highest dose (25000 µg/kg body weight (bw) per day) at 1 year. However, no effect was demonstrated in the related study (Leung et al., 2020 [RefID 13789]). Based on these divergent results from animal (rat) studies, the overall likelihood of effects of BPA for uterine proliferative and apoptotic changes was ALAN for apoptosis in the developmental until adult exposure period. *In vitro* MoA studies do not support/explain the increase of apoptosis observed in BPA-treated animals in one *in vivo* study.

Chou et al. (2017) [RefID 1210] suggested that BPA exposure might alter miRNA expression to activate the hedgehog signalling pathway for promoting cell proliferation and decreasing apoptosis in endometrial tumorigenesis. Ponniah et al. (2015) [RefID 5876] showed that treatment of BeWo cells with BPA during stress-induced paradigms led to a reduction in apoptosis. Wang KH et al. (2015) [RefID 7676] reported that BPA increases cell migration and invasion ability in human endometrial carcinoma cell line possibly through MAPK pathway-dependent upregulation of COX-2 gene expression. Wang KH et al. (2015) [RefID 7676] reported that BPA induces upregulation of COX-2 gene expression that can involve multiple physiological functions including resistance to apoptosis.

In conclusion, MoA studies do not support/explain the increase of apoptosis observed in BPA-treated animals in one *in vivo* study.

Implantation incidence

As indicated in the WoE chapter, the CEP Panel considered that the evidence from the studies available indicated a Likely effect of BPA on implantation incidence. No human evidence on mechanisms of action was available.

In animals, the *in vivo* studies showed an effect of BPA on the number of embryo implantations and implantation loss (Li et al., 2016 [RefID 4128]; Yuan et al., 2018 [RefID 13593]). BPA caused a change in tight junction (TJ) proteins in the uterine epithelium during early pregnancy (Martínez-Peña et al., 2013 [RefID 4909]). Exposure to BPA interfered with ERα-mediated and PGR-mediated signalling pathways in the uterus during early pregnancy. In *in vitro* studies BPA can downregulate SGK1 and ENaCα protein expression through ERs in Ishikawa cells which may lead to embryo implantation failure through the ER/SGK1/ENaCα signalling pathway (Yuan et al., 2018 [RefID 13593]). BPA did not affect cell proliferation, but arrested ESCs at G2/M phase of cell cycle enhancing cell migration (Forte et al., 2016 [RefID 2056]). BPA also increased gene expression and protein levels of some decidualisation markers, such as insulin growth factor binding protein 1 (IGFBP1) and prolactin (PRL), amplifying the effect of progesterone alone. Data suggest that BPA might alter human endometrium physiology, hence, affecting fertility and pregnancy outcome. In another *in vitro* study (Olson et al., 2017 [RefID 5518]) BPA impaired steroid-hormone receptor expression at very high concentrations but not at lower concentrations. Cell proliferation and cyclin D2 expression in the high-dose groups was decreased compared with controls. These findings demonstrate that BPA disrupts *in vitro* decidualisation of uterine stromal fibroblasts by altering steroid-hormone receptor expression at higher concentrations but not at lower physiological doses. BPA treatment could reduce the invasion ability of BeWo human endometrial cells (model mimicking embryo implantation) and alter the expression level of E-cadherin, DNMT1, TIMP-1, TIMP-2, MMP-2 and MMP-9 (Wang ZY et al., 2015 [RefID 7852]). BPA activated ERK through nuclear and membrane ERs and inhibited CXCL8 expression in DSCs, thereby affecting their regulation of trophoblast invasion (Li X et al., 2018 [RefID 11157]).

Overall, BPA interfered with ERα-mediated and PGR-mediated signalling pathways in the uterus during early pregnancy *in vivo*. *In vitro* BPA interfered with ERα-mediated and PGR-mediated signalling pathways in the uterus during early pregnancy BPA, can alter expression of genes associated with decidualisation, cell cycle regulation and several proliferative markers.

Male reproductive toxicity

After the integration of the human and animal evidence, the overall likelihood of effects of BPA for the cluster male reproductive toxicity was scored ALAN.

Effects on prostate

No evidence was available regarding the MoA of BPA for prostate effects in the human studies. In animals, for histology, the following subclusters were considered if available: non-neoplastic changes, pre-neoplastic lesions, neoplastic lesions, proliferation and apoptosis.

Seventeen MoA studies specifically addressing carcinogenic effects on the prostate were considered, five within the mammalian stream and 12 in the *in vitro* stream. MoA studies from the *in vitro* stream often used prostate cancer cell lines (in seven studies) and in particular LNCaP, PC3 and C4-2 cell lines, in some cases stem cells (in four studies) and normal cells (in 2 studies). Other studies related to non-neoplastic changes have also been taken in consideration.

Prostate histology (*in vivo* studies)

Multifocal inflammation and reactive hyperplasia in the ventral prostate were reported in two single-dose-level Tier 1 rat studies (Bernardo et al., 2015 [RefID 533] and Brandt et al., 2014 [RefID 700], both from the same institution).

Bernardo et al. (2015) [RefID 533] suggested there was 'stimulated growth of the fetal/neonatal prostate' and reported that AR expression was increased on PND21 in the ventral prostate at the lower but not the higher dose (25 not 250 μg/kg bw per day); they suggested that this was consistent with pre-natal BPA-induced androgen sensitivity leading to an increased expression of AR in the prostate in pubertal and young adults rodents. Brandt et al. (2014) [RefID 700] suggested that prostate morphological changes seen on PND21 (but not PND180) after gestational BPA exposure may be related to delayed prostate morphogenesis, but also that the data are consistent with stimulated growth of the fetal/neonatal prostate.

Bernardo et al. (2015) [RefID 533] reported that co-treatment with genistein attenuated the BPA effects and suggested that a mechanism might be ERβ-mediated antagonism of AR function and AR-dependent proliferation. Brandt et al. (2014) [RefID 700] reported attenuation by co-treatment with indole-3-carbinol and suggested that this might possibly be related to suppressed responsiveness to oestrogen and decreased expression of oestrogen receptor alpha (ERα). A detailed rationale for choice of these bioactive plant compounds and for the doses used was not provided in the papers, but the data do suggest that oestrogen/androgen balance is involved in BPA early developmental effects.

Mechanisms involved in oestrogen/androgen balance are explored in a number of studies, including gene and protein expression related to oestrogen/androgen balance in the adult rodent prostate and developing rodent epididymis:

Castro et al. (2018) [RefID 11674] reported increased plasma E2 and aromatase expression in prostate, leading to an increase in intraprostatic E2, in juvenile rats after gestational exposure to BPA 10 μg/kg bw per day.

Wong et al. (2015) [RefID 7997] reported that after neonatal (PND1, 3 and 5) BPA exposure to 2, 10 or 50 μg/kg bw per day, adult rats had upregulated expression of the secretoglobin polypeptide family member Scgb2a1, with a parallel increase in histone H3 lysine 9 acetylation (H3K9Ac). They noted that *Scgb2a1* is an AR-regulated gene that exhibits androgenic responsiveness in *in vitro* reporter assays. but that functional consequence of BPA-mediated developmental reprogramming of Scgb2a1 in the prostate is not clearly defined.

Wu et al. (2016) [RefID 8036] concluded that low-dose BPA (10–90 μg/kg bw per day) may induce proliferation of ventral prostate in adult rats by increasing the oestrogen-to-androgen ratio and upregulating expression of prostaglandin D2 synthase (Ptgds) to promote production of dihydrotestosterone.

Huang DY et al. (2017) [RefID 2869] concluded that BPA 0.1–1 nM directly promotes the in-vitro-proliferation of rat prostate cells through increasing the expression of ERs, reducing the expression of ARs and thus decreasing apoptosis-induced cell death.

Huang DY et al. (2018) [RefID 12167] reported that BPA increased the oestrogen-to-androgen ratio and upregulated ERα and AR expression, and subsequently further induced the occurrence of epithelial–mesenchymal transition.

It seems plausible that at low doses, reported BPA effects on developing prostate could involve oestrogen/androgen balance in some way. However, explicit upstream mechanisms have not been proposed.

Olukole et al. (2018) [RefID 12841] reported rat prostate hyperplasia and inflammation after high-dose BPA (10000 μg/kg bw per day for 14 days). Effects were ameliorated by melatonin, which is considered to be an antioxidant substance (endogenous free radical scavenger, e.g. Hu C et al. 2021). These findings are consistent with generation of oxidative stress as a mechanism for high-dose BPA effects on adult prostate.

At lower doses, there may be other mechanisms. Wu et al. (2016) [RefID 8036] and Huang DY et al. (2018) [RefID 12167] (from the same laboratory) reported prostate proliferation after 10, 30 and 90 μg/kg bw per day for 1 or 3 months, respectively. Wu et al. (2016) [RefID 8036] reported that 90 μg/kg bw per day BPA had less effects on prostate than 10 μg/kg bw per day. They concluded that BPA increased the oestrogen-to-androgen ratio and upregulated prostaglandin D2 synthase (Ptgds) expression to transport testosterone into the prostate and convert into dihydro-testosterone, which would cause the prostatic epithelium to proliferate. They noted that it is unclear how BPA affects Ptgds gene expression, but this is a downstream effect.

In vivo studies on mechanisms for rodent developmental BPA-induced prostate inflammation and reactive hyperplasia have explored a number of endpoints, including alterations in ER-related and AR-related gene and protein expression in the prostate. Proposed mechanisms include increased expression of AR, decreased expression of ERα, increased prostate aromatase expression leading to increased intraprostatic E2, and altered expression of other androgen-related genes (e.g. Scgb2a1, Ptgds).

In conclusion, despite the relatively large amount of data available, there is no consensus on mechanisms underlying developmental BPA-induced prostate effects, in particular whether effects are upstream or downstream.

Prostate histology (*in vitro* studies)

- Prostate histology (non-neoplastic changes)

No evidence was available regarding the MoA of BPA for this effect in the human studies. Two histological non-neoplastic findings (inflammation and hyperplasia) were evaluated in animal studies: based on the inconsistencies in the outcomes and the diversity of the effects, the CEP Panel judged the prostatic non-neoplastic changes as ALAN.

Regarding inflammation, the results of two Tier 1 related studies (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]; Prins et al., 2018 [RefID 13779]) indicated no effect on inflammation. An increase in multifocal inflammation was observed in the two other related Tier 1 studies (Bernardo et al., 2015 [RefID 533]; Brandt et al., 2014 [RefID 700]) and a decrease in lymphocytic infiltration was reported in another study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]). Regarding hyperplasia, two Tier 1 studies (Bernardo et al., 2015 [RefID 533]; Brandt et al., 2014 [RefID 700]) showed an increase, whereas one Tier 1 study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) showed a decrease. Another related Tier 1 study (Prins et al., 2018 [RefID 13779]) showed no effect.

Some lines of research have pointed to a potential role for inflammation in prostatic carcinogenesis and tumour progression in humans. The cause of prostatic inflammation is often unknown, but might be caused by infection, exposure to chemical, physical trauma, hormonal variations and/or exposures, or dietary factors. The resultant epithelial cellular injury might cause a loss of tolerance to normal prostatic antigens, resulting in a self-perpetuating autoimmune reaction. No MoA studies have specifically investigated *in vitro* the role of BPA in inducing prostate inflammation.

Regarding hyperplasia, a small number of *in vitro* MoA studies indicate that BPA can enhance proliferation of prostate (see the subcluster proliferation and apoptosis).

In conclusion, *in vitro* MoA studies indicate that BPA may increase the proliferative rate of different types of prostate cells while no data are available for inflammation.

- Prostate histology (pre-neoplastic lesions)

No evidence was available regarding the MoA of BPA for this effect in the human studies. Two histological pre-neoplastic findings (atypical hyperplasia and dysplasia) were evaluated in animal studies in the developmental exposure period and were judged as ALAN.

No *in vitro* MoA study specifically addressed these outcomes.

- Prostate histology (neoplastic lesions)

No evidence was available regarding the MoA of BPA for this effect in the human studies. In animal studies, no neoplastic lesions of prostate were reported.

Sex hormones play a role in prostate cancer development. Prostate cancer is initially androgen-dependent and relies on AR activation. Increasing evidence has demonstrated that *in utero* exposure to oestrogenic compounds may have a significant role decade after these initial exposures in promoting prostate cancer development through a synergistic effect with testosterone. However, all prostate cancers eventually become androgen-independent with the participation of many other signalling molecules.

Some MoA studies have focused their attention on androgen and oestrogen signalling. Prins et al. (2014) [RefID 5929] assessed whether BPA has oestrogen-like effects on human prostate stem-progenitor cells. They demonstrated that BPA increases stem-progenitor cell self-renewal and expression of stem-related genes as well as signalling activation of Akt ERK. The authors proposed that early-life perturbations in oestrogen signalling, including exposure to BPA, can amplify and modify the stem-progenitor cell populations of the prostate gland and increase the hormone-dependent cancer risk. Tarapore et al. (2014) [RefID 7129] showed that BPA can disrupt the centrosome duplication cycle and maturation, which underlies genomic instability, neoplastic transformation and cancer progression in the prostate. It was also suggested from the changes in microtubulin dynamics that in the non-tumorigenic cells, BPA may initiate or promote prostate cancer progression by interfering with AR function. Ho et al. (2017) [RefID 2727] investigated BPA effects on centrosome amplification, showing that BPA disrupts centrosome function and microtubule organisation, likely mediated via ER 1 signalling. Burton et al. (2015) [RefID 763] looked at the effect of E2 and BPA on the expression of histone modifying enzymes in prostate cancer cell lines. BPA exposure resulted in differential expression, and the use of an ER antagonist reversed the effects, indicating that BPA can regulate gene expression in prostate cancer via ER signalling.

Other *in vitro* MoA studies considered epigenetic events or other mechanisms such as modulation of gene expression, protein expression, signalling pathways, stem cell homeostasis or centrosome activity. Ho et al. (2015) [RefID 2726] determined whether epigenetic events mediating the action of BPA on human prostaspheres (PS) enriched in epithelial stem-like/progenitor cells are linked to prostate cancer. A set of non-coding small nucleolar RNAs with C/D motifs, known as SNORDs, were identified to be repressed by BPA. The authors considered the action of BPA to be mediated by histone modification of the exonic/regulatory sequences of the affected SNORDs and not by aberrant methylation of SNORD-specific CpG sites which might be relevant to prostate cancer carcinogenesis. Prins et al. (2018) [RefID 13779] reported that chronic low-dose BPA exposure in a model of epithelial stem and progenitor cells isolated by PS culture reprogrammes adult rat prostate stem cell homeostasis with increases in stem cell numbers and a shift in lineage commitment toward basal progenitor cells, while reducing luminal progenitor cells. This may underpin the increased carcinogenic risk with ageing. Calderon-Gierszal and Prins (2015) [RefID 796] reported effects of BPA in a model of prostatic organoids. Budding structures increased with 1 nM but decreased with 10 nM with a parallel increase in degraded structures. Vimentin and 963 mRNA expression were also increased at 10 nM as were prostate epithelial cell stemness genes NANOG, CD49 and OCT4. The authors concluded that nanomolar levels of BPA could modify embryonic prostate morphogenesis and disrupt prostate stem cell homeostasis in the maturing prostate. Such stem cell perturbations were considered to potentially increase the risk of human prostate cancer with ageing. Derouiche et al. (2013) [RefID 1548] reported that environmentally relevant concentrations of BPA (1–10 nM) induce migration of prostate cancer cells by modulating the cell calcium signalling.

In conclusion, data obtained from *in vitro* MoA studies indicate that BPA can enhance prostate cancer susceptibility, while its direct carcinogenicity is still debated. However, the results of studies carried out in laboratory rodents do not demonstrate a clear and direct evidence of tumorigenic activity of BPA.

- Prostate histology (proliferation and apoptosis)

No evidence was available regarding the MoA of BPA for this effect in the human studies. In animal studies carried out in rats, the CEP Panel considered proliferation and apoptosis in the developmental exposure period ALAN. Brandt et al. (2014) [RefID 700] showed an increase in proliferation at the dose of 25 µg/kg/bw from GD10 to GD21. This result was not supported by a Tier 2 study (Hass et al., 2016 [RefID 2610]). Brandt et al. (2014) [RefID 700] showed an increase in apoptosis at the highest dose (250 µg/kg/bw from GD10 to GD21).

A BPA-related increase of proliferation was supported by a few *in vitro* MoA studies. Prins et al. (2018) [RefID 13779] reported that stem cells, assessed by PS number, doubled with chronic 2.5 µg BPA/kg bw per day exposures. Moreover, PS size, reflecting progenitor cell proliferation, was greater at 25 and 250 µg BPA doses. Wu et al. (2013) [RefID 8034] conclude that BPA is the androgenic effectors of cell proliferation. Huang DY et al. (2017) [RefID 2869] investigated the proliferative effect and possible mechanisms of action of nanomolar BPA on the prostatic epithelium from rats. The results from BPA doses of 0.1–1 nM showed increased proliferation and upregulated ER but downregulated AR expression. It was concluded that BPA promotes the proliferation of prostate cells through increasing the expression of ERs, reducing the expression of ARs.

Regarding apoptosis, Urriola-Muñoz et al. (2018) [RefID 13285] showed that BPA induces apoptosis in prostate and ovary cancer cell lines, in a process dependent on ADAM17 activation. By contrast, lower rates of apoptosis were reported by Huang DY et al. (2017) [RefID 2869] on the prostatic epithelium from rats.

In conclusion, data obtained from a small number of *in vitro* MoA studies indicate that BPA can enhance proliferation of the prostate supporting the weak evidence gathered from studies in animals. Data from *in vitro* MoA studies regarding apoptosis do not shed light on this effect.

Effects on testis

Chevalier et al. (2015) [RefID 1148] reported a case–control study undertaken with 52 cases of cryptorchidism (26 transient, 26 persistent) in young boys born after 34 weeks of gestation compared with 128 healthy young boys matched for gestational age, birthweight and, when possible, parental geographical origin. Insulin-like peptide 3 (INSL3), a major regulator of testicular descent, was decreased in idiopathic undescended testis and inversely related, in the whole population of newborn males, to umbilical cord BPA concentrations. This negative correlation provides indirect evidence for an impact of BPA on INSL3 Leydig production during fetal development. It suggests that INSL3 is a possible target of fetal exposure to BPA. However, the deleterious impact of BPA on fetal testicular descent, via the disturbance of the INSL3 pathway, has yet to be demonstrated directly.

At relatively high dose (>1,000 µg/kg bw per day), NTP Clarity Report (2018)/Camacho et al. (2019) [RefID 11370] reported polyarteritis in adult rat testis (and in pancreas) after dosing from GD6 at 2,500 µg/kg bw per day only (not at lower or higher doses). They did not comment on the isolated finding; it is unclear whether this is a treatment effect or a random variation.

Quan et al. (2017) [RefID 6025] reported increased apoptosis in testis at all doses 1,000–100000 µg/kg bw per day, without dose–response. The proposed mechanism was BPA-induced ROS which attacks cells in testes directly and induces cell apoptosis.

Xie et al. (2016) [RefID 8130] reported spermatogenic arrest at the spermatocyte level and dose-related increased germ cell apoptosis, at high subcutaneous doses equivalent to oral 2220, 22220 and 1111000 µg/kg bw per day. In the same study they reported elevated expression levels of ERα and ERβ and proposed that this likely contributed to the abnormal proliferation of germ cells which then triggered increased apoptosis. They made no reference to ROS.

At lower doses, Shi et al. (2018) [RefID 13099] reported increased apoptosis at 20 and 50 µg BPA/kg bw per day with no dose–response, and decreased testis stages VII and VIII seminiferous epithelial cells only at 20, not at 0.5 or 50 µg/kg bw per day, in neonatal mice. They also measured markers of

oxidative stress and methyltransferases for DNA methylation and histone modification. It is not entirely clear from their discussion, but it appears that they concluded that increased ROS from damaged mitochondria was a mechanism in the BPA effects.

Gurmeet et al. (2014) [RefID 2502] reported reduced seminiferous tubule diameter at 100000 but not at 5,000 or 1,000 μg/kg bw per day in rats dosed from PND28–70. The study also reported significant lower level of free plasma testosterone and 17β-E2 in the BPA-treated animals; they speculated that the low testosterone level might have caused failure of spermatogenesis and disruption of the seminiferous epithelium, and that the low plasma testosterone level in BPA-treated animals was probably due to interference of proliferative activity and development of Leydig cells. No upstream mechanism was proposed.

Brouard et al. (2016) [RefID 734] reported increased incidences of seminiferous tubules with lumen, acrosomal vesicles and acrosome reaction after subcutaneous BPA equivalent to oral 1,800 μg/kg bw per day (the only dose tested) PND15–30. They suggested that BPA could be promoting spermatocyte and spermatid formation via activation of receptors in testis other than classical ERs, such as androgen (AR), oestrogen-related (ERRγ) and PPARγ receptors. They also reported decreased expression of genes encoding for blood-testis barrier (BTB) proteins, thus, suggested that BPA could be disrupting the BTB, presumably as a downstream effect.

Given the relatively high doses in Gurmeet et al. (2014) [RefID 2502] and Brouard et al. (2016) [RefID 734] (>1,000 μg/kg bw per day), it is possible that BPA-induced oxidative stress was involved in inducing Leydig cell and androgen/oestrogen changes.

However, also at lower doses, BPA-induced oxidative stress is implicated in testis effects.

Ullah et al. (2018a) [RefID 13281] reported reduced rat testis epithelial height and increased peroxidase in testis of rats dosed 50000 but not 5,000 or 25,000 μg/kg bw per day for 28 days. Although they reported that BPA did not significantly induce signs of oxidative stress *in vitro* (1, 10 or 100 ng/mL = 4.4, 44 or 440 pM), the authors concluded that *in vivo* BPA induces oxidative stress, which reduces testosterone secretion and thus spermatogenesis.

Ullah et al. (2018b) [RefID 13282] reported that markers of oxidative stress in the testis were significantly elevated and sperm motility and daily sperm production were reduced after dosing 50 but not 25 or 5 μg/L in drinking water (at oral dose ~2.5, but not at 1.25 or 0.25 μg/kg bw per day) from PND23 for 48 weeks. They also reported reductions in LH, FSH and testosterone, and concluded that low concentrations of BPA suppress gonadotropin secretion from pituitary, has oestrogenic and antiandrogenic effects, induces oxidative stress in the testis and affects spermatogenesis by causing arrest at the spermatogonial and spermatid stage (but the exact mechanism of action is unknown).

Effects on sperm

Two publications coming from the same study assessed whether occupational BPA exposure is associated with altered LINE-1 methylation bearing inconclusive results. Miao et al. (2014) [RefID 5073] investigated 77 male factory workers routinely exposed to BPA compared with 72 male workers not routinely exposed, to investigate if BPA exposure is associated with LINE-1 methylation changes in spermatozoa. The researchers concluded that LINE-1 hypomethylation does not appear to play a major role for the reported associations between BPA exposure and poor semen quality. In a different analysis, Tian et al. (2018) [RefID 13256] used 72 make workers routinely exposed via their employment compared with 86 unexposed workers and found a statistically significant association with LINE-1 methylation changes in spermatozoa. Finally, Zheng et al. (2017) [RefID 8992] considered if BPA exposure is associated with alteration in DNA hydroxymethylation, a marker for epigenetic modification, in human sperm and also used a case–control approach with male workers routinely exposed to BPA as part of their employment compared with those that were not. It was reported that BPA exposure likely interferes with gene expression via affecting DNA hydroxymethylation in a way partially dependent on trimethylation of histone 3 in human spermatogenesis.

Park et al. (2018) [RefID 12869] reported decreased sperm motility and increased abnormal sperm in mice at a single-dose-level (10000 μg BPA/kg bw per day) and increased serum markers of oxidative stress. BPA effects were ameliorated by a plant extract (*Lespedeza cuneata*) which has been reported to have anti-oxidative properties.

Dobrzynska et al. (2014) [RefID 1645] reported reduced mouse sperm counts at 5,000–20000 µg BPA/kg bw per day. They speculated that the mechanism might be oxidative stress disrupting cell junctions and adhesion between Sertoli and germ cells, leading to decreased number or dysfunction of Leydig and Sertoli cells.

These effects were seen at relatively high BPA doses (>1,000 µg/kg/d) but other studies reported effects on sperm at lower doses.

Wang HF et al. (2016) [RefID 7618] reported dose-related reduction in sperm motility in mice dosed 10, 50 or 250 µg BPA/kg bw per day for 8 weeks. They also reported that BPA dose-relatedly reduced CatSper expression and selectively and transiently inhibited CatSper currents in in mature mouse spermatozoa (maximum inhibition at about 100 pM). They therefore proposed that the mechanism involved the CatSper channel, either by downregulating the expression of CatSper or by inhibiting CatSper currents, although they could not exclude the involvement of other receptors such as ER. They also reported a dose-related decrease in sperm acrosome reaction at 50 and 250 µg BPA/kg bw per day. This is likely to be a downstream effect, since the acrosome reaction is dependent on CatSper (Tamburrino et al., 2014).

Rahman et al. (2015) [RefID 6061] reported that BPA at up to 1,000 nM did not produce significant or partial toxic effects on spermatozoa; however, at very high concentration (100 µM) BPA inhibited sperm motility and reduced intracellular ATP levels in spermatozoa, and increased levels of glycolysis/electron transport chain enzymes (GAPDHS/SDHB), for which the authors could not find a clear mechanistic explanation.

Rahman et al. (2016) [RefID 6062] reported that BPA 1–100 µM negatively affected *in vitro* mouse spermatozoa motility, viability, mitochondrial functions and intracellular ATP levels by activating the MAPK, phosphatidylinositol 3-kinase (PI3K) and protein kinase-A (PKA) pathways. They concluded that BPA adversely affects sperm function by activating several kinase pathways.

Skibinska et al. (2016) [RefID 6776] reported that BPA increased sperm mitochondrial membrane potential significantly at 1 nM but significantly decreased at 1,000 nM, with no change in mitochondrial superoxide formation, sperm vitality or phosphatidylserine membrane translocation. They concluded that BPA may target mitochondria by increasing the intracellular calcium ion concentration which may activate mitochondrial protein phosphorylation and in turn dephosphorylates cytochrome *c* oxidase. Consequently, membrane mitochondrial potential and ROS may increase.

Liu C et al. (2014) [RefID 4378] reported that in the testes of adult male rats dosed oral BPA 20 µg/kg bw per day daily for 60 days, initiation of meiosis (programmed DNA DSBs) was delayed in the early meiotic stage (spermatids), and chromosomal abnormalities and meiotic DSBs accumulated in the late meiotic stage (pachytene spermatocytes), suggesting that spermiation was inhibited and spermatogenesis was disrupted. The authors had no explanation for the spermiation delay but suggested that meiotic DSB accumulation in spermatocytes might be caused by BPA-induced inhibition of DNA break repair (by some unknown mechanism).

Chen et al. (2017) [RefID 1121] reported that testis of adult rats dosed BPA 50 µg/kg bw per day for 35 weeks had altered histone acetylation and upregulated ERβ with no obvious change in ERα or GPER. They concluded that ERβ is likely involved, at least in part, in the BPA-induced epigenetic changes in testis tissue (cell type(s) unknown).

Li Y et al. (2018a) [RefID 12491] reported *in vitro* DNA methylation and histone responses to BPA in spermatogonial cells (mouse cell line GC-1, immortalised by transformation with the plasmid pSV3-neo in type B spermatogonia). Expression of DNMT1 (DNA methylation enzyme) was increased 1.5-fold at 1 and 10 ng/mL (4.4 and 44 nM); decreased DNMT1 and changes in histone and DNA methylation were seen only at higher concentrations at which cell growth was inhibited.

Effects on Epididymis

NTP Clarity Report (2018)/Camacho et al. (2019) [RefID 11370] reported increased epididymis exfoliated germ cells and inflammation at 25,000 µg/kg bw per day at interim sacrifice (1 year) but not at terminal sacrifice (2 years); lower doses (2.5–2,500 µg/kg bw per day) did not affect these endpoints. This is a high-dose effect; possible mechanisms include both oestrogenic effects and oxidative stress.

Cluster overview for Reproductive and developmental toxicity

BPA effects are reported over a huge range of effective concentrations/doses (low nM and μg/kg bw per day to high μM and >1,000 μg/kg). This always needs to be considered; there may be qualitative as well as quantitative differences in mechanisms, depending on dose.

It is clear that many of the reported effects of BPA on male reproductive endpoints are measuring downstream intermediate pleiotropic effects (e.g. altered expression of many genes and proteins, DNA methylation and histone changes, activation of MAPK, PI3K, PKA, Akt/mTOR pathways, INSL3, StAR, etc.).

Details of upstream mechanisms, which are more likely to be BPA specific, are unclear in many cases. For example, mechanisms for BPA-induced sperm mitochondrial membrane potential changes could include altered ion channels, receptors (and receptor cross-talk) and ROS.

Receptor interactions are an obvious candidate mechanism for early BPA effects (AR, ERα, ERβ, GPER, ERRγ, PPARγ, etc.). Some studies have reported receptor-dependency of BPA effects, but the results are difficult to generalise, because steroid receptor properties and interactions are dynamic; substance effects can vary by e.g. tissue, life-stage and dose. In fact, much of the complexity of steroid receptor mechanisms has been revealed by research with BPA.

A plausible upstream mechanism for BPA effects is oxidative stress generation, at both low and high doses.

In summary, it seems plausible that BPA-induced oxidative stress (with modulation of androgen and oestrogen pathways, and downstream inflammation) is an early key event in the adverse effects of BPA on adult male and female reproductive organs.

3.1.6.5. Conclusion on hazard identification for Reproductive and developmental toxicity of BPA

In the previous opinion on BPA assessment (EFSA CEF Panel, 2015), the CEF Panel concluded that the evidence is not sufficient to infer a causal link between BPA exposure and reproductive and developmental effects in humans but re-confirmed that BPA is a reproductive toxicant in experimental animal studies at high doses (above a HED of 3.6 mg/kg bw per day, corresponding to the NOAEL HED for General toxicity). The CEF Panel assigned a likelihood level of ALAN to reproductive and developmental effects of BPA in animals at low doses (below HED 3.6 mg/kg bw per day).

In adult animals exposed at doses lower than 3.6 mg/kg bw per day, reproductive effects were considered to be ALAN; the data suggested that low-dose BPA may have adverse effects on testis function, especially various measures of spermatogenesis, although these effects were modest, and in several multigeneration studies no effects were observed at dose levels from as low as 3 μg/kg bw per day up to at least 50 mg/kg bw per day. There was less evidence that BPA will significantly impair testis morphology or reproductive endocrinology, especially in the longer term.

In animals exposed *in utero* at doses lower than 3.6 mg/kg bw per day, reported effects on reproductive function were contradictory and highly variable between studies. A likelihood level of Likely was assigned to BPA-induced proliferative changes in the mammary gland. The CEF Panel established a tolerable daily intake which was designated as temporary (t-TDI), pending the outcome of a long-term study in rats involving pre-natal as well as post-natal exposure to BPA then being undertaken by NTP/FDA.

Based on the human data, none of the reproduction clusters was considered Likely. Female fertility and pre-eclampsia after adult exposure, pubertal development after exposure during pregnancy were considered ALAN. Male fertility after exposure during adulthood, prematurity, fetal and post-natal growth after exposure during pregnancy and pubertal development after exposure during childhood were considered Not Likely.

In the animal studies, the likelihood of reproductive effects was assessed by WoE in three clusters (developmental toxicity, male reproductive toxicity, female reproductive toxicity), each subdivided according to exposure periods (developmental, developmental and adult, growth phase/young age, adult and indirect (germline) exposure).

Based on the animal data, several endpoints in both female and male reproductive toxicity clusters were judged as Likely.

In the female reproductive toxicity cluster, there were Likely effects on ovary weight and histology after developmental exposure, on implantation rate after growth phase/young age exposure and on follicle counts after adult exposure. Therefore, these endpoints were taken forward for BMD analysis (see Chapter 3.2.1).

In the male reproductive toxicity cluster, there were Likely effects on epididymis (exfoliated germ cells and inflammation) after developmental and adult exposure, on testis histology (increased seminiferous tubules with lumen and acrosomal vesicles) after growth phase/young age exposure and effects on sperm motility, morphology, viability and acrosome reaction after adult exposure. Therefore, these endpoints were taken forward for BMD analysis (see Chapter 3.2.1).

In all three clusters studied in animal studies (developmental toxicity, male reproductive toxicity, female reproductive toxicity), several endpoints were rated ALAN.

In the Developmental Toxicity cluster, mammary gland and bone development (both sexes) and body weight (described in Metabolic hazard identification section) after developmental exposure, and also for age at first oestrus and body weight effects after exposure during growth phase, were judged as ALAN.

In the Female reproductive toxicity cluster, effects on uterus histology (increase in apoptosis and squamous metaplasia) after developmental and adult exposure, and on oestrus cyclicity after adult exposure, were judged as ALAN.

In the Male reproductive toxicity cluster, effects on prostate (inflammation, reactive hyperplasia and apoptosis) and testis (polyarteritis, inflammation, reduced stage VIII seminiferous epithelial cells and increased germ cell degeneration) after developmental exposure were judged as ALAN.

After the integration of the human and animal evidence, the overall likelihood of BPA effects was considered Likely for the clusters Female reproductive toxicity and Male reproductive toxicity, and ALAN for Developmental toxicity.

Based mainly on the animal data and in reasonable agreement with the human data, a female reproduction hazard is identified in terms of likely effects on ovary weight and histology after developmental exposure, on implantation rate after growth phase/young age exposure and on follicle counts after adult exposure.

Male reproductive toxicity effects identified from the animal data as Likely were epididymis (exfoliated germ cells and inflammation) after developmental and adult exposure, testis histology (increased seminiferous tubules with lumen and acrosomal vesicles) after growth phase/young age exposure and sperm (motility, morphology, viability and acrosome reaction) after adult exposure. This is broadly in agreement with the previous EFSA conclusion (EFSA CEF Panel, 2015) that doses of BPA below 3.6 mg/kg bw per day may have modest adverse effects on testis function, especially on various measures of spermatogenesis.

Mechanisms of action for the identified BPA reproductive toxicity endpoints have been non-systematically explored in the literature. They include oestrogen and AR interactions and associated downstream and cross-stream effects, including epigenetic changes. Other possible mechanisms, including notably BPA-induced generation of oxidative stress, have been less explored.

3.1.7. Cardiotoxicity

3.1.7.1. Epidemiological studies

For the HOC Cardiotoxicity, no information on endpoints related to this HOC was found in the case–control or cohort studies considered.

The endpoints for each study identified as relevant in this opinion are reported in Annex C. Myocardial infarction, coronary artery stenosis and peripheral artery disease were key endpoints in the 2015 EFSA opinion (EFSA CEF Panel, 2015, see Section 3.6.4, WoE of Cardiovascular effects of BPA in humans).

Identification of the clusters to be considered for WoE

On the basis on the approach described in Chapter 2.3.2 'Definition of Health Outcome Categories and Clusters ', no clusters related to Cardiotoxicity were identified.

WoE of the relevant clusters

No information on endpoints related to Cardiotoxicity was found in the case–control or cohort studies considered.

Overall conclusions

On the basis of the above, the CEP Panel concluded that the evidence for a positive association between BPA exposure and Cardiotoxicity is Inadequate.

Cross-sectional studies

One cross-sectional study was identified (Xiong et al., 2015 [RefID 8164]) that determined serum BPA concentrations in patients with dilated cardiomyopathy (DCM, n = 88) and a group of 88 healthy individuals. BPA levels in the DCM group were significantly higher compared with that in the controls (6.9 ± 2.7 ng/mL vs. 3.8 ± 1.9 ng/mL, $p < 0.001$).

3.1.7.2. Animal studies

For the HOC Cardiotoxicity, a total of 22 studies were appraised by the CEP Panel. The details of the appraisals (internal and external validity) are reported in Annex E.

The endpoints for each study identified as relevant are reported in Annex F.

Identification of clusters of relevant endpoints

In the HOC cardiotoxicity, several clusters were determined and extracted from the available literature after assessment by the experts: absolute and relative heart weight, incidence of cardiac lesions, cardiac structural changes as measured by echocardiography, effects on cardiac function as measured by echocardiography, blood pressure and atherosclerotic lesions.

Elevated blood pressure is the starting event, which if persistent over long time and having a large effect size will eventually lead to hypertrophy of ventricular myocardium and increased heart weight. Hypertrophy of ventricular myocardium due to high blood pressure will cause changes of left ventricular structures, (measured by echocardiography) as well as cardiomyopathy (diagnosed by histopathology). Both may be the underlying cause of a failing heart function, which can be measured by echocardiography. Persistent elevated blood pressure is a well known and accepted risk factor for a number of serious and potentially life-threatening cardiovascular diseases in humans, such as stroke, heart failure, reduced kidney function and vascular dementia. Persistent elevated blood pressure is also contributing to the development of atherosclerotic lesion and is a known risk factor for it whereby the mechanism by which elevated blood pressure contributes to the development of atherosclerosis is not fully understood.

Oxidative stress as could be discussed as a common underlying molecular mechanism for the described effects. Oxidative stress is an inducer of cardiac lesions by initiating inflammatory processes which may lead to atherosclerosis, fibrosis and cardiomyopathy. The link to cardiac hypertrophy, heart weight increase, cardiac structural changes and increased blood pressure are, however, less clearly understood. Details of the involved pathways and their messengers can be taken from Elahi et al. (2009). However, the relevance of this mechanism for humans, especially for females, is currently unclear (Reckelhoff et al., 2019).

WoE of the clusters of relevant endpoints

The main information extracted from the studies addressing relevant endpoints in the HOC Cardiotoxicity are summarised in Annex G. The outcome of the weight of the evidence is described in the text below and presented in a tabulated format in Annex H.

The clusters of the effects of BPA on cardiotoxicity considered for this assessment were the following:

- Blood pressure
- Absolute and relative heart weight
- Incidence of cardiac lesions
- Cardiac structural changes (as measured by echocardiography)
- Effects on cardiac function (as measured by echocardiography)
- Atherosclerotic lesions.

Blood pressure

Developmental exposure (pre-natal and/or post-natal until weaning)

No studies were available for this exposure period.

Developmental and adult (pre-natal and post-natal in pups until adulthood)

For this exposure period, two studies, one in rats, Desai et al. (2018) [RefID 11817] (Tier 2), and one in mice, Patel et al. (2013) [RefID 5697] (Tier 3), were identified. In the rat study, an increase in blood pressure was seen in male rats only, however, no effect was observed in female rats at the single dose administered of 600 µg/kg bw per day. In the mouse study, the systolic blood pressure was increased at a dose of 5 µg/kg bw per day but not at 0.5 µg/kg bw per day and not at 200 µg/kg bw per day with females. Furthermore, in female mice, the diastolic blood pressure was increased at the doses of 0.5, 5, and 200 µg/kg bw per day. However, no dose–response could be identified. In this study, no effect on blood pressure was observed in male animals. The effect in female animals might be related to the fact that in female controls, both systolic and diastolic blood pressure was lower than in the male controls.

Because of the inconsistent results, effects in males in one study and not in the second study, and the reverse for females and the inconsistency in the dose–response, overall, the CEP Panel considered the evidence Inadequate to assign a likelihood level to the effect of BPA on blood pressure in the developmental and adult exposure period, so, this endpoint was not taken forward for BMD analysis.

Growth phase/young age exposure

No studies were available for this exposure period.

Adult exposure (after puberty)

For this exposure period three studies, one in rats (Jiang et al., 2015 [RefID 3189], Tier 2), and two in mice (Saura et al., 2014 [RefID 6494]; Belcher et al., 2015 [RefID 490], both Tier 3), were identified.

In rats, no effect on the blood pressure was observed in the single-dose study of 50 µg/kg bw per day. In one of the mouse studies, Saura et al. (2014) [RefID 6494] (sex of the animals not given), the systolic and the diastolic blood pressure was elevated at 16, 164, 1641 and 16416 µg/kg bw per day. In the second mouse study, Belcher et al. (2015) [RefID 490], male animals had a decreased systolic blood pressure at doses of 4.3, 43, 430, 4490 and 44900 µg/kg bw per day, whereas no effect was seen on the diastolic blood pressure. In female animals, the systolic blood pressure decreased with a dose of 44,900 µg/kg bw per day and no effect on the diastolic blood pressure was seen. Thus, whereas no effect was seen on the blood pressure in rats, contradictory results are reported for the two mouse studies with overlapping dosing, which is considered as unexplained inconsistency.

The CEP Panel assigned a likelihood level of Not Likely to the effect of BPA on blood pressure in the adult exposure period, so, this endpoint was not taken forward for BMD analysis.

Indirect (germline) exposure

No studies were available for this exposure period.

Overall cluster selection of the endpoints/studies for BMD analysis for Blood pressure

Overall, the CEP Panel assigned a likelihood level of Inadequate evidence/Not Likely to the effects of BPA on the cardiovascular function (blood pressure) in the exposure periods developmental and adult and adulthood (after puberty), respectively.

The CEP Panel considered that the evidence from the studies available did not show a Likely or Very Likely effect of BPA in any exposure period, therefore, none of the endpoints was taken forward for BMD analysis.

Absolute and relative heart weight

Developmental exposure (pre-natal and/or post-natal until weaning)

For this exposure period, five studies in rats were identified, three in Tier 1 (Cao et al., 2015 [RefID 831]; Gear et al., 2017 [RefID 2229]; NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]), and two in Tier 2 (Lejonklou et al., 2017 [RefID 3975]; Dunder et al., 2018 [RefID 11866]).

The doses tested encompass five doses in rats in the dose range between 2.5 and 25,000 µg/kg bw per day in rats (Gear et al., 2017 [RefID 2229] and NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) and 0.5 and 50 µg/kg bw per day in the studies of Lejonklou et al. (2017) [RefID 3975] and Dunder et al. (2018) [RefID 11866]. Cao et al. (2015) [RefID 831] investigated one single dose of 240 µg/kg bw per day. Male and female rats were tested.

As no effects were noted at any dose in any study, the CEP Panel assigned a likelihood level of Not Likely to the effect of BPA on relative heart weight during the developmental exposure period, so, this endpoint was not taken forward for BMD analysis.

Developmental and adult exposure (pre-natal and post-natal in pups until adulthood)

For this exposure period, five studies in rats were identified, three in Tier 1 (Cao et al., 2015 [RefID 831]; Gear et al., 2017 [RefID 2229]; NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]), one in Tier 2 (Dunder et al., 2018 [RefID 11866]) and one in Tier 3 (Patel et al., 2013 [RefID 5697]). The doses tested encompass five doses in the dose range between 2.5 and 25000 µg/kg bw per day in rats (Gear et al., 2017 [RefID 2229] and NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) and 0.5 and 50 µg/kg bw per day in the studies of Lejonklou et al. (2017) [RefID 3975] and Dunder et al. (2018) [RefID 11866]. Cao et al. (2015) [RefID 831] investigated one single dose of 240 µg/kg bw per day. Male and female animals were tested. In the Tier 3 study Patel et al. (2013) [RefID 5697] in mice, inconsistent results were obtained with a decrease at 5 µg/kg bw per day and no effect at the others with doses tested of 0.5, 5 and 50 µg/kg bw per day.

As no effects at any dose in any study were noted, the CEP Panel assigned a likelihood level of Not Likely to the effect of BPA on relative heart weight during the developmental and adult exposure period, so, this endpoint was not taken forward for BMD analysis.

Growth phase/young age exposure

No studies were available for this exposure period.

Adult exposure (after puberty)

For this exposure period, two studies in rats were identified, both in Tier 2 (Hu et al., 2016 [RefID 2843]; Jiang et al., 2015 [RefID 3189]). Furthermore, results from one Tier 3 study in mice (Belcher et al., 2015 [RefID 490]) were available.

In Jiang et al. (2015) [RefID 3189] (Tier 2 study) in rats with testing of a single dose (50 µg/kg bw per day), no effect was observed after 24 weeks but a marginal increase of relative heart weight was seen after exposure for 48 weeks. In the other Tier 2 study in rats (Hu et al., 2016 [RefID 2843]), a statistically non-significant increasing trend (doses of 20000 and 100000 µg/kg bw per day) was seen. In the Tier 3 study in mice, inconsistent results were obtained with a increase in two middle doses

without a clear dose–response with doses tested of 4.3, 43, 430, 4490 and 44900 µg/kg bw per day (Belcher et al., 2015 [RefID 490]).

The CEP Panel assigned a likelihood level of Not Likely to the effect of BPA on relative heart weight during the adult exposure (after puberty) period, so, this endpoint was not taken forward for BMD analysis.

Indirect (germline) exposure

No studies were available for this exposure period.

Overall cluster selection of the endpoints/studies for BMD analysis for absolute and relative heart weight

Overall, the CEP Panel assigned a likelihood level of Not Likely to the effects of BPA on relative heart weight in the exposure periods developmental, developmental and adult and adulthood (after puberty).

The CEP Panel considered that the evidence from the studies available did not show a Likely or Very Likely effect of BPA in any exposure period, therefore, none of the endpoints was taken forward for BMD analysis.

Incidence of cardiac lesions

Developmental exposure (pre-natal and/or post-natal until weaning)

For this exposure period two studies in rats were identified, all in Tier 1. Increases at 2.5 and 25 µg/kg bw per day in males only and no effect of the other three doses in the study of NTP Clarity Report (2018)/Camacho et al. (2019) [RefID 11370] (Tier 1) were observed. No effect in females was noted. The doses ranged between 2.5 and 25000 µg/kg bw per day. In the other Tier 1 study (Gear et al., 2018 [RefID 2229]) no effect was shown in males, but an increase in females at 2.5, 25 and 250 µg/kg bw per day. This effect was judged not treatment related.

Because of the inconsistency of the results, the CEP Panel assigned a likelihood level of Not Likely to the effect of BPA on the incidence of cardiac lesions in the developmental exposure period, so, none of the endpoints was taken forward for BMD analysis.

Developmental and adult exposure (pre-natal and post-natal in pups until adulthood)

In this exposure period, two studies in rats were identified, both in Tier 1 (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]; Gear et al., 2017 [RefID 2229]). The doses tested were the same as described above (2.5, 25, 250, 2500, 25000 µg/kg bw per day in both sexes).

No effect was seen in both sexes in both Tier 1 studies over a dose range between 2.5 and 25000 µg/kg bw per day.

The CEP Panel assigned a likelihood level of Not Likely to the effect of BPA on the incidence of cardiac lesions in the developmental and adult exposure period, so, none of the endpoints was taken forward for BMD analysis.

Growth phase/young age exposure

No studies were available for this exposure period.

Adult exposure (after puberty)

For this exposure period, two studies in rats (Hu et al., 2016 [RefID 2843]; Jiang et al., 2015 [RefID 3189]) were identified, both in Tier 2, and one study in rabbits (Fang et al., 2014 [RefID 1914]) allocated to Tier 2.

In one rat study (Hu et al., 2016 [RefID 2843]), an increase in fibrosis was seen at doses of 20000 and 100000 µg/kg bw per day, but there was no effect at the only dose (5000 µg/kg bw per day) below the cut-off (10000 µg/kg bw per day). In this study, only male animals were tested. In the other rat study (Jiang et al., 2015 [RefID 3189]), cardiac hypertrophy was seen male animals, the only sex tested, at the only dose tested of 50 µg/kg bw per day. In the rabbit study (Fang et al., 2014 [RefID

1914]), fibrosis was increased at the only dose tested of 400 µg/kg bw per day in male animals. Female animals were not tested.

As no response was seen in the relevant dose range below the cut-off of 10000 µg/kg bw per day or only Inadequate evidence was available from single-dose studies, the CEP Panel assigned a likelihood level of Not Likely to the effect of BPA on the incidence of cardiac lesions in the adult exposure (after puberty) period, so, none of the endpoints was taken forward for BMD analysis.

Indirect (germline) exposure

No studies were available for this exposure period.

Overall cluster selection of the endpoints/studies for BMD analysis for incidence of cardiac lesions

Overall, the CEP Panel assigned a likelihood level of Not Likely to the effects of BPA on incidence of cardiac lesions in the exposure periods developmental, developmental and adult and adulthood (after puberty).

The CEP Panel considered that the evidence from the studies available did not show a Likely or Very Likely effect of BPA in any exposure period, therefore, none of the endpoints was taken forward for BMD analysis.

Cardiac structural changes

Developmental exposure (pre-natal and/or post-natal until weaning)

For this exposure period, one study in rats was identified, allocated to Tier 1 (Gear et al., 2017 [RefID 2229]). The endpoint measured was left ventricular wall thickness (LVWT). Male and female animals were exposed since GD6 at doses of 2.5, 25, 250, 2500, 25000 µg/kg bw per day. Examination took place on PND21.

As no effect was observed at any doses in both sexes in the only study available, the CEP Panel assigned a likelihood level of Not Likely to the effect of BPA on cardiac structural changes (i.e. LVWT) in the developmental exposure period, so, this endpoint was not taken forward for BMD analysis.

Developmental and adult (pre-natal and post-natal in pups until adulthood)

In this exposure period, two studies were identified, one study in rats allocated to Tier 1 (Gear et al., 2017 [RefID 2229]) and one study in mice allocated to Tier 3 (Patel et al., 2013 [RefID 5697]).

In Gear et al. (2017) [RefID 2229], the endpoint measured was LVWT. Doses of 2.5, 25, 250, 2500, 25000 µg/kg bw per day were investigated. Exposure was in male and female animals from GD6 to parturition followed by observational period until termination at 2 years (called stop group) and exposure GD6 until termination at 2 years (called continuous group). Examination took place at PND90 and 6 months. Male animals had in increase at 250 µg/kg bw per day and female animals had a decrease at PND90 at 2.5 µg/kg bw per day. No consistent effects were seen in both sexes.

Patel et al. (2013) [RefID 5697] measured left ventricular internal dimension, at diastole (LVIDd) and found no effect in males and females at doses of 0.5, 5 and 200 µg/kg bw per day. Mice were treated with BPA 0.5 or 5 µg/kg bw per day from GD11.5 until euthanasia at 4 months. A separate group was treated with BPA 200 µg/kg bw per day from GD11.5 until weaning. After weaning of the progeny, dams were treated with vehicle water. In this study relative wall thickness (RWT) was increased in male animals at all doses, but the effect was in the order 5 > 0.5 > 200 µg/kg bw per day; in female animals 0.5 µg/kg bw per day had no effect, at 5 µg/kg bw per day an increase was observed and at 200 µg/kg bw per day, again no effect was observed.

The CEP Panel assigned a likelihood level of Not Likely to the effect of BPA on cardiac structural changes (LVW, LVIDd, RWT) in the developmental and adult exposure period, so, none of the endpoints was taken forward for BMD analysis.

Growth phase/young age exposure

No studies were available for this exposure period.

Adult exposure (after puberty)

For this exposure period, two studies (Hu et al., 2016 [RefID 2843]; Jiang et al., 2015 [RefID 3189]) in rats were identified, both allocated to Tier 2. One further study in mice, Tier 3 (Belcher et al., 2015 [RefID 490]), was also found. The endpoints measured were indices of diameters of wall or calculated derived parameters (in systole or in diastole) such as LVWT, interventricular septum (IVS), left ventricular internal dimension at diastole (LVIDd), left ventricular internal dimension at systole (LVIDs), left ventricle wall thickness (LVPW) measured by echocardiography.

In the two Tier 2 rat studies, inconsistent effects (only in one dose) or at high doses were observed. In the study by Hu et al. (2016) [RefID 2843], doses of 5000, 20000 and 100000 µg/kg bw per day were given for 30 days to male animals. IVS was increased at 100000 µg/kg bw per day (above the cut-off dose), but no effect was observed at 5000 and 20000 µg/kg bw per day. No effect was shown for LVIDd and LVIDs, whereas left ventricle posterior wall thickness, at diastole (LVPWd) was increased at 20000 µg/kg bw per day (above the cut-off dose), no effect of 5000 and 100000 µg/kg bw per day were observed. In Jiang et al. (2015) [RefID 3189], male animals were dosed with 50 µg/kg bw per day for 48 weeks. No other doses were tested. LVIDd was increased at 48 weeks with 50 µg/kg bw per day, but not at 24 weeks; the same findings were described for LVIDs and LVPW with increase at 48 weeks, but not at 24 weeks.

In the Tier 3 mouse study of Belcher et al. (2015) [RefID 490], doses of 50, 500, 5000 and 50000 µg/kg bw per day did not show statistically significant effects on LW thickness at PND90.

The CEP Panel assigned a likelihood level of Not Likely to the effect of BPA on cardiac structural changes in the adult exposure period, so, none of the endpoints was taken forward for BMD analysis.

Indirect (germline) exposure

No studies were available for this exposure period.

Overall cluster selection of the endpoints/studies for BMD analysis for cardiac structural changes as measured by echocardiography

Overall, the CEP Panel assigned a likelihood level of Not Likely to the effects of BPA on cardiac structural changes in the exposure periods developmental, developmental and adult and adulthood (after puberty).

The CEP Panel considered that the evidence from the studies available did not show a Likely or Very Likely effect of BPA in any exposure period, therefore, none of the endpoints was taken forward for BMD analysis.

Effects on cardiac function

Developmental exposure (pre-natal and/or post-natal until weaning)

No studies were available for this exposure period.

Developmental and adult (pre-natal and post-natal in pups until adulthood)

No studies were available for this exposure period.

Growth phase/young age exposure

No studies were available for this exposure period.

Adult exposure (after puberty)

Two studies, both allocated to Tier 2 (Hu et al., 2016 [RefID 2843]; Jiang et al., 2015 [RefID 3189]), were available in which ejection fraction (EF) and fractional shortening (FS) were measured by echography in rats.

In one of the studies (Jiang et al., 2015 [RefID 3189]), a single dose of 50 µg/kg bw per day, administered daily in feed to male rats from PND22 to 24 weeks or 48 weeks, respectively, resulted in

no effect at 24 weeks, and a small, clinically not relevant effect, at 48 weeks, on the EF and the FS. The study was performed in male animals only. Females were not investigated.

In the second study (Hu et al., 2016 [RefID 2843]), at 30 days no effects were observed at doses of 5000 and 20000 µg/kg bw per day and a small effect, a clinically not relevant decrease on EF, was observed at 100000 µg/kg bw per day. This study was also performed in male animals only.

In the second study, an indication of a small but clinically not relevant decrease in FS and EF is described. However, no effects at the cumulative dose (dose × duration of exposure) of 8400 µg/kg (at 24 weeks) and of 150000 µg/kg and 600000 µg/kg (at 30 days) were seen. At cumulative doses of 16,600 µg/kg (48 weeks) and of 3000 mg/kg (at 30 days) a decrease of both related endpoints was seen.

When taking together the evidence from the two studies, using the cumulative doses, no clear dose–response was identified; the effect size was small and clinically not relevant.

Given the fact that the effect size was small and clinically not relevant, the CEP Panel assigned a likelihood level of Not Likely to the effect of BPA on cardiac function in the adult exposure period, so, none of the endpoints was taken forward for BMD analysis.

Indirect (germline) exposure

No studies were available for this exposure period.

Overall cluster selection of the endpoints/studies for BMD analysis for effects on cardiac function as measured by echocardiography

The CEP Panel considered that the evidence from the studies available did not show a Likely or Very Likely effect of BPA in any exposure period, therefore, none of the endpoints was taken forward for BMD analysis.

Atherosclerotic lesions

Developmental exposure (pre-natal and/or post-natal until weaning)

No studies were available for this exposure period.

Developmental and adult exposure (pre-natal and post-natal in pups until adulthood)

No studies were available for this exposure period.

Growth phase/young age exposure

No studies were available for this exposure period.

Adult exposure (after puberty)

One study in rabbits was allocated to Tier 2 (Fang et al., 2014 [RefID 1914]). As the study which found an increase in atherosclerotic lesions in the aorta at 400 µg/kg bw per day was a single-dose study not supported by findings in other studies, the CEP Panel considered the evidence Inadequate to assign a likelihood level to the effect of BPA on atherosclerotic lesions in the adult exposure period, so, this endpoint was not taken forward for BMD analysis.

Indirect (germline) exposure

No studies were available for this exposure period.

Overall cluster selection of the endpoints/studies for BMD analysis for atherosclerotic lesions

The CEP Panel considered that the evidence from the studies available did not show a Likely or Very Likely effect of BPA in any exposure period, therefore, the endpoint atherosclerotic lesion (arch, thoracic and abdominal) was not taken forward for BMD analysis.

Overall evidence on Cardiotoxicity

After evaluating the risk of bias of the studies and performing a WoE analysis, the evidence for an adverse effect was graded in all clusters as Not Likely. Hence, none of the endpoints were carried forward for BMD analysis.

3.1.7.3. Integration of likelihoods from human and animal studies

The integration of the likelihood per cluster from both the human and animal studies available showed an overall likelihood as Not Likely.

3.1.7.4. In vitro and Mechanistic studies

Considering that the evidence of effects of BPA on the cardiotoxcity clusters in human and animal studies were judged as Not Likely or as Inadequate Evidence, the section on *in vitro* and mechanistic studies was not considered for this HOC.

3.1.7.5. Conclusions on hazard identification for Cardiotoxicity of BPA

In the 2015 EFSA opinion (EFSA CEF Panel, 2015), BPA attenuation of repeated and acute exposure to BPA was demonstrated in adult female rats on cardio-respiratory reflexes elicited by phenylbiguanide (PBG) (Pant et al., 2012). 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. In the 2010 EFSA opinion (EFSA CEF Panel, 2010), two cross-sectional studies reporting associations between BPA exposure and cardiovascular outcomes. Cardiovascular endpoints were therefore considered relevant for the new evaluation.

There were indications in the 2015 EFSA opinion (EFSA CEF Panel, 2015) from one prospective study that BPA exposure may be associated with cardiovascular effects, but it could not be ruled out that the effect was confounded by diet or other concurrent exposure factors. This association did not provide sufficient evidence to infer a causal link between BPA exposure and cardiovascular effects in humans. Potential effects were considered to be ALAN.

In the current assessment, no case–control or cohort studies were available on BPA cardiotoxicity; only one cross-sectional study was identified (Xiong et al., 2015 [RefID 8164]) that determined serum BPA concentrations in patients with dilated cardiomyopathy (DCM, n = 88) and a group of 88 healthy individuals. BPA levels in the DCM group were significantly higher compared with that in the controls. On the basis of this finding, the CEP Panel concluded that the evidence for an adverse association between BPA exposure and cardiotoxicity in human is Inadequate.

As for the animal evidence, overall the likelihood for an adverse effect was graded in most the cardiotoxicity clusters as Not Likely and the others as Inadequate evidence. Hence, none of the endpoints were brought forward for BMD analysis.

In accordance with the results from 2015, in the current risk assessment of BPA, no clear causal association was found for cardiotoxicity effects of BPA based on WoE of animal data.

3.1.8. Carcinogenicity and mammary gland proliferative effects

3.1.8.1. Epidemiological studies

Two human studies were available addressing effects of BPA on carcinogenicity: Tse et al. (2017) [RefID 7312] on prostate and Costas et al. (2015) [RefID 10171] on lymphoid tissue.

However, the criteria to bring forward to WoE analysis the clusters/exposure periods were not met: i.e. there were not clusters/exposure periods for which at least two studies were available and at least one was showing a statistically significant effect for one of the endpoints measured.

3.1.8.2. Animal studies

For the HOC Carcinogenicity and mammary gland proliferative effects a total of 46 studies were appraised by the CEP Panel. The details of the appraisals (internal and external validity) are reported in Annex E.

The endpoints for each study identified as relevant are reported in Annex F.

Identification of clusters of relevant endpoints

BPA-induced proliferative effects in the mammary gland were already considered key in the uncertainty analysis in the 2015 EFSA opinion (EFSA CEF Panel, 2015, Section 4.3.2) For more details see Annex A, Section 2.5.

From the newly available BPA literature, data on mammary gland weight and histology, on uterus weight and histology and on prostate gland histology were identified as relevant endpoints. As a general approach, histological changes were subdivided into the following subclusters:

- Non-neoplastic changes: that includes endpoints such as hyperplasia, inflammation and other endpoints which might be related to pathological conditions that ultimately could result in neoplasia.
- Pre-neoplastic lesions: that includes endpoints such as atypical hyperplasia or dysplasia.
- Neoplastic lesions: which includes both benign and malignant tumours as endpoints.
- Proliferation and apoptosis: which considers data obtained by the quantitative evaluation of histological sections stained with specific markers of proliferation (e.g. Ki67, proliferating cell nuclear antigen (PCNA)) or apoptosis (e.g. caspase 3, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)).

Even if the endpoints taken into consideration have been divided into different subclusters, it must be considered that many of these are related/connected to each other (e.g. hyperplasia seen with standard histology and proliferation detected with immunohistochemistry) or biologically (dysplasia that can progress in a tumour).

For the histologic evaluation, also whole mount techniques were described in some studies. These techniques consist in the microscopic examination of an entire organism or organ small enough or thin enough to be placed directly onto a slide and then stained. Along with histology and molecular biology techniques, the whole mount evaluation of mammary glands has proven useful in detecting changes on the entire 3D epithelial structure of the mammary gland related to the treatment with endocrine disrupting chemicals in rodents (Mandrup et al., 2015).

Effects on Mammary gland weight

In addition to physiological changes during pregnancy and lactation, pathological processes or xenobiotic-induced increases may affect mammary gland weights (Greaves, 2007). An abnormal increase of mammary gland weight is a parameter that can be related to proliferative processes and especially neoplasia (Sacco et al., 2000).

Effects on Mammary gland histology

The following relevant endpoints were retrieved for the cluster mammary gland effects:

- non-neoplastic changes;
- pre-neoplastic lesions;
- neoplastic lesions;
- proliferation and apoptosis.

Given the similarities in alterations in growth and differentiation induced by environmental chemicals in mammary gland development and carcinogenesis in humans and experimental animals, rodent models can be used as reasonable surrogates for human mammary gland development (Rudel et al., 2011). Transient or persistent developmental effects can be induced by such chemicals depending on their

dose and the exposure period (Fenton, 2006). Undifferentiated mammary gland structures in particular TEBs are considered sensitive to chemical carcinogens and eventually may give rise to carcinomas, whereas benign neoplastic lesions such as adenomas arise from more differentiated structures. TEBs are characterised by a high rate of cell proliferation which decreases progressively towards the ductal portions, the more differentiated alveolar buds and lobes (Russo, 2015). An increased number of TEBs and ducts due to lateral branching as well as increased gland density are associated with an increased risk of breast cancer (Muñoz-de-Toro et al., 2005). Therefore, non-neoplastic findings detected in mammary glands of rodents following BPA treatment (such as changes in TEBs, TDs, lobular hyperplasia, longitudinal growth, branching, gland density and ductal dilatation) were considered relevant endpoints for human risk assessment of BPA.

Usually, long-term studies have to be conducted to detect the induction of mammary neoplastic lesions by non-genotoxic chemicals in rodents which also spontaneously develop such lesions during ageing. Adenocarcinomas are known to arise either spontaneously or be induced by chronic administration of estrogens and xenobiotics in rodents (Greaves, 2007; Russo, 2015). Therefore, a chronic study with BPA included in the current evaluation was particularly relevant for detecting pre-neoplastic (atypical foci) and neoplastic lesions (adenomas and adenocarcinomas). Epithelial foci with unusual growth patterns are considered as pre-neoplastic lesions which may develop into adenocarcinomas (Russo, 2015). Adenomas are glandular neoplastic lesions without atypical cytological features of malignancy while adenocarcinomas infiltrate the surrounding tissue in rodents (Greaves, 2007).

For the carcinogenic process the ratio of proliferation to apoptosis is important. The administration of genotoxic carcinogens in rodents is known to induce excessive proliferation of the glandular epithelium (Russo, 2015), the intraductal proliferation with multiple layers of epithelial cells is considered a pre-neoplastic lesion. For BPA, marked decreases of apoptotic cells in TEBs was reported (Muñoz-de-Toro et al., 2005). Thus, both processes proliferation and reduced apoptosis may result in hyperplastic changes in the mammary gland.

Effects on Prostate histology

The following relevant endpoints were retrieved for the cluster prostate effects:

- non-neoplastic changes;
- pre-neoplastic lesions;
- proliferation and apoptosis.

Among non-neoplastic changes, inflammation was reported as a relevant endpoint in a number of studies carried out on rats. In some cases, the type or the distribution of the inflammatory process was specified as 'suppurative', 'lymphocytic infiltration', 'multifocal' or 'reactive atypia'. The findings were different depending on the anatomical part of the prostate gland considered (ventral vs dorsolateral). Exposure to chemicals and hormonal exposures are considered among the numerous causes of prostatitis in humans. Moreover, a potential role for inflammation in prostatic carcinogenesis and tumour progression in humans has been proposed. Hyperplasia was also reported as a relevant endpoint in a number of studies carried out on rats. In some cases, this finding was further specified as 'epithelial' or 'reactive' or 'functional'. Reactive hyperplasia is a reparative process characterised by hyperplasia of acinar epithelium in response to degeneration and inflammation, which is generally caused by an infection (Creasy et al., 2012). Functional hyperplasia is an enlargement of the prostate gland caused by hypertrophy and hyperplasia of the epithelial cells in response to increased demand or hormonal stimulation (Creasy et al., 2012). Other endpoints were reported in a single study: atrophic tubules, glandular diameter and vascular congestion.

Pre-neoplastic lesions were considered as relevant endpoints in few rat studies. In this subcluster atypical hyperplasia and dysplasia (that can be considered synonym of atypical hyperplasia (Creasy et al., 2012) were included. Atypical hyperplasia is considered a pre-neoplastic lesion in mouse and rat prostate cancer models since there appears to be a morphologic continuum between atypical hyperplasia and benign adenoma of the prostate and the distinction between the two is not always clear (Creasy et al., 2012).

Proliferation and programmed cell death (apoptosis) were investigated and reported in some studies. Dysregulations if these processes can create a favourable environment for tumour development and

progression. An increase in the proliferation rate is a key event in the following, already mentioned endpoints: all types of hyperplasia (including atypical hyperplasia/dysplasia) and neoplasia.

Effects on Uterus weight

Uterus weight was considered as relevant endpoint in a number of studies in rats, mice and in one study in hamsters. An increase of uterus weight is a sensitive method to detect ER agonists. This can be used to evaluate the ability of a test chemical to elicit biological activities consistent with oestrogenic agonists (uterotrophic assay) (Marty and O'Connor, 2014).

Effects on Uterus histology

The following relevant endpoints were retrieved for the cluster uterus histology:

- non-neoplastic changes;
- neoplastic lesions;
- proliferation and apoptosis.

Among non-neoplastic changes, squamous metaplasia was reported as a relevant endpoint. Squamous metaplasia is characterised by the presence of stratified squamous epithelium that is replacing the normal uterine columnar lining epithelium. Squamous metaplasia often develops in the rat and mouse uterus under oestrogen dominance, for example in response to elevated endogenous oestrogen level or when treated with high doses of oestrogenic compounds (Dixon et al., 2014). Endometrial (cystic) hyperplasia is characterised by an increase of proliferating endometrial cells. Prolonged oestrogen excess or administration of xenobiotics with oestrogenic effects can induce endometrial hyperplasia (Dixon et al., 2014). Dilation of the uterine horns is a common finding in laboratory rodents and is due to the accumulation of watery fluid in the uterine lumen. This change is a physiological feature that occur during the pro-estrus and estrus phases under the influence of oestrogen. In *in vivo* studies, an increased incidence of luminal dilation can indicate a drug-induced alteration of the hormonal status (e.g. relative oestrogen dominance) (Dixon et al., 2014).

A neoplastic lesion, stromal polyps, was considered as relevant endpoint in one study in rats. Stromal polyps are polypoid masses protruding into the uterine lumen; the predominant bulk of the lesion is composed of stromal spindle-shaped or stellate cells. Growth is expansive without invasion (Dixon et al., 2014). Stromal polyps are a common spontaneous uterine lesion in rats. There is limited information concerning the aetiology and significance of these polyps as an endpoint in toxicology and carcinogenicity studies. Uterine endometrial polyps that occur in women and the uterine stromal polyps that occur in rodents have distinct characteristics; both lesions are benign (Davis, 2012).

Proliferation and programmed cell death (apoptosis) were investigated and reported in some studies using different histological techniques: 5-bromo-2'-deoxyuridine (BrdU) incorporation for proliferation, standard histology or TUNEL for apoptosis. Dysregulations of these processes can promote the development and progression of pre-neoplastic and neoplastic lesions.

WoE of the clusters of relevant endpoints

The clusters of the effects of BPA on Carcinogenicity and mammary gland proliferation effects in this assessment were the following:

- mammary gland weight
- mammary gland histology
- prostate histology;
- uterus weight;
- uterus histology.

For histology, the following subclusters were considered if available:

- non-neoplastic changes;

- pre-neoplastic lesions;
- neoplastic lesions;
- proliferation and apoptosis (immunohistochemistry).

Effects on mammary gland weight

Developmental exposure (pre-natal and/or post-natal until weaning)

For effects on mammary gland weight in this exposure period only one Tier 1 study in rats was identified (Montévil et al., 2020 [RefID 13788]).

The mammary gland weight in female rats was determined at PND90 (Montévil et al., 2020 [RefID 13788]). Based on the authors' description it appears that a data-driven approach was used for identifying a NMDR by defining a step function around the doses of 25 and 250 µg/kg bw per day. However, when using more conventional approaches for risk assessment, e.g. modelling the data in PROAST (Hill and Exponential models) or using spline and polynomial fit (without overfitting the data), no dose–response was identified by the CEP Panel. The CEP Panel considered that alternative interpretations of the data may be plausible, e.g. that a significant difference between the dose group with 25 µg/kg bw per day and the controls, in the absence of dose–response, may be likely explained by random fluctuations and variability in the data. These considerations also apply to other NMDRs for histological findings in this study.

Developmental and adult exposure (pre-natal and/or post-natal in pups until adulthood)
No studies were available for this exposure period.

Growth phase/young age exposure
No studies were available for this exposure period.

Adult exposure (after puberty)
No studies were available for this exposure period.

Indirect (germline) exposure
No studies were available for this exposure period.

Overall cluster selection of the endpoints/studies for BMD analysis for mammary gland weight effects:

The CEP Panel assigned a likelihood level of Not Likely to the mammary gland weight effects of BPA in the developmental exposure period.

The CEP Panel considered that the evidence from the study available did not indicate a Likely or Very Likely effect of BPA in any exposure period, therefore this endpoint was not taken forward for BMD analysis.

Effects on mammary gland histology

Developmental exposure (pre-natal and/or post-natal until weaning)

For histological effects in this exposure period seven studies were identified, i.e. one Tier 1 study in mice (Tucker et al., 2018 [RefID 13275]), five Tier 1 studies in rats (Grassi et al., 2016 [RefID 2387]; Kass et al., 2015 [RefID 3402]; Mandrup et al., 2016 [RefID 4831]; NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]; Montévil et al., 2020 [RefID 13788] and one Tier 3 study in rats (Leung et al., 2017 [RefID 3990]).

Numerous histological non-neoplastic changes were evaluated using the whole mount technique. No change in mammary gland density was seen at PND30 in female and male rats by Kass et al. (2015) [RefID 3402]. Montévil et al. (2020) [RefID 13788], who assessed samples from the CLARITY study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]), reported several effects at single doses in female F1 pups mammary glands: there were increases of gland density, Fractal dimension 3D, Dim 3 (third dimension from Principal Component Analysis) and angles of branches between beginning and

end with a breaking point between 25 and 250 µg/kg bw per day, thickness of the epithelium and variation of ductal thickness at 25 µg/kg bw per day and an increased average branch length at 250 µg/kg bw per day. In addition, there were decreases of the gland depth, the proportion of the small and very small branches, the maximum branch length and topological asymmetry of the epithelial trees (i.e. they were more symmetric in the 250 µg BPA group) the lateral branching at 250 µg/kg bw per day and the aspect ratio at 2.5 and 250 µg/kg bw per day (smaller AR means that the glands were rounder). Some of these results (gland density, Dimension 3D and angles of branches between beginning and end, thickness of epithelium, variation of ductal thickness, aspect ratio) were re-analysed by the CEP Panel. This re-analysis revealed that a formal dose–response is not identified by fitting flexible biologically based functions or polynomials that are commonly used to describe biological systems. Without further biological explanations justifying a NMDR instead of a deviation due to chance, it would be reasonable to conclude that there is no dose–response but rather single-dose effects, apart for variation for ductal thickness and aspect ratio where a potential NMDR may occur.

In the CLARITY study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) independent changes in mammary glands of female rats were observed at 1 year at some doses only, such as decreased incidences of ductal dilatation and lobular hyperplasia, which was not considered adverse. However, no effect on ductal dilatation (3, 8 and 14 months) was found in female mice (Tucker et al., 2018 [RefID 13275]) and no lobular hyperplasia was observed by Montévil et al. (2020) [RefID 13788] at PND90 in female rats with the same treatment as used in the CLARITY study.

No effects on lobuloalveolar structures were found in another Tier 1 study (Mandrup et al., 2016 [RefID 4831]) in female rats but a decrease was seen in male rats at PND100 along with a potential increase in the tubuloalveolar pattern ('in very few structures in each gland') indicating a female-like morphology at high doses (5000 µg and 50000 µg/kg bw per day). At the lowest dose (25 µg/kg bw per day) used in this study, mammary glands of males at PND22 showed a higher longitudinal growth and a lower distance to lymph node. A reduction of the distance between lymph node and final edge (ductal growth) was reported at PND30 in male rats exposed to 64 µg/kg bw per day via drinking water (average oral dose) (Kass et al., 2015 [RefID 3402]). Additionally, in Kass et al. (2015) [RefID 3402] male pups were exposed to BPA by s.c. injection of 25 and 250 µg/kg bw per day (converted to the correspondent oral doses 892.5 and 8,925 µg/kg bw per day) from GD9 until GD23, resulting in an increased ductal growth at PND5 (transient effect) and a delay in ductal growth when assessed on PND30, both at the higher dose. No changes in ductal length were observed at PND21 in female rats when exposed to 25 or 250 µg/kg bw per day from GD10–GD21 (Grassi et al., 2016 [RefID 2387]). No changes in longitudinal growth at PND20 or in lobuloalveolar hyperplasia at 8 and 14 months were reported in female mice exposed to high doses (500, 5000 or 50000 µg/kg bw per day) during gestation (Tucker et al., 2018 [RefID 13275]). In the same study, there was an increase in branching density at the low dose at PND20. The branching score in female rats on PND21 increased at a low dose (25 µg/kg bw per day; Grassi et al., 2016 [RefID 2387]) while no changes in branching occurred in females at PND100 (Mandrup et al., 2016 [RefID 4831]). At 6 months reduced lateral branching was observed at 250 µg/kg bw per day in female rats (Montévil et al., 2020 [RefID 13788]). In female mice increases in TEB length and TEB counts along with higher developmental scores were observed in the mid-dose group (Tucker et al., 2018 [RefID 13275]). An increase in the number of TEB was also reported in female rats fed a high butter fat (HBF) diet at low BPA doses (2.5 and 25 µg/kg bw per day) compared with HBF controls in a Tier 3 study (Leung et al., 2017 [RefID 3990]). However, decreases in the number of TEBs were reported (at PND15 and PND30) along with a reduced ductal growth (at PND30) in male but not in female rats exposed via drinking water to 64 µg/kg bw per day (Kass et al., 2015 [RefID 3402]), possibly indicating a delayed gland development in male rats. At PND21 the number of TDs increased in female rats at a low dose (25 µg/kg bw per day) without changes in the number of TEBs (Grassi et al., 2016 [RefID 2387]). In summary, for three non-neoplastic endpoints (ductal dilatation, TEBs and effects on branching/branches) significant effects were observed in females but not with consistent findings in studies with different study designs. Additionally, in two Tier 1 studies on male rats effects (ductal growth and alveolar morphology) were observed (Mandrup et al., 2016 [RefID 4831]; Kass et al., 2015 [RefID 3402]). While no effects in alveolar dilatation were reported on mammary glands in males in the NTP Clarity Report (2018)/Camacho et al. (2019) [RefID 11370]. Taking the diversity of the effects and their time and dose dependency into account, the CEP Panel judged the induction of non-neoplastic changes in mammary gland of female and male rats as ALAN.

Pre-neoplastic lesions in mammary glands were examined in two Tier 1 studies with female rats: a significant increase in intraductal hyperplasia at the second lowest dose (250 µg/kg bw per day) at PND400 was reported by Kass et al. (2015) [RefID 3402] but no changes in pre-neoplastic lesions including atypical foci were observed at 1 or 2 years in the CLARITY study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]). Based on the outcomes in these two studies the CEP Panel judged the induction of pre-neoplastic lesions in mammary glands in female rats as Not Likely in this exposure period.

Neoplastic lesions were reported in female rats at 2 years in the CLARITY study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]). In the lowest dose group (2.5 µg/kg bw per day) the incidence of adenocarcinomas (22% vs 6% in controls) and of combined adenomas and adenocarcinomas (24% vs 8%) was significantly increased, while incidences of adenocarcinomas of 10%, 14%, 18% and 11% were observed at 25, 250, 2500 and 25000 µg/kg bw per day, respectively, and did not differ significantly from controls. No adenomas or adenocarcinomas were observed at 1 year. Also at 6 months, there were neither adenomas nor adenocarcinomas in females while ductal carcinomas *in situ* (in 2 out of 10 females) were reported at an early timepoint of PND90 in the mid-dose group treated with 250 µg/kg bw per day (Montévil et al., 2020 [RefID 13788]). Also, no carcinomas were found in any dose group of female mice at 14 months (Tucker et al., 2018 [RefID 13275]). Based at the age-dependent findings of adenomas or adenocarcinomas at 2 years the CEP Panel judged the neoplastic lesions in female rats as Likely.

Immunohistochemistry results did not indicate significant changes in proliferation in TEBs of mammary glands in female rats at PND21 ($p = 0.056$ only at 25 µg/kg bw per day: Grassi et al., 2016 [RefID 2387]) or in glands of female and male rats at PND30 (Kass et al., 2015 [RefID 3402]). Based on these studies the CEP Panel judged the induction of proliferation in mammary gland of female and male rats as Not Likely.

The CEP Panel assigned a likelihood level of ALAN to the mammary gland histological effects of BPA in the developmental exposure period, based on the non-neoplastic histological findings in female and male rats (ALAN) and neoplastic (Likely) histological findings in studies with female rats, while no clear evidence of proliferation (immunohistochemistry) and pre-neoplastic outcomes was reported Consequently, none of these endpoints was taken forward for BMD analysis. However, the Likely and ALAN endpoints were considered in the uncertainty analysis (see Appendix D).

<u>Developmental and adult exposure (pre-natal and post-natal in pups until adulthood)</u>

For histological effects in this exposure period two Tier 1 studies in rats were identified (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]; Montévil et al., 2020 [RefID 13788]).

Among the non-neoplastic effects identified in this exposure group several changes were only reported for one dose group in female rats, i.e. an increase in lobular alveolar budding at 250 µg/kg bw per day at PND90 (Montévil et al., 2020 [RefID 13788]), changes in ductal dilatation (increase at 1 year; decrease at 2 years) and a decrease in lobular hyperplasia, both at 25 µg/kg bw per day (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]); therefore, no dose–response could be established. An increase in alveolar dilatation was only reported in males at the lowest dose after 2 years (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) while the effect was not significant in females in both studies. Montévil et al. (2020) [RefID 13788] reported a NMDR for mammary gland scores at 2.5 (accelerated gland development) and 25 µg/kg bw per day (no significantly increased proliferation) on PND90 in rats from a 90-day pilot study (Delclos et al., 2014) for females in oestrus. No changes in lobular hyperplasia were observed at any dose on PND90 in Montévil et al. (2020) [RefID 13788]. The same authors reported significant decreases in average gland density in the rostral area (area 1) and in the middle of the gland (area 2) at 250 µg/kg bw per day on PND90 which were reported to be significant when compared with controls. Montévil et al. (2020) [RefID 13788] reported that gland density in the anterior area (area 3) showed a NMDR with a breaking point between 25 and 250 µg/kg bw per day. However, these data were re-analysed by the CEP Panel, revealing that a formal dose–response was not identified by fitting flexible biologically based functions or polynomials that are commonly used to describe biological systems. Without further biological explanations justifying a NMDR instead of a deviation due to chance, it would be reasonable to conclude that there is no dose–response but single-dose effects. Regarding mammary gland score, the data were in line with the definition by the CEP Panel of indications for a NMDR. Taking this and the diversity of the effects into

account the CEP Panel judged the induction of non-neoplastic changes in mammary gland of female and male rats as ALAN.

Regarding pre-neoplastic lesions the only reported effect was a significant increase in atypical foci in the 2.5 µg/kg bw per day dose group of females after one and 2 years of treatment. In addition, there were non-significant increases in the 25 and 250 µg/kg bw per day dose groups (9% and 8%, respectively, vs 0% in controls) at 1 year (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]). The CEP Panel judged the lesions in this exposure group as ALAN.

No significant effects on neoplastic lesions were observed in the two studies. The incidences of adenocarcinomas in the two lowest dose groups (2.5 and 25 µg/kg bw per day) were both 4% compared with 0% in controls at 1 year and 18–20% compared with 12% in controls at 2 years (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]). At 6 months no adenomas or adenocarcinomas were observed in any dose group (Montévil et al., 2020 [RefID 13788]). Therefore, the CEP Panel judged the neoplastic effects in this exposure group as Not Likely.

The CEP Panel assigned a likelihood level of ALAN to the mammary gland effects of BPA during the developmental and adult exposure period, based on some non-neoplastic changes in female and male rats (ALAN) and pre-neoplastic findings in female rats (ALAN). Hence, none of the endpoints was taken forward for BMD analysis. However, they were considered in the uncertainty analysis (see Appendix D).

Growth phase/young age exposure

No studies were available for this exposure period.

Adult exposure (after puberty)

No studies were available for this exposure period.

Indirect (germline) exposure

No studies were available for this exposure period.

Overall cluster selection of the endpoints/studies for BMD analysis for mammary gland histological effects:

The CEP Panel noted the inconsistency in the likely pre-neoplastic lesions (ALAN) in the absence of neoplastic lesions during developmental to adult exposure, while in the developmental exposure period there was only evidence of neoplastic lesions (Likely) but not of pre-neoplastic lesions. Therefore, the overall likelihood in the cluster mammary gland effects across all exposure periods was ALAN.

The CEP Panel considered that the evidence from the studies available did not show a Likely or Very Likely effect of BPA in the cluster mammary gland histological effects in any exposure period, therefore, none of the endpoints was taken forward for BMD analysis.

Effects on prostate gland histology

Developmental exposure (pre-natal and/or post-natal until weaning)

For effects on prostate following developmental exposure of BPA, four Tier 1 studies in rats (Bernardo et al., 2015 [RefID 533]; Brandt et al., 2014 [RefID 700]; NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]; Prins et al., 2018 [RefID 13779]), one Tier 2 study in rats (Hass et al., 2016 [RefID 2610]) and one Tier 3 study in rats (Prins et al., 2017 [RefID 5930]) were identified.

Two histological non-neoplastic changes were evaluated: inflammation and hyperplasia. Regarding inflammation, the results of a Tier 1 (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) for suppurative inflammation and of a Tier 1 Grantee study (Prins et al. (2018) [RefID 13779]), were supported by a Tier 2 study (Hass et al., 2016 [RefID 2610]) and by a Tier 3 study (Prins et al., 2017 [RefID 5930]) indicating no effects on inflammation. An increase in multifocal inflammation was observed at PND18 in the other two Tier 1 studies from the same laboratory with two doses tested, i.e. 25 and 250 µg/kg bw per day (Bernardo et al., 2015 [RefID 533]; Brandt et al., 2014 [RefID 700]). In addition, an increase in inflammatory reactive atypia was observed in the ventral prostate at 25 (not

statistically significant) and 250 µg/kg bw per day (Brandt et al., 2014 [RefID 700]). In another study a decrease in lymphocytic infiltration was reported (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]); this decrease was not considered to be adverse. Regarding hyperplasia, two Tier 1 studies (Bernardo et al., 2015 [RefID 533]: reactive hyperplasia; Brandt et al., 2014 [RefID 700]: hyperplasia/dysplasia) showed an increase at the lowest dose only or at the two doses tested with same effect size; it should be mentioned that in the study of Brandt et al. (2014) [RefID 700], data on incidence of hyperplasia and dysplasia were reported together as one endpoint. One Tier 1 study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) showed a decrease of hyperplasia in the animals treated with 2.5 µg/kg bw from GD6 to PND21 at PND730. A Grantee Tier 1 study (Prins et al., 2018 [RefID 13779]) showed no effect. Based on the inconsistencies in the outcomes and the diversity of the effects, the CEP Panel judged the prostatic non-neoplastic changes in the developmental exposure period as ALAN.

Two histological **pre-neoplastic lesions** were evaluated: atypical hyperplasia and dysplasia. Regarding atypical hyperplasia, one Tier 1 study (Bernardo et al., 2015 [RefID 533]) showed an increase at the lowest dose only (25 µg/kg bw per day from GD10 to GD21). This result was not supported by a Tier 2 study (Hass et al., 2016 [RefID 2610]) and a Tier 3 study (Prins et al., 2017 [RefID 5930]) that showed no effect on this endpoint. Regarding dysplasia, one Tier 1 study (Brandt et al., 2014 [RefID 700]) showed an increase at the lowest dose only (25 µg/kg bw per day from GD10 to GD21) but it should be mentioned that in this study data on incidence of hyperplasia and dysplasia were reported as together as one endpoint. Based on these results, the CEP Panel judged the pre-neoplastic lesions in prostate in the developmental exposure period as ALAN.

Proliferation was evaluated in two studies. One Tier 1 study (Brandt et al., 2014 [RefID 700]) showed an increase at the lowest dose only (25 µg/kg bw per day from GD10 to GD21). This result was not supported by a Tier 2 study (Hass et al., 2016 [RefID 2610]) that showed no effect on this endpoint. Apoptosis was evaluated in one study (Brandt et al., 2014 [RefID 700]) that showed an increase at the highest dose only (250 µg/kg bw per day from GD10 to GD21). Based on these results, the CEP Panel judged the prostatic changes regarding proliferation and apoptosis in the developmental exposure period as ALAN.

The CEP Panel assigned a likelihood level of ALAN to the prostate histological effects of BPA during the developmental exposure period, hence, none of the endpoints was taken forward for BMD analysis. However, they were considered in the uncertainty analysis (see Appendix D).

<u>Developmental and adult exposure (pre-natal and post-natal in pups until adulthood)</u>

For effects on prostate histology following developmental and adult exposure, two Tier 1 studies (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]; Prins et al., 2018 [RefID 13779]) in rats were identified.

Two histological non-neoplastic changes were evaluated: inflammation and hyperplasia. Regarding inflammation, the results of one study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) showed two opposite effects for dorso-lateral (increase) and for ventral prostate (decrease, not adverse) both for suppurative inflammation and lymphocytic infiltration. The second study showed no effect (Prins et al., 2018 [RefID 13779]). Regarding hyperplasia, the results of one study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) showed an effect at one intermediate dose only (250 µg/kg bw per day) out of five doses at PND730. The related study showed no effect (Prins et al., 2018 [RefID 13779]).

Based on the inconsistencies in the outcomes, the diversity of the effects and no clear MDR or NMDR, the CEP Panel assigned a likelihood level of Not Likely to the prostate histological effects of BPA during the developmental and adult exposure period, hence, none of the endpoints was taken forward for BMD analysis.

<u>Growth phase/young age exposure</u>

No studies were available for this exposure period.

<u>Adult exposure (after puberty)</u>

For effects on prostate histology following adult exposure, one Tier 2 study (Olukole et al., 2018 [RefID 12841]) and two Tier 3 studies (Wu et al., 2016 [RefID 8036]; Huang DY et al., 2018 [RefID 12167]) in rats were identified.

The following non-neoplastic changes were increased in a Tier 2 study (Olukole et al., 2018 [RefID 12841]) in which a single-dose was used (10000 µg/kg bw per day; 14 days of treatment, starting at 16 weeks of age): inflammation, hyperplasia (reactive), hyperplasia (functional), atrophic tubules, glandular diameter and vascular congestion. Proliferation was evaluated in two Tier 3 studies (Wu et al., 2016 [RefID 8036]; Huang DY et al., 2018 [RefID 12167]) from the same research group. Both studies showed an increase in all BPA dose groups (10, 30, 90 µg/kg bw per day for 3 months in Huang DY et al., 2018 [RefID 12167] and for 1 month in Wu et al., 2016 [RefID 8036]).

Among pre-neoplastic lesions, atypical hyperplasia was observed in a Tier 2 study (Olukole et al., 2018 [RefID 12841]) in which a single dose was used (10000 µg/kg bw per day; 14 days of treatment, starting at 16 weeks of age).

Based on the limited data from one Tier 2 single-dose study and two Tier 3 studies, the CEP Panel considered the evidence on prostatic histological changes in the adult exposure period as Inadequate to assign a likelihood of the effects. Therefore, none of the endpoints was taken forward for BMD analysis.

Indirect (germline) exposure

No studies were available for this exposure period.

Overall cluster selection of the endpoints/studies for BMD analysis for effects on prostate gland histology

Overall, the CEP Panel assigned a likelihood level of ALAN to the prostate gland histological effects of BPA in the developmental exposure period, Not Likely in the developmental and adult exposure, and considered the evidence Inadequate for the adult exposure period, based on non-neoplastic changes, pre-neoplastic lesions and proliferation/apoptosis reported in some studies but with no clear dose–response and/or inconsistent results between different studies.

Therefore, the overall likelihood across all exposure periods, i.e. the highest likelihood given in the cluster prostate gland histology, was ALAN.

The CEP Panel considered that the evidence from the studies available did not show a Likely or Very Likely effect of BPA in any exposure period, therefore, none of the endpoints was taken forward for BMD analysis.

Effects on uterus weight

Developmental exposure (pre-natal and/or post-natal until weaning)

For this exposure period one Tier 1 study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) in rats, one Tier 1 study in hamsters (Radko et al., 2015 [RefID 6046]) and one Tier 3 study in mice (Patel et al. (2013) [RefID 5697]) were identified.

In the Tier 1 rat study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]), animals were dosed with 2.5, 25, 250, 2500 and 25000 µg/kg bw per day and no change in uterus weight was observed. In the Tier 1 hamster study uterus weight (wet and dry) on PND21 was statistically significantly increased at 160000 µg/kg bw per day; female hamster pups were dosed with three daily oral doses (PND18–20) of 8000, 40000 and 160000 µg/kg bw per day.

The CEP Panel assigned a likelihood level of ALAN to the uterus weight effects of BPA during the developmental exposure period, as no effect was observed in the Tier 1 in (young) adult rats and an increase in weanling hamsters (uterotropic assay) was only seen at the highest dose (160000 µg BPA/kg bw per day). Therefore, this endpoint was not taken forward for BMD analysis. However, it was considered in the uncertainty analysis (see Appendix D).

Developmental and adult exposure (pre-natal and post-natal in pups until adulthood)

For this exposure period two Tier 1 rat studies (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]; Leung et al., 2020 [RefID 13789]) and one Tier 3 mouse study (Patel et al., 2013 [RefID 5697]) were identified. No effect was seen on uterus weight in the Tier 1 rat study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]). Rats were dosed with 2.5, 25, 250, 2500 or 25000 µg/kg bw per day 1 year. In addition, no effects were observed on uterus weight in the other Tier 1 study (Leung et al., 2020 [RefID 13789]) at PND90, 6 months and 1 year.

Based on these two Tier 1 rat studies, the CEP Panel assigned a likelihood level of Not Likely to the effects on uterus weight. Therefore, this endpoint was not taken forward for BMD analysis.

Growth phase/young age exposure

No studies were available for this exposure period.

Adult exposure (after puberty)

No studies were available for this exposure period.

Indirect (germline) exposure

No studies were available for this exposure period.

Overall cluster selection of the endpoints/studies for BMD analysis for effects on uterus weight

Overall, the CEP Panel assigned an overall likelihood level of ALAN to effects of BPA on uterus weight during the developmental exposure period, and Not Likely during developmental and adult exposure period. Therefore, the overall likelihood across all exposure periods, i.e. the highest likelihood given in the cluster effects on uterus weight, was ALAN.

The CEP Panel considered that the evidence from the studies available did not show a Likely or Very Likely effect of BPA in any exposure period, therefore, this endpoint was not taken forward for BMD analysis.

Effects on uterus histology

Developmental exposure (pre-natal and/or post-natal until weaning)

For effects on uterus histology following developmental exposure of BPA, two Tier 1 studies in rats (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]; Vigezzi et al., 2015 [RefID 7472]) were identified.

Several **non-neoplastic** changes were evaluated: uterine dilatation, luminal epithelium anomalies, gland cell anomalies, cystic endometrial hyperplasia, endometrial hyperplasia and squamous metaplasia. Regarding uterine dilatation, an increase was observed in one Tier 1 study at an intermediate dose out of five doses tested (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]). Increases in luminal epithelium anomalies and gland cell anomalies at PND360 were reported in the other Tier 1 study (Vigezzi et al., 2015 [RefID 7472]) with the two doses tested (i.e. 0.5 and 50 µg/kg bw per day) and with the higher dose, respectively. In one Tier 1 study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370], cystic endometrial hyperplasia was increased with the highest dose after 1 year and at the highest two doses after 2 years. In the same study, no changes were seen for endometrial hyperplasia, whereas a slight increase in squamous metaplasia was observed at the highest dose (25000 µg/kg bw per day) after 1 year (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]). In the other Tier 1 study no squamous metaplasia was detected in animals treated with lower doses (0.5 µg/kg and 50 µg/kg bw per day) (Vigezzi et al., 2015 [RefID 7472]). Based on these results, the CEP Panel judged the non-neoplastic changes of the uterus in the developmental exposure period as Likely.

One **neoplastic** lesion was reported, i.e. stromal polyps. For this endpoint, a decreased incidence was seen in a Tier 1 study at the highest dose after 2 years (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]). Given that a decreased incidence of stromal polyps is not adverse, the CEP Panel judged uterine neoplastic changes in the developmental exposure period as Not Likely.

No statistically significant effect on **apoptosis** was reported in one study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]). Therefore, the CEP Panel judged the uterine proliferative/apoptotic changes in the developmental exposure period as Not Likely.

The CEP Panel assigned a likelihood level of Likely to the uterine histological effects of BPA based on non-neoplastic changes (gland cellular anomalies, squamous metaplasia and cystic endometrial hyperplasia) observed in two rat Tier 1 studies (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]; Vigezzi et al., 2015 [RefID 7472]). Therefore, these endpoints were taken forward for BMD analysis (see Chapter 3.2.1) and for uncertainty analysis (see Appendix D).

Developmental and adult exposure (pre-natal and post-natal in pups until adulthood)

For effects on uterus histology following developmental and adult exposure to BPA, two Tier 1 studies in rats were identified: NTP Clarity Report (2018)/Camacho et al. (2019) [RefID 11370] and the Clarity-BPA Consortium grantee study of Leung et al. (2020) [RefID 13789].

The following **non-neoplastic** changes were evaluated: uterine dilatation, cystic endometrial hyperplasia, endometrial hyperplasia and squamous metaplasia. Regarding uterine dilatation, a significant dose positive 'trend' at 2 years was reported in one study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]). In the Camacho et al. study, cystic endometrial hyperplasia was decreased in animals treated with the lowest dose (2.5 µg/kg bw/day; not adverse) at 1 year, while an increase of endometrial hyperplasia was seen in animals treated with 2.5 and 250 µg/kg bw per day at 1 year but not at 2 years. Additionally, a significant positive dose trend was reported for the incidence of squamous metaplasia at 1 year (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) but no statistically significant effect on this endpoint (% of animals with squamous metaplasia) was reported in the other Tier 1 study, neither at PND90 nor at 1 year (Leung et al., 2020 [RefID 13789]). Based on the results from one Tier 1 study with no effect and another Tier 1 study with a statistical trend and no evident MDR or NMDR, the CEP Panel judged the non-neoplastic changes of the uterus in the developmental and adult exposure period as ALAN.

One **neoplastic** lesion was evaluated, i.e. stromal polyps. For this endpoint, a significant dose trend towards increased incidence at the higher doses at 1 year was observed, while a negative trend (no adverse effect) was observed at 2 years in a Tier 1 study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]). Based on this implausible biological result, the CEP Panel judged the uterine neoplastic changes in the developmental and adult exposure period as Not Likely.

Apoptosis was evaluated with standard histology in one study (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) that reported an increase (trend) at the highest dose (25,000 µg/kg bw per day) only at 1 year. However, no effect was demonstrated in the other study (Leung et al., 2020 [RefID 13789]) in which apoptosis at PND90 and at 1 year was evaluated with a specific and sensitive method (TUNEL). Based on these divergent results the CEP Panel judged the changes in uterine proliferation/apoptosis in the developmental until adult exposure period as ALAN.

Based on non-neoplastic changes and apoptosis, the CEP Panel assigned a likelihood level of ALAN to the uterine histological effects of BPA during the developmental and adult exposure period, hence, none of the endpoints was taken forward for BMD analysis. However, they were considered in the uncertainty analysis (see Appendix D).

Growth phase/young age exposure

No studies were available for this exposure period.

Adult exposure (after puberty)

For effects on uterus histology following adult exposure to BPA, one study was considered (Kendriorski and Belcher, 2015 [RefID 3453]). In this study, two strains of mice were used in different experiments. Due to the low number of animals for one strain (C57Bl/6J) part of the study was considered Tier 3, while the other part performed on CD-1 mice was considered Tier 1.

The following **non-neoplastic** changes were evaluated: gland nests and gland nest density. An increase in gland nests and gland nest density was reported in the Tier 1 experiment on CD-1 mice treated with the second highest dose (4000 µg/kg bw per day). Effects on gland density (with a high

incidence in controls) was not confirmed by the Tier 3 part (C57Bl/6J) of the experiment, whereas an increase of gland nest density was seen with a U-shaped NMDR (in the lowest and the two highest doses). Based on the results without clear dose–responses from one Tier 1 part of the experiment and from the Tier 3 part, the CEP Panel judged the non-neoplastic changes of the uterus in the adult exposure period as ALAN.

Based on non-neoplastic changes, the CEP Panel assigned a likelihood level of ALAN to the uterine histological effects of BPA during the adult exposure period. This endpoint was considered for uncertainty analysis (see Appendix D).

Indirect (germline) exposure

No studies were available for this exposure period.

Overall cluster selection of the endpoints/studies for BMD analysis for effects on uterus histology

Overall, the CEP Panel assigned a likelihood level of Likely to the uterine histological effects of BPA in the developmental exposure period and ALAN in the developmental and adult and in the adult exposure periods. The overall likelihood across all exposure periods, i.e. the highest likelihood given in the cluster uterus, was Likely.

The CEP Panel considered that the evidence from the studies available showed a Likely effect for gland cell anomalies, squamous metaplasia and endometrial cystic hyperplasia. Therefore, these endpoints were taken forward for BMD analysis (see Chapter 3.2.1).

3.1.8.3. Integration of likelihoods from human and animal studies

There was no human evidence available for this category. Thus, the overall likelihood of effects of BPA was based on the animal evidence.

Table 14 presents the likelihoods of effect for each cluster and the overall likelihood in the animal stream in Carcinogenicity and mammary gland proliferative effects.

Table 14: Overall likelihood from the animal studies for Carcinogenicity and mammary gland proliferative effects.

Human stream	Animal stream		Integrated likelihood
Cluster: Effects on Mammary gland weight:	**Cluster: Effects on Mammary gland weight:**		
Not applicable	Developmental exposure (pre-natal and/or post-natal until weaning)	Not Likely	
	Overall likelihood:		*Not likely*
Cluster: Effects on Mammary gland histology	**Cluster: Effects on Mammary gland histology**		
Not applicable	Developmental exposure (pre-natal and/or post-natal until weaning)	ALAN	
	Developmental and adult exposure (pre-natal and/or post-natal in pups until adulthood)	ALAN	
	Overall likelihood:	*ALAN*	*ALAN*
Cluster: Effects on Prostate histology	**Cluster: Effects on Prostate histology**		
Not applicable	Developmental exposure (pre-natal and/or post-natal until weaning)	ALAN	

	Developmental and adult exposure (pre-natal and/or post-natal in pups until adulthood)	Not likely	
	Adult exposure (after puberty)	Inadequate evidence	
	Overall likelihood:	*ALAN*	*ALAN*
Cluster: Effects on Uterus weight	**Cluster: Effects on Uterus weight**		
Not applicable	Developmental exposure (pre-natal and/or post-natal until weaning)	ALAN	
	Developmental and adult exposure (pre-natal and/or post-natal in pups until adulthood)	Not likely	
	Overall likelihood	*ALAN*	*ALAN*
Cluster: Effects on Uterus histology	**Cluster: Effects on Uterus histology**		
Not applicable	Developmental exposure (pre-natal and/or post-natal until weaning)	Likely	
	Developmental and adult exposure (pre-natal and/or post-natal in pups until adulthood)	ALAN	
	Adult exposure (after puberty)	ALAN	
	Overall likelihood	*Likely*	*Likely*

3.1.8.4. *In vitro* and Mechanistic studies

Regarding scoring of likelihood of effects in the WoE for the HOC Carcinogenicity, no relevant clusters were available from the human studies. A few subcluster/endpoints were scored Likely and several were scored ALAN. In the following, MoA studies for these subclusters are considered.

Effects on Mammary gland

Among the HOC Carcinogenicity, the cluster mammary gland histology was considered with the following subclusters: non-neoplastic changes, pre-neoplastic lesions, neoplastic lesions, proliferation and apoptosis.

Mammary gland histology

Based on the integration of the evidence from human and animal studies, the overall likelihood of effects of BPA for mammary gland histology was scored ALAN. This scoring was mainly based on evidence from the subclusters neoplastic changes, proliferation and apoptosis while the subclusters pre-neoplastic lesions (only for the developmental exposure period) and neoplastic lesions (only for the developmental until adulthood exposure period) were considered Not Likely and Likely, respectively.

Several *in vivo* studies addressed the role of proliferation and ERα in the action of BPA in different compartments of the mammary gland. Oral treatment of adult female rats with BPA (5000 µg/kg bw per day for 8 weeks) resulted in significant increases in proliferation and apoptosis indices in epithelial cells but not in ERα expression (Ibrahim et al., 2016 [RefID 2972]). An increase in proliferation and in the proliferation-to-apoptosis ratio was also observed in female rats at PND50 exposed to BPA (250 µg/kg bw per day) via lactating dams (Wang J et al., 2014 [RefID 7640]). BPA exposure did not alter ERα expression but reduced the expression of ERβ which was shown to reduce breast cancer growth. Hindman et al. (2017) [RefID 2711] studied the role of ERα activation in fetal stroma of *in utero* treated female mice (i.p. 25 µg/kg bw per day equivalent to the oral dose 2790 µg/kg bw per day) on histological changes in the mammary gland. In mammary gland sections of 4.5-week-old mice epithelial elongation was directly correlated with proliferation and inversely correlated with ERα expression in the stroma indicating a critical role of the stroma in BPA effects on mammary gland morphology. *In utero*

exposure to BPA (250 ng/kg bw per day, s.c. equivalent to the oral dose 55.5 µg/kg bw per day) of wild-type and ERα knockout mice (Wadia et al., 2013 [RefID 7531]) indicated that BPA-induced changes in the expression of genes involved in the mammary stromal–epithelial interactions were ERα dependent. Gomez et al. (2017) [RefID 2308] studied the effects of perinatal BPA treatment (GD9 to PND21 with 0.5 µg or 50 µg/kg bw per day) on 17β-E2 treated ovariectomised rats. While the histological findings showed a BPA (0.5 µg)-induced increase in ductal and atypical lobular hyperplasia, the immunohistochemistry data did not demonstrate increases in the proliferation index or significant changes in the expression levels of ERα or the progesterone receptor except for a decrease in ERα in ducts from the low BPA dose group.

Data on BPA-induced epigenetic changes in mammary glands were reported in several *in vivo* studies. Camacho et al. (2015) [RefID 802] addressed the BPA effects on gene expression and global genomic DNA methylation in mammary glands of female rats exposed to a wide range of BPA doses (0.5 to 300000 mg/kg bw per day from GD6 to PND90). While only limited BPA effects were observed on DNA methyltransferase coding genes and ER and ERR genes at PND4 and PND90, genome-wide gene expression data analysed by DNA microarray technology (PND4) showed effects of the highest BPA dose (300000 mg/kg bw per day) similar to those in tissues of oestrogen-treated rats. In addition, the expression levels of several genes were modulated in the low-dose range of BPA (0.5 to 2700 µg/kg bw per day) but mostly without dose–response. In another study BPA effects on the methylation pattern were reported in female rats exposed *in utero* to BPA (250 µg/kg bw per day from GD9 to PND1, subcutaneously equivalent to the oral dose 8925 µg/kg bw per day) at different time points, i.e. PND4, PND21 and PND50 (Dhimolea et al., 2014 [RefID 1576]). According to the authors the time-dependent changes in the methylation pattern of genomic DNA may reflect the developmental morphological changes observed in BPA-treated animals rather than causal events resulting in carcinogenesis later in life. Epigenetic effects of BPA were also studied in female rat offspring exposed via lactating dams (250 µg/kg bw per day, PND2 to PND1) at PND100 (Jadhav et al., 2017 [RefID 3045]). The authors found a large number of genes with hypermethylated loci following pre-pubertal BPA exposure. Using network and pathway analysis enriched networks related to cancer, cell death and proliferation were identified. Bhan et al. (2014a) [RefID 557] studied altered epigenetic programming by BPA in mammary glands of ovariectomised adult female rats. Their findings indicate that BPA (two subcutaneous injections with 25 µg/kg bw equivalent to the oral dose 892.5 µg/kg bw per day) induced long non-coding RNA HOTAIR, a regulatory RNA associated with breast cancer. In an additional study with the same study design the authors Bhan et al. (2014b) [RefID 558] provided evidence of BPA-induced expression of the methyltransferase EZH2 which is overexpressed in breast cancer and associated with proliferation, tumour invasiveness and metastasis. In the same laboratory the expression levels of HOXC6 (Hussain et al., 2015 [RefID 2960]) and HOXB9 (Deb et al., 2016 [RefID 1483]), oestrogen-regulated homeobox-containing genes (HOX genes) which are overexpressed in breast cancer, were studied with the same design. The results indicate that the expression levels of HOXC6 and HOXB9 were increased by BPA treatment.

Expression of proteins was studied by Lee et al. (2017) [RefID 3907] in the human MCF-7 breast cancer cell line and in a xenograft mouse model of breast cancer (mice injected s.c. with MCF-7 cells). It was shown that treatment of xenografted mice with BPA (50 mg/kg bw, injected s.c. for 10 weeks) induced breast cancer growth and proteins related to epithelial–mesenchymal transition and metastasis. In a rodent cancer model with DMBA (at PND50) Leung et al. (2017) [RefID 3990] observed that exposure of rats to a low pre-natal dose of BPA (25 µg/kg bw per day) together with a HBF diet fed to the dams increased the tumour incidence (at PND140) and shortened the tumour-free survival time. The increase in tumour incidence was not observed at higher BPA doses, i.e. 250 and 2500 µg/kg bw per day, and in BPA (25 µg) exposed rats from dams fed a control diet. The authors also studied the methylation pattern (PND21) in offspring exposed to BPA and HBF and concluded that some of the differentially expressed genes may be related to breast cancer. Using quantitative proteomic techniques Betancourt at al. (2014) [RefID 548] reported on the expression of proteins in sera of rats exposed via lactating dams (250 µg/kg bw per day). The authors discussed the altered protein expression in serum (at PND21 and PND35) in the context with their potential roles in proliferation/apoptosis and carcinogenesis in the mammary gland.

Wang DH et al. (2014) [RefID 7598] reported increases in lateral branching and hyperplasia in mammary glands of 4-month-old female mice exposed to BPA (25 µg/kg bw per day from PND21 for 3

weeks). The authors suggest that BPA affects mammary stem cells by altering the expression of genes associated with early neoplastic lesions.

A role of increased production of ROS in female rat following long-term treatment with BPA (10, 50 or 100 µg/kg bw per day for 12 weeks) was addressed in a study with female rats (Thilagavathi et al., 2017 [RefID 9247]). Dose-related decreases in SOD and CAT activities and GSH levels along with increased TBARS in the mammary glands were observed. At the lowest BPA dose there was an increase of Enos synthase associated with decreased levels of steroidogenic enzymes and decreased levels of serum E2. Histological analysis revealed hyperplasia in mammary epithelial cells in all dose groups.

Only some changes in nuclear receptor expression (decreased ERα and AR) were observed in a study on female mice at 8 months after treatment with BPA from GD10.5 to GD17 (Tucker et al., 2018 [RefID 13275]). The histological changes in this study are described in 3.1.8.2.

In male rat offspring a delayed mammary gland development was reported by Kass et al. (2015) [RefID 3402] who observed at PND30 a reduced ductal growth along with a reduced expression of the AR following pre-natal exposure to BPA (64 µg/kg bw per day orally or 250 µg/kg bw per day s.c. equivalent to an oral dose 8925 µg/kg bw per day; see 3.1.8.2).

In an *ex vivo* experiment morphological changes were studied in embryonic mouse mammary buds dissected at embryonic day 14 and cultured for 5 days (Speroni et al., 2017 [RefID 6866]). Ductal growth and branching were increased following treatment with 1 nM BPA but inhibited by a high concentration of BPA (1,000 nM). The increase in ductal growth was inhibited by a nuclear ER antagonist (fulvestrant, ICI 182,780). The results suggest a direct oestrogenic action of BPA on the gland development in a concentration dependent manner. In another study using primary organotypic 3D cultures of mammary glands from 6-week-old to 8-week-old mice (Williams et al., 2016 [RefID 7958]) an altered branching pattern was observed along with proteomic changes after treatment with 20 nM BPA for 6 days. Such culture models may be useful to elucidate further the mechanisms of action of BPA involved in morphological changes in the mammary gland.

Potential MoAs for BPA-induced effects in the mammary gland were addressed inter alia by studies with breast cancer or normal breast epithelial cell lines on signalling pathways, ROS, epigenetic processes, gene and protein expression. Several of the studied mechanisms were associated with proliferation or apoptosis, cell migration and cell invasion.

Recent *in vitro* studies on signalling pathways confirmed previous findings that BPA acts via ERα/ERβ (Lee et al., 2014 [RefID 3922]; Potratz et al., 2017 [RefID 5906]; Li Y et al., 2018b [RefID 12494]) and non-classical ERs or ER-independent mechanisms. Chronic exposure to BPA (50 nM) may decrease the expression of ER target genes and initiate transcriptional reprogramming of breast cancer cells (Patterson et al., 2015 [RefID 5725]). An increased proliferation and anchorage-independent growth in ER-negative breast cancer cells by BPA (40 nM) was reported to be mediated via eGFR activation (Sauer et al., 2017 [RefID 6493]). The induction of rapid signalling via the G protein-coupled oestrogen receptor (GPER) was reported to be involved in breast cancer cell adhesion (Magruder et al., 2014 [RefID 4777]), migration (Sanchez et al., 2016 [RefID 6427]) and proliferation under hypoxia (Xu FY et al., 2017 [RefID 8183]). Other BPA effects are dependent on the ERRγ, e.g. proliferation of ER-positive (MCF-7) and ER-negative (SkBr3) cells (Song et al., 2015 [RefID 6818]) or migration and invasion of the triple-negative breast cancer cells (Zhang et al., 2016 [RefID 8865]) induced by nanomolar concentrations of BPA (10 nM). In summary, the data confirm that different signalling pathways can contribute to BPA-stimulated effects at nanomolar concentrations.

A variety of *in vitro* studies with breast cancer cells showed alterations of gene and protein expression by BPA. The expression of HOX genes which are of particular interest in the regulation of perinatal development and known to be overexpressed in breast cancer were reported to be transcriptionally regulated by nanomolar concentrations of BPA (Bhan et al., 2014a [RefID 557]; Bhan et al., 2014b [RefID 558]; Deb et al., 2016 [RefID 1483]; Hussain et al., 2015 [RefID 2960]). BPA (10 nM) enhanced cancer stem cell activity via induction of the transcription factor SOX2 (Lillo et al., 2017 [RefID 4277]) and upregulated the octamer-binding transcription factor 4 (Oct4) in human embryonic stem cells indicating the disturbance of expression of markers for stem cells and mammary epithelial cells (Yang LQ et al., 2013 [RefID 8370]). Results from proteomic analyses of mouse mammary tissue cultured in a 3D model (Williams et al., 2016 [RefID 7958]) showed that 20 nM BPA altered proteins involved in

pathways related to different cellular processes such as proliferation, focal adhesion assembly, substrate adhesion-dependent cell spreading/cell migration and epithelium morphogenesis. Using a reporter gene assay (VM7Luc4E2) in MCF-7 cells the EC50 value of 260 nM was derived for BPA-induced RNA expression (Peng et al., 2018 [RefID 12887]). The induction of the expression of cell cycle genes (Kim et al., 2017 [RefID 3525]; Lee et al., 2014 [RefID 3922]; Li XT et al., 2014 [RefID 4187]) and ER-regulated key proteins (Potratz et al., 2017 [RefID 5906]; Wang T et al., 2018 [RefID 13374]) were tested only at micromolar BPA concentrations (e.g. 10 µM). At a high concentration (100 µM) BPA induced the expression of COX-2 via the transcription factor NF-κB (Song et al., 2017 [RefID 6815]). Overall, the expression of regulatory proteins critical for cell differentiation may be affected by low and high concentrations of BPA.

Two *in vitro* studies investigated the formation of ROS by BPA. Lei et al. (2017) [RefID 3960] observed in MCF-7 cells a slight increase of the viability up to 1 µM BPA while ROS levels were enhanced at 50–100 µM where cell viability was markedly decreased and LDH release was increased. Similarly, in a study using a normal mammary epithelial cell line (MCF10A) no increase of ROS production was detected with 1 and 10 nM BPA (Kang et al., 2013 [RefID 3348]), i.e. in a concentration in which enhanced proliferation was observed in another study (Pfeifer et al., 2015 [RefID 5815]). Based on these *in vitro* findings intracellular ROS formation may be linked rather to cytotoxicity than to physiological processes, e.g. proliferation.

Mammary gland proliferation and apoptosis

Proliferation was studied in numerous *in vitro* studies using ER-positive breast cancer cells (e.g. MCF-7 and T47D cells) which usually require ≥100 nM BPA for growth stimulation (Katchy et al., 2014 [RefID 3410]; Kim et al., 2017 [RefID 3525]; La Rosa et al., 2014 [RefID 3794]; Lee et al., 2014 [RefID 3922]; Li XT et al., 2014 [RefID 4187]; Mesnage et al., 2017 [RefID 5057]; Potratz et al., 2017 [RefID 5906]; Rotroff et al., 2013 [RefID 6302]; Wang T et al., 2018 [RefID 13374]). Related oestrogenic effects such as the expression of cell cycle genes (Kim et al., 2017 [RefID 3525]; Lee et al., 2014 [RefID 3922]; Li XT et al., 2014 [RefID 4187]) and ER-regulated key proteins (Potratz et al., 2017 [RefID 5906]; Wang T et al., 2018 [RefID 13374]) were tested at micromolar concentrations of BPA. Induction of proliferation and apoptosis at 1 µM BPA was observed (Sengupta et al., 2013 [RefID 6567]), while a reduction of apoptosis and ROS production was reported in ER-positive VM7Luc4E2 breast cancer cells (Lee GA et al., 2018 [RefID 12427]). BPA-induced proliferation was mainly induced by an ER-dependent mechanism and was not observed in ER-negative cells, i.e. MDA-MB-231 cells (Li XT et al., 2014 [RefID 4187]; Mesnage et al., 2017 [RefID 5057]). However, Pfeifer et al. (2015) [RefID 5815] using ERα-negative normal breast epithelial cell lines (MCF10A, 184A1) showed that 10 nM BPA induced proliferation via upregulation of the c-Myc protein. Using the same low BPA concentration (10 nM) Song et al. (2015) [RefID 6818] also reported an increased proliferation in ER-positive (MCF-7) and ER-negative (SkBr3) cells mediated by the receptor ERRγ. Additionally, it was reported that BPA (≥100 nM) stimulated proliferation and migration of ER-negative breast cancer cells (SkBr3, MDA-MB-231) via the G-protein-coupled oestrogen receptor (GPER) pathway and under hypoxic culture (Xu FY et al., 2017 [RefID 8183]). In summary, the results from *in vitro* studies suggest that ERα-dependent as well as ER-independent mechanisms lead to BPA-induced proliferation in breast cancer cells, also depending on the study design, cell lines and culture conditions used.

Effects related to Carcinogenicity

Two *in vitro* studies reported DNA damage only at high concentrations of BPA (10 µM) which also induced cytotoxicity (Aghajanpour-Mir et al., 2016 [RefID 57]; Lei et al., 2017 [RefID 3960]). Therefore, these genotoxic effects were considered by the CEP Panel in the Genotoxicity chapter (Chapter 3.1.9).

In several other studies BPA promoted altered cell adhesion, migration, epithelial–mesenchymal transition and invasion mediated via various signalling pathways at micromolar concentrations (Kim et al., 2017 [RefID 3525]; Lee et al., 2017 [RefID 3907]; Magruder et al., 2014 [RefID 4777]; Sanchez et al., 2016 [RefID 6427]; Zhang et al., 2016 [RefID 8865]), except for two studies which showed an enhanced migration potential at 10 nM BPA (Zhang et al., 2015 [RefID 8866]) or 100 nM (Cao et al., 2017 [RefID 837]) in ER-negative breast cancer cells. No effect on migration and cell invasion was observed following long-term exposure (at least 4 weeks) to 250 nM BPA in MCF-7 cells (Norberto et al., 2017 [RefID 5440]). The reported findings on cellular cancer-related endpoints indicate that BPA may contribute to tumorigenesis in breast cancer cells.

In conclusion, the CEP Panel considered that the *in vivo* studies show evidence that stromal–epithelial interactions may play a crucial role in the BPA-induced developmental changes in the mammary gland. Epigenetic effects of BPA including changes in methylation patterns are frequently reported along with altered expression of genes involved in proliferation/apoptosis and other cellular functions. Results from two breast cancer models (i.e. xenograft mouse model and chemically (DMBA) induced cancer) suggest that BPA may enhance the susceptibility to carcinogenic stimuli. In addition, there is some evidence from *in vitro* studies with breast cancer cells which supports the hypothesis that BPA promotes changes in proliferation and pathological processes which ultimately could contribute to carcinogenesis in the mammary gland.

Effects on prostate

Among the HOC Carcinogenicity, the cluster 'prostate histology' was considered and the following subclusters were considered if available: non-neoplastic changes, pre-neoplastic lesions, neoplastic lesions, proliferation and apoptosis.

Eighteen MoA studies specifically addressing the carcinogenic effects on the prostate were considered, five within the mammalian stream, one human and 12 in the *in vitro* stream. MoA studies from the *in vitro* stream often used prostate cancer cell lines (in seven studies) and in particular LNCaP, PC3 and C4–2 cell lines, in some cases stem cells (in four studies) and normal cells (in two studies). Other studies related to non-neoplastic changes have also taken in consideration.

Prostate histology (*in vivo* studies)

In vivo studies on mechanisms for rodent developmental BPA-induced prostate inflammation and reactive hyperplasia have explored a number of endpoints, including alterations in ER-related and AR-related gene and protein expression in the prostate. Proposed mechanisms include increased expression of AR, decreased expression of ERα, increased prostate aromatase expression leading to increased intraprostatic E2, and altered expression of other androgen-related genes (e.g. Scgb2a1, Ptgds).

In conclusion, despite the relatively large amount of data available, there is no consensus on mechanisms underlying developmental BPA-induced prostate effects, in particular whether effects are upstream or downstream. More details are reported in Chapter 3.1.6.4, *In Vitro* and Mechanistic studies on Reproductive and developmental Toxicity.

Prostate histology (*in vitro* studies)

- Prostate histology (non-neoplastic changes)

Two histological non-neoplastic findings (inflammation and hyperplasia) were evaluated in animal studies. Based on the inconsistencies in the outcomes and the diversity of the effects, the CEP Panel judged the prostatic non-neoplastic changes as ALAN. MoA *in vitro* studies indicate that BPA may increase the proliferative rate of different types of prostate cells while no data are available for inflammation.

- Prostate histology (pre-neoplastic lesions)

Two histological pre-neoplastic findings (atypical hyperplasia and dysplasia) were evaluated in animal studies in the developmental exposure period and were judged as ALAN.

No MoA *in vitro* study specifically addressed these outcomes.

- Prostate histology (neoplastic lesions)

In animal studies, no neoplastic lesions of prostate induced by BPA without additional hormonal treatment (testosterone and oestrogen) were identified by the CEP Panel.

Data obtained from MoA studies indicate that BPA can enhance prostate cancer susceptibility while its direct carcinogenicity is still debated. However, the results of studies carried out in laboratory rodents do not demonstrate a direct evidence of tumorigenic activity of BPA alone. More details can be found in the Chapter 3.1.6.4 in the HOC Reproductive and developmental toxicity.

- Prostate histology (proliferation and apoptosis)

In animal studies carried out in rats, the CEP Panel judged proliferation and apoptosis in the developmental exposure period as ALAN.

Data obtained from a few *in vitro* MoA studies indicate that BPA can enhance proliferation of the prostate cells supporting the weak evidence from studies in animals. Data from *in vitro* MoA studies regarding apoptosis do not shed light on this aspect. More details can be found in the Chapter 3.1.6.4 in the HOC Reproductive and developmental toxicity.

Effects on uterus

Among the HOC Carcinogenicity, the clusters 'uterus weight' and 'uterus histology' were considered. The CEP Panel judged the likelihood for an increase in uterus weight ALAN during developmental exposure.

For histology, the following subclusters were considered if available: non-neoplastic changes, pre-neoplastic lesions, neoplastic lesions, proliferation and apoptosis. The CEP Panel considered the overall likelihood of this cluster Likely during the developmental exposure based on effects on non-neoplastic changes and ALAN during the developmental and adult exposure period.

Uterus weight

The CEP Panel considered the likelihood for an increase in uterus weight ALAN during developmental exposure. The possible MoA may be mediated by ERs. No MoA study specially addressed this outcome.

Uterus histology (non-neoplastic changes)

Most MoA studies indicate that BPA may increase the proliferative rate of different types of uterine cells. More details can be found in the Chapter 3.1.6.4 in the HOC Reproductive and developmental toxicity.

Uterus histology (pre-neoplastic lesions)

No pre-neoplastic lesions were evaluated in human or animal studies and no MoA study specifically addressed this outcome.

Uterus histology (neoplastic lesions)

No neoplastic lesions were evaluated in human studies. Based on evidence from animal (rats and mice) studies, the overall likelihood of effects of BPA for uterine neoplastic lesions was considered Not Likely**.**

Uterus histology (proliferation and apoptosis)

MoA studies do not support/explain the increase of apoptosis observed in BPA-treated animals in one *in vivo* study. More details can be found in the Chapter 3.1.6.4 in the HOC Reproductive and developmental toxicity.

3.1.8.5. Conclusion on hazard identification for Carcinogenicity and mammary gland proliferative effects of BPA

In the 2015 EFSA opinion on BPA (EFSA CEF Panel, 2015), BPA effects on mammary gland proliferation were considered Likely based on results from a subchronic rat study with pre-natal exposure to BPA as well as proliferative and related morphological changes in the mammary gland reported in other studies. The data on mammary gland proliferation were taken forward for a BMD–response modelling but these data were not suitable to provide a RP (BMDL) due to considerable uncertainty in the outcome of modelling. However based on the WoE evaluation, effects related to proliferation in the mammary gland were taken into account in the evaluation of uncertainty for hazard characterisation and included in the risk assessment. The evidence for proliferative changes in prostate and other organs (testis, liver) was evaluated as too weak to reach a definite conclusion. Overall, the findings in mammary gland, prostate and other organs were considered 'insufficient to conclude that there is a link to cancer development in later life' and the likelihood level of 'unlikely to ALAN' was assigned to carcinogenic effects of BPA (EFSA CEF Panel, 2015).

A new pre-natal and chronic toxicity study of BPA in rats and additional studies in rodents were conducted to further clarify whether changes in proliferation and differentiation in mammary gland, prostate, uterus and other organs eventually result in an increased incidence of tumours. Having assessed these data in the current evaluation the CEP Panel considered that the new findings support

the earlier observations. While no effects on mammary gland weight of female rats treated with BPA at doses from 2.5 to 25,000 µg/kg bw per day were observed up to 2 years of age, several histological changes, e.g. in longitudinal growth, TEBs, branching, gland density and mammary gland scores were reported in studies with female and/or male rats and mice after BPA exposure during the developmental until weaning or adulthood period. Due to the diversity of these outcomes assessed at different timepoints and doses the CEP Panel considered the induction of these non-neoplastic effects ALAN. Regarding pre-neoplastic lesions the CEP Panel noted an increase of atypical foci at the lowest dose (2.5 µg/kg bw per day) in female rats following treatment during developmental until adult exposure (Likely) but no effect after only developmental exposure until weaning (Not Likely) in female rats in the chronic study. On the other hand, regarding neoplastic lesions in the same study there was an increase of adenomas and/or adenocarcinomas at the lowest dose in female rats following treatment during developmental until weaning period (Likely) but no statistically significant effect was found after long-term exposure from *in utero* until adulthood (Not Likely) in female rats.

Overall, the CEP Panel considered the sum of the histopathological findings ALAN and therefore, the effects were taken into account in the evaluation of uncertainty for hazard characterisation and included in the risk assessment.

MoA studies addressing epigenetic effects, changes in gene expression and hormone receptor levels *in vivo* and *in vitro* suggest various mechanisms of action of BPA possibly involved in the induction of proliferative/morphological changes in the mammary gland. Some evidence from *in vitro* studies with breast cancer cells supports the hypothesis that BPA promotes changes in proliferation and pathological processes which ultimately contribute to a higher susceptibility to mammary gland carcinogenesis.

In the current evaluation also data on histological effects in prostate from the new pre-natal and chronic toxicity study of BPA in rats and additional studies in rodents were considered in the WoE approach. Non-neoplastic changes related to inflammation and hyperplasia following developmental exposure until weaning were graded as ALAN due to inconsistencies in the outcome among studies. In the developmental and adult exposure period these effects were considered as Not Likely and in the adult exposure period the non-neoplastic changes were Inadequate due to the limitations in the study database. Pre-neoplastic lesions, i.e. atypical hyperplasia and dysplasia were considered as ALAN after developmental exposure and Unlikely after developmental until adult exposure in rat studies. Moreover, proliferation and apoptosis were considered as ALAN following developmental exposure in two rat studies. No evidence of induction of neoplastic lesions were seen in any of the exposure periods.

MoA studies confirmed the role of sex hormones in prostate cancer development. In a rat model using testosterone and two-fold elevated oestrogen levels, developmental BPA treatment increased the severity score of prostatic intraepithelial neoplasia (PIN) and adenocarcinoma multiplicity. BPA treatment alone was reported to increase prostate stem cell proliferation and disrupt prostate stem cell homeostasis. Via epigenetic or additional mechanisms BPA can modulate gene and protein expression, signalling pathways, stem cell homeostasis or centrosome activity. In conclusion, MoA studies indicate that BPA can enhance the susceptibility to prostate cancer while the results of studies in rodents do not demonstrate a direct tumorigenic effect of developmental and chronic exposure to BPA.

Regarding uterus, several non-neoplastic changes were considered as Likely after developmental exposure to BPA in rats: gland cell anomalies, endometrial cystic hyperplasia and squamous metaplasia. Therefore, these endpoints were taken forward for BMD analysis (see Chapter 3.2.1). Uterine dilatation, endometrial hyperplasia and squamous metaplasia were considered ALAN after developmental and adult exposure. Gland nests and gland nest density were non-neoplastic changes observed in the adult exposure period. Apoptosis was considered ALAN after developmental and adult exposure period and Not Likely after developmental exposure. No evidence of induction of neoplastic lesions were seen in any of the exposure periods, since a negative dose trend (no adverse effect) was observed at two years with a significant decrease at the highest dose (developmental exposure), while a positive trend without statistical significance at the higher doses was observed at one year (developmental and adult exposure). Therefore, neoplastic lesions in the uterus were considered as Not Likely.

Most MoA studies indicate that BPA increases the proliferative rate of different types of uterine cells. Data obtained from *in vitro* MoA studies indicate that BPA can modulate many mechanisms underlying the onset of growth and invasion of uterine tumours. However, the results of rodent studies do not provide evidence of a carcinogenic activity of BPA.

3.1.9. Genotoxicity

In the present assessment, the CEP Panel examined whether new data from the published literature could provide new evidence on the potential genotoxicity of BPA. To this aim, a literature search was performed as reported in Annex A. Also the references from the previous CEF Panel opinion (EFSA CEF Panel, 2015) have been included in the current assessment using the same appraisal criteria applied to the newly published data and considering the EFSA Scientific Committee guidance documents on genotoxicity published after 2015 (EFSA Scientific Committee, 2017c, 2021a).

Genotoxicity studies considered for this assessment are:

- *in vitro* and *in vivo* studies (88 publications) retrieved from the literature search (Annex A and Annex L),
- *in vitro* and *in vivo* studies (15 publications) considered in the Sscientific opinion on the risks to public health related to the presence of BPA in foodstuffs (EFSA CEF Panel, 2015) (Appendix E and Annex L).

In vitro and *in vivo* studies were grouped based on the genotoxicity endpoint investigated:

- gene mutations (e.g. bacterial reverse mutation assay);
- chromosomal damage (CA and micronucleus assays);
- DNA damage (comet assay).

These studies were summarized in synoptic tables (Annex L), evaluated for reliability and relevance and grouped into lines of evidence in a WoE approach (see Chapter 2.3.5).
The genotoxicity studies have been assessed using a scoring system for reliability based on criteria published by Klimisch et al. (1997) as explained in Chapter 2.3.5. In a second step, the relevance (high, limited, low) of study results was assessed based on reliability of the study and some general aspects e.g. genetic endpoint, purity of the test substance, route of administration and status of validation of the assay (see Chapter 2.3.5).
Genotoxicity studies evaluated as of low relevance have not been further considered in the assessment. Studies not investigating classical genotoxicity endpoints (e.g. γH2AX, oxidative DNA damage, DNA binding, ROS generation) and studies in humans are considered in the MoA and as supportive evidence. All the studies evaluated were summarised in a narrative form (Appendix E).

3.1.9.1 WoE

1. Gene mutations *in vitro* and *in vivo*

1a. *In vitro* gene mutation

Of the six available studies of the mutagenicity of BPA in bacteria, only one describes the application of the Ames test in a comprehensive battery of *Salmonella* Typhimurium strains (TA1535, TA97, TA98, TA100 and TA102) at a range of concentrations up to 5000 µg/plate. It reports negative results both in the presence and absence of metabolic activation (Xin et al., 2015 [RefID 8150]). Three studies reported negative results in TA98 and TA100 (Masuda et al., 2005; Fic et al., 2013; Zemheri and Uguz, 2016 [RefID 9535]). A study shows negative results in TA98, TA100 and TA102 strains (Tiwari et al., 2012). The sixth used the bacterial SOS/umuC assay with a range of concentrations from 1 to 1000 µg/L in presence and absence of S9 mix. It also reported negative results (Balabanič et al., 2021 [RefID 224-G]).

The CEP Panel concluded that BPA does not induce gene mutations in bacteria.

No studies on gene mutation assays in mammalian cells following the OECD guidelines were available.

1b. *In vivo* gene mutation

No studies on gene mutation assays *in vivo* were available.

2. Induction of chromosomal aberrations/micronuclei *in vitro* and *in vivo*

2a. *In vitro* chromosomal aberrations/micronuclei

Fifteen *in vitro* studies of micronuclei (MN) and structural CA induction in different cell lines were available for evaluation. Of these, nine were further considered in the assessment (Figure 2), classified as having high (1 study) or limited relevance (8 studies).

All showed positive results in both blood cells and established cell lines. In the single study classified as of high relevance, a concentration-dependent increase of MN frequency over a wide range of concentrations (1.5 to 37 µg/ml corresponding to 6.6 µM and 162 µM) was observed in the AHH-1 human lymphoblastoid cell line (Johnson and Parry, 2008). Positive CA results were also reported from cultures of human peripheral lymphocytes in two studies with limited relevance (Santovito et al., 2018 [RefID 11220]; Di Pietro et al., 2020 [RefID 258-G]). In one of these (Santovito et al., 2018 [RefID 11220]), MN frequency was also measured. A study of MN in bovine peripheral blood lymphocytes also reported positive findings (Šutiaková et al., 2014 [RefID 7026]).

In murine macrophage RAW264.7 cells, positive MN results were associated with an increase inreactive oxygen species (ROS), and a decreased level of antioxidant enzymes (GPx, SOD and CAT. Concomitant phosphorylation of P53 and release of cytochrome C from mitochondria were detected along with increased apoptosis. Pretreatment with N-acetylcysteine (NAC) reduced BPA-induced cytotoxicity, apoptosis and genotoxicity (MN frequency was reduced by 30%). These results indicate that the toxic effect of BPA in macrophages was mainly through the oxidative stress-associated mitochondrial apoptotic pathway (Huang FM et al., 2018 [RefID 296-G]).

Finally, two studies in the Chinese hamster ovary (CHO) and V79 cell lines reported positive results (Xin et al., 2015 [RefID 8150]; Yu et al., 2020 [RefID 475-G]). Xin and co-workers reported a concentration dependent increase of both MN and CAs in CHO cells in the absence of metabolic activation. In contrast, the BPA-induced increase in MN frequency in V79, reported by Yu and colleagues, apparently required CYP1A1 and CYP1B1 expression.

Overall, the significant increases of chromatid and chromosome breaks observed in several studies *in vitro* indicated that BPA has clastogenic activity also at non-cytotoxic concentrations. Two reports indicated that oxidative stress is implicated in the observed induction of chromosomal damage. In addition, Johnson and Parry (2008) reported the formation of aberrant mitotic spindles, with multiple poles, in cells treated with BPA.

In conclusion, the *in vitro* studies on CA and MN induced by BPA indicated that both clastogenic and aneugenic mechanisms may operate.

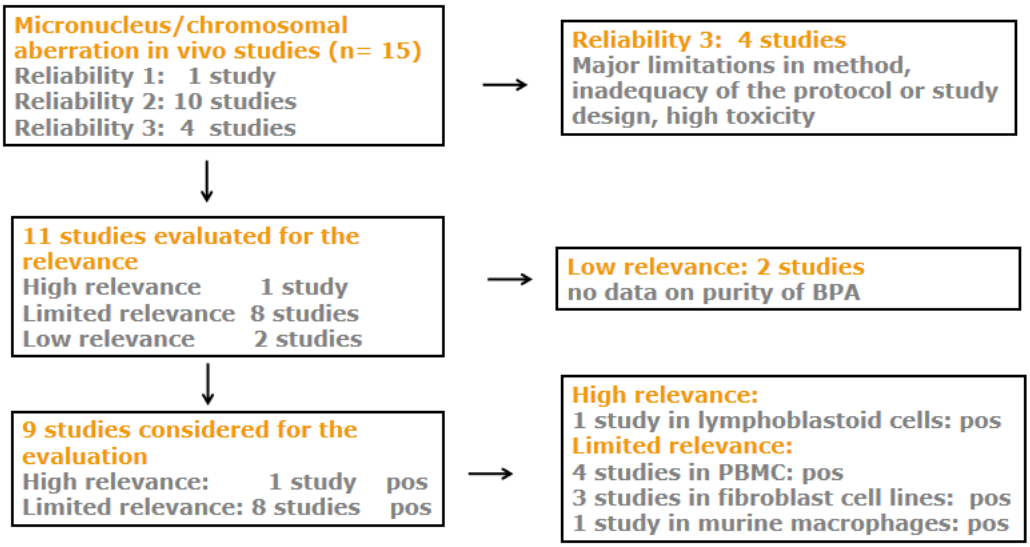


Figure 2: *In vitro* chromosomal aberration and micronucleus studies: evaluation and summary of test results from 15 studies.

2b. *In vivo* chromosomal aberrations/micronuclei

Eleven *in vivo* studies addressing BPA-induced MN and structural CA after oral exposure were evaluated. After a screening for the reliability and relevance of the results, six studies from four publications, all ranked as of limited relevance, were selected for further consideration (Figure 3 and Table 15). Of these, three studies were considered positive for the induction of MN and CA in the same publication (Tiwari et al., 2012) or of MN (Panpatil et al., 2020 [RefID 379-G]) in rats following daily oral BPA administrations for 6 and 28 days, respectively. Tiwari et al. (2012) applied a range of doses from 2.4 µg up to 50 mg/kg bw per day. In a separate publication, the same authors (Tiwari and Vanage, 2017) reported that these experimental conditions were associated with the induction of lipid peroxidation (malonaldehyde, MDA) and oxidative stress (decreased SOD, CAT, GSH) in rat bone marrow and peripheral blood lymphocytes. In Panpatil et al. (2020) [RefID 379-G] the dose range was much lower (50 and 100 µg/kg bw per day). A fourth study tested positive in the mouse bone marrow MN test after the administration of a daily dose of 50 mg/kg bw for 28 days in presence of high level of cytotoxicity (Fawzy et al., 2018 [RefID 270-G]). A study by Naik and Vijayalaxmi (2009) reported negative findings in the mouse bone marrow MN test and CAs following a single dose in the range 10 to 100 mg/kg bw.

Overall, the available data provided evidence of chromosomal damage after multiple oral administrations but not after single oral administration of BPA.

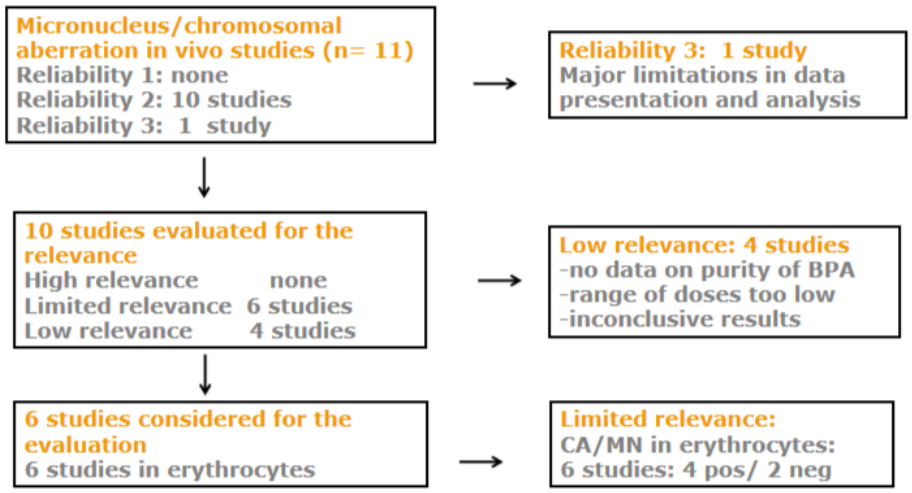


Figure 3: *In vivo* chromosomal aberration and micronucleus studies: evaluation and summary of test results from 11 studies.

Table 15: Summary table of test results of MN and CAs *in vivo* studies.

Test system	Dose	Results	Reference
MN and CA in bone marrow Swiss albino mice 6 animals/group	10, 50 and 100 mg/kg bw, single dose by gavage; 10 mg/kg bw for 5 days (50 mg) by gavage	**NEGATIVE** No significant decrease of PCE/NCE ratio, but significant increase of gaps and C-mitoses	Naik and Vijayalaxmi, 2009

MN and CA in bone marrow Holtzman rats 10 animals/group	2.4 µg, 10 µg, 5 mg and 50 mg/kg bw per day orally for 6 days	**POSITIVE** Dose-related increase of CA and MN-PCEs starting from 10 µg	Tiwari et al., 2012
MN in bone marrow Male Swiss albino mice 10 animals/group	50 mg/kg bw per day orally for 28 days	**POSITIVE** Significant reduction in the ratio of PCE/NCE	Fawzy et al., 2018 [RefID 270-G]
MN in bone marrow Male Wistar rats 6 animals/group	50 and 100 µg/kg bw per day orally for 28 days	**POSITIVE** Dose-related increase of MDA in blood and of urinary 8-OHdG	Panpatil et al., 2020 [RefID 379-G]

3. Comet assay

3a. *In vitro* comet assay

Twenty-two *in vitro* studies using a comet assay in different cell lines were available for evaluation. Twelve were classified as of limited relevance and further considered in the assessment. Most cell lines used in these studies were of human origin from blood, mammary gland and prostate. Rodent cell lines from rat, mouse and hamster and one cell line from monkey were also considered.

Eleven of the 12 studies reported positive results. Three studies on HepG2 cell line yielded both positive (Li XH et al., 2017 [RefID 4176]; Balabanič et al., 2021 [RefID 224-G]) and negative (Fic et al., 2013) results. In a non-tumorigenic human prostatic cell line, BPA induced a significant increase in DNA strand breaks paralleled by a decrease in total GSH, antioxidant capacity, glutathione peroxidase 1 (GPx1) and SOD activity and an increase in glutathione reductase (Kose et al., 2020 [RefID 325-G]). Positive results were also reported in CHO cells (Xin et al., 2015 [RefID 8150]). Positive results were reported from two studies in which human PBMC were analysed by both alkaline and neutral comet assays (Mokra et al., 2017 [RefID 5170]). Evidence of oxidative damage to DNA bases was provided by the addition of endonuclease III (Nth) and 8-oxoguanine DNA glycosylase (hOGG1) DNA repair enzymes (Mokra et al., 2018 [RefID 364-G]). DNA strand breaks induction by BPA was associated with increased ROS, MDA and reduced SOD activity in HepG2 (Li XH et al., 2017 [RefID 4176]). In murine macrophage RAW264.7 cells, positive DNA strand breaks were associated with an increase in ROS and decreased level of antioxidant enzymes (Huang FM et al., 2018 [RefID 296-G]). In Marc-145 rhesus monkey embryo renal epithelial cells, DNA strand breaks induction was associated with increased ROS and TBARS and decrease in GSH and SOD activity (Yuan et al., 2019 [RefID 478-G]).

DNA strand breaks induction in mouse embryonic fibroblast cell line (NIH3T3) is associated with elevated ROS and a modest increase in DNA 8-hydroxy-2′-deoxyguanosine (8-OHdG) at the highest concentration tested (Chen et al., 2016 [RefID 1130]). In rat INS-1 insulinoma cells DNA strand breaks and ROS level increased in parallel along with the induction of DNA damage-associated proteins (p53 and p-Chk2). At the highest concentration of 100 µM, pre-treatment with NAC reduced the number of induced DNA strand breaks by two-fold (Xin et al., 2014 [RefID 8147]). Finally, ER-positive MCF-7 cells were more sensitive than ER-negative MDA-MB-231 cells to BPA-induced DNA damage, as measured by comet assay (Iso et al., 2006).

The available *in vitro* studies provided evidence that BPA induces DNA strand breaks most likely related to the induction of oxidative stress.

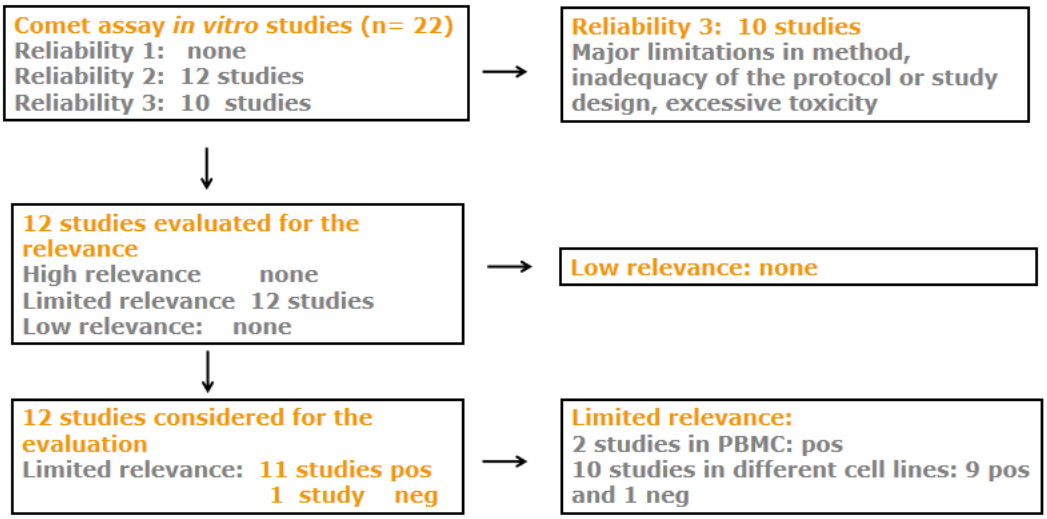


Figure 4: *In vitro* comet assay: evaluation and summary of test results from 22 studies.

3b. *In vivo* comet assay

In the current assessment only five of 21 *in vivo* comet assay studies of DNA strand breaks induction by BPA were classified as of high (one study) or limited relevance and have been considered for evaluation. Among the five oral studies selected, three were positive and two were negative. A single study of high relevance reported negative results in multiple mouse organs (liver, kidney, testes, urinary bladder, colon and lungs) after single treatment at three doses up to the MTD of 500 mg/kg bw (Sharma et al., 2018 [RefID 662-G]). Negative results were also reported in rats exposed to 200 mg/kg bw per day orally for 10 days (De Flora et al., 2011). In contrast, dose-related increases in DNA strand breaks were reported at doses greater than 10 µg/kg bw in rats treated for 6 days with a range of doses between 2.4 µg and 50 mg/kg bw per day (Tiwari et al., 2012). A weak and dose-dependent increase in liver DNA strand breaks was observed at 50 and 100 mg/kg bw per day, whereas the increase in kidney was limited to 50 µg/kg bw (Panpatil et al., 2020 [RefID 379-G]). Finally, in a study on BPA neurotoxicity, a significant increase of strand breaks in brain cells was observed after treatment in a range of doses from 0.5 to 5000 µg/kg bw per day for 8 weeks (Zhou YX et al., 2017 [RefID 9083]).

Overall, the comet assays provided only limited evidence of DNA damage following multiple administrations of BPA, but not following single dose administrations.

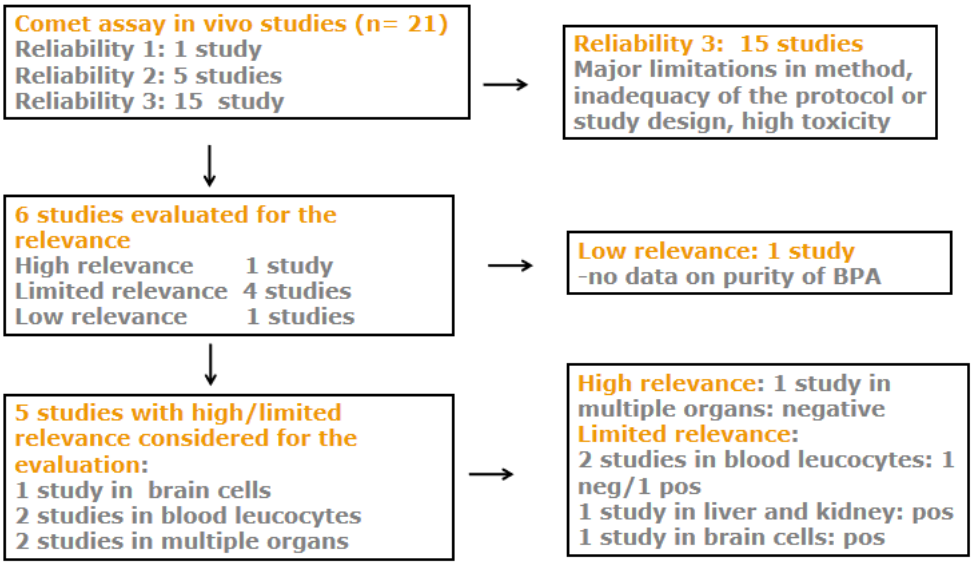


Figure 5: *In vivo* comet assay: evaluation and summary of test results from 21 studies.

Table 16: Summary table of test results of Comet *in vivo* studies.

Test system	Dose	Results	Reference
Comet assay in liver, kidney, testes, urinary bladder, colon and lungs			
CD-1 male mice 5 animals/group	125, 250 and 500 (MTD) mg/kg bw **Single dose by gavage**	**NEGATIVE**	Sharma et al., 2018 [RefID 662-G]
Comet assay in blood leukocytes			
Sprague-Dawley rats 8 animals/group	200 mg/kg bw per day orally **for 10 days**	**NEGATIVE**	De Flora et al., 2011
Holtzman rats 10 animals/group	2.4 µg, 10 µg, 5 mg and 50 mg/kg bw per day orally **for 6 days**	**POSITIVE** Dose-related increase starting from 10 µg/kg	Tiwari et al., 2012
Comet assay in liver and kidney			
Male Wistar rats (WNIN) 6 animals/group	50 and 100 µg/kg orally **for 4 weeks**	**POSITIVE** Weak dose-related in liver; only at 50 µg/kg in kidney	Panpatil et al., 2020 [RefID 379-G]
Comet assay in brain cells			
KM male mice 11 animals/group	0.5, 50 and 5000 µg/kg bw per day orally **for 8 weeks**	**POSITIVE**	Zhou YX et al., 2017 [RefID 9083]

4. Effects in germ cells

A dominant mutation test carried out in Holtzman rats examined the mutagenic activity of BPA in male germ cells (Tiwari and Vanage, 2013). In this study a decrease in total implants/female and live implants/female, with a concurrent significant increase in the number of resorbed embryos per female, was observed during the fourth week and sixth week in females mated with males treated with 5.0 mg BPA/kg bw per day for 6 days. The timing of response may indicate the induction of post-implantation loss due to dominant lethal mutations in mid-spermatids and spermatocytes. However, the CEP Panel noted that the limited protocol of the study, with less analysable total implants and resorptions than

recommended, as well as the lack of positive and historical controls, prevents to reach a firm conclusion on the dominant lethal effect of BPA on male germ cells.

A comprehensive evaluation of the aneugenic potential of BPA in rodent germ cells was carried out in the framework of a European collaborative project on aneugenic chemicals (Pacchierotti et al., 2008). Hyperploidy and polyploidy were evaluated in metaphase II (MII) oocytes and zygotes following acute (0.2 and 20 mg/kg bw), subacute (0.04 mg/kg bw for 7 days) and subchronic (0.5 mg/L equivalent to 0.09 mg/kg[19] for 7 weeks) administration of BPA to young adult (4 or 9 weeks old) females, and in epididymal sperm after subacute treatment of adult males (six daily administrations of 0.002, 0.02 and 0.2 mg/kg bw). The results of the project did not provide any evidence of increased frequency of aneuploidy in mouse oocytes and zygotes and in sperm cells following low dose BPA exposure.

Other studies on male and female germ cells investigated the effect of BPA exposure on meiotic progression and recombination.

In a study on adult (8 weeks old) male rats (Liu C et al., 2014 [RefID 4378]), the administration of BPA (20 µg/kg bw per day) by gavage for 60 consecutive days (one spermatogenesis cycle) inhibited spermiation and delayed meiosis initiation. An accumulation of unresolved DSBs and chromosomal abnormalities (asynapsis, end-to-end associations and altered synaptonemal complex staining) was observed in the late pachytene stage. These data indicate that the inhibition of meiotic DSBs repair following activation of checkpoints may halt meiotic progression and inhibit meiotic entry.

In mice, oral exposure to BPA in neonatal life (20 or 500 µg/kg bw per day on days 1-12 post-partum) resulted in a reduction of meiotic recombination in pachytene cells, which persisted in the adult males (Vrooman et al., 2015 [RefID 7521]). A reduction in meiotic recombination (measured by the number of MLH1 foci in pachytene stage meiocytes) was also observed in another study in young (6 weeks old) male adult mice following oral administration of BPA (20 µg/kg bw per day) on days 1-8 post-partum (Horan et al., 2018 [RefID 291-G]). In the same study, administration of BPA (20 µg/kg bw per day) to pregnant females on days 14-15 post-coitum, to coincide with the time of meiotic entry in the fetal ovary, resulted in an increase of meiotic recombination in developing oocytes.

It was suggested that a decreased level of recombination in spermatocytes may be lethal in these cells due to the actions of the spindle assembly checkpoints (SAC). In contrast, increased meiotic recombination, which is compatible with continued oocyte survival, may increase the frequency of aneuploid eggs and embryos (Horan et al., 2018 [RefID 291-G]). However, the CEP Panel noted that adequately conducted studies in male and female mouse germ cells failed to demonstrate an increase in aneuploid gametes following oral exposure to low doses of BPA under several exposure regimens (Pacchierotti et al., 2008).

Overall, the CEP Panel concluded that there is no evidence for aneugenic activity in germ cells in studies in adult animals using low doses of BPA.

[19] According to EFSA Scientific Committee (2012)

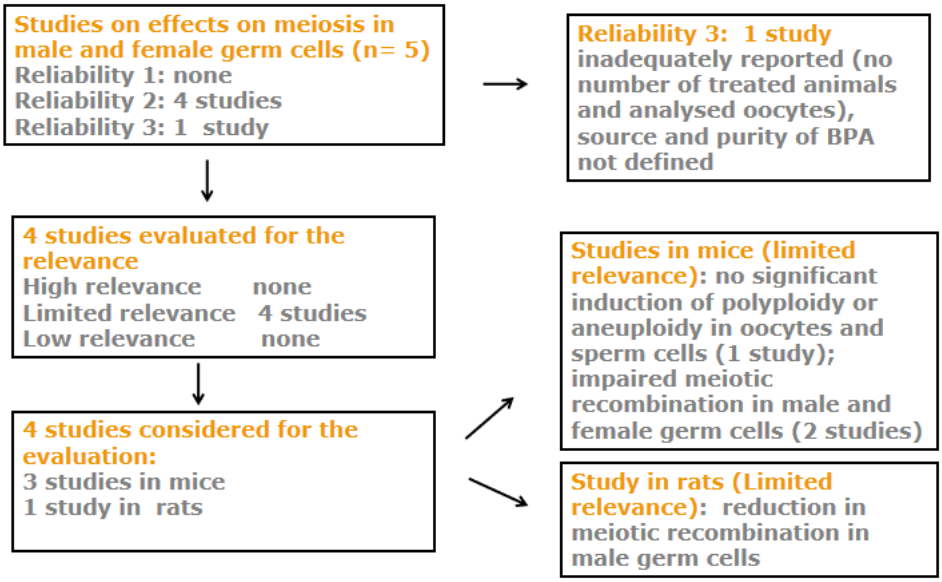


Figure 6: Effects in germ cells: summary of evaluation and results from five studies.

5. Other studies

5a. DNA adducts

In early publications, ^{32}P post-labelling indicated the formation of several DNA adducts of BPA *in vitro* in the presence of a microsomal activation system and *in vivo* in mouse and rat liver and in mouse mammary tissue (Atkinson and Roy, 1995a,b; Izzotti et al., 2009). These reactions were proposed to occur *via* oxidation of BPA to bisphenol-*o*-quinone. Formation of DNA adducts was confirmed in human prostate cell lines (De Flora et al., 2011). No further characterisation of BPA-induced DNA adducts has been reported since the last EFSA opinion.

The possible biological significance of these DNA adducts is undermined by the limitation of the postlabelling technique together with multiple studies that indicate that BPA induces mutagenic and clastogenic DNA damage in the absence of metabolic activation.

5b. Induction of γH2AX foci

Several studies have investigated the induction of γH2AX foci (generally regarded as a marker of DNA DSBs) following BPA treatment (Iso et al., 2006; Pfeifer et al., 2015 [RefID 5815]; George and Rupasinghe, 2018 [RefID 277-G]; Kim et al., 2018b [RefID 11137]; Mahemuti et al., 2018 [RefID 11171]; Hercog et al., 2019 [RefID 278-G]; Hercog et al., 2020 [RefID 288-G]; Nair et al., 2020 [RefID 367-G]; Yin et al., 2020 [RefID 474-G]; Escarda-Castro et al., 2021 [RefID 266-G]; Yuan et al., 2021 [RefID 477-G]).

Iso et al. (2006) reported increased levels of γH2AX foci after treatment with 17β-E2 or BPA in ER-positive MCF-7 cells (1000x higher concentrations of BPA were needed to induce the same levels of effects as E2). Induction was less severe in ER-negative MDA-MB-231 cells and the ER antagonist ICI182780 blocked BPA-induced γH2AX focus formation in MCF-7 cells. Taken together, these findings indicate that BPA-induced genotoxicity is ER-dependent.

The effects of low-dose BPA were studied in the ERα-negative MCF10A and in 184A1 normal breast epithelial cell lines and the ERα-positive MCF7 and MDA-MB-231 human breast epithelial adenocarcinomas. Low doses (10 and 100 nM) induced DSBs as measured by γH2AX foci in all cell lines and increased the level of c-Myc and of the cell-cycle regulatory proteins cyclins D1 and E and E2F1. Silencing c-Myc reduced BPA-induced γ-H2AX foci and abolished BPA-mediated mitochondrial ROS production. BPA also induced proliferation in ERα-negative 184A1 mammary cells. The authors conclude that low-dose BPA exerts a c-Myc–dependent genotoxicity and mitogenicity in ERα-negative mammary cells (Pfeifer et al., 2015 [RefID 5815]).

A concentration-dependent increase in γ-H2AX foci and ROS levels was reported following exposure to BPA in the sub-micromolar range in HepG2 and NKNT human hepatocyte cell lines. Similar effects were also observed in liver tissue of juvenile rats exposed to a relatively low dose of BPA (0.5 mg/kg bw for 90 days). These effects were correlated to the BPA-associated promotion of cell proliferation (Kim et al., 2018b [RefID 11137]).

A 2-month exposure of HME1 mammary epithelial cells and MCF7 breast cancer cell line to low, non-toxic BPA concentration (0.0043 nM) increased levels of DNA damage as shown by upregulation of DNA damage markers (γH2AX, pCHK1, pCHK2, p-P53) and disruption of the cell cycle progression. In addition, hypomethylation was observed in a panel of 24 tumour suppressor genes consistent with an epigenetic mechanism (Nair et al., 2020 [RefID 367-G]).

Together these data suggest that the BPA-mediated increase in γH2AX foci is associated with an increased rate of cell proliferation induced by exposure to low concentrations of this compound. In contrast, only the highest tested concentration (20 µg/ml for 72 h) of BPA induced a significant increase in γH2AX foci in HepG2 cells (Hercog et al., 2019 [RefID 287-G], 2020 [RefID 288-G]). This increase was accompanied by changes in the expression of some genes involved in xenobiotic metabolism and response to oxidative stress (but no changes in any of the genes involved in the DNA damage response DDR), although the relevance of these changes is uncertain.

Mahemuti et al. (2018) [RefID 11171] examined γH2AX foci and changes in global gene expression induced by BPA in cultured human foetal lung fibroblasts. BPA (100 µM) increased DNA DSBs as shown by induction of γH2AX foci. Activated ATM signalling (increased phosphorylation of p53) resulted in increased cell cycle arrest at G1 phase accompanied by senescence, autophagy and decreased cell proliferation. Finally, BPA increases cellular ROS and activates the Nrf2-regulated stress response and xenobiotic detoxification pathways. The unclear levels of toxicity in this study does not allow any clear conclusion to be reached (Mahemuti et al., 2018 [RefID 11171]).

The quality of some of the studies (including undefined levels of toxicity) precludes any evaluation of the relevance of the findings reported by George and Rupasinghe (2018) [RefID 277-G], Yin et al. (2020) [RefID 474-G], Escarda-Castro et al. (2021) [RefID 266-G] and Yuan et al. (2021) [RefID 477-G].

In conclusion, γH2AX foci are induced by BPA exposure, both at low and high concentrations. These foci may however arise from different mechanisms (increased cell proliferation with concurrent ROS production as well as DSB associated with toxic events).

5c. Oxidized DNA bases

A significant increase in DNA 8-OHdG was observed in sperm exposed to high concentrations of BPA (Barbonetti et al., 2016 [RefID 419]). The significance of changes in oxidative DNA damage in the extreme experimental conditions of sperm with no motility and/or viability is, however, questionable.

In a short report published in conference proceedings, 8-OHdG was produced *in vitro* by reacting dG with BPA in the presence of the Fenton reagent. The authors conclude that BPA may act as a prooxidant

(Budiawan et al., 2018 [RefID 757-G]) although the very low levels of 8-OHdG formation (42.26 ppb, analyzed by HPLC/EC) raise questions about the relevance of this short report.

No convincing evidence of BPA induction of oxidized DNA base(s) is available.

5d. Analysis of mutations by whole genome sequencing (WGS)

In comparison with untreated controls, increased levels of single and double base substitutions, small insertion/deletions and structural variants (mainly translocations) were identified by WGS in human embryonic HEK 293T kidney cells following exposure to 100 μM BPA (Hu et al., 2021 [RefID 295-G]). In these experimental conditions, a large increase of γH2AX foci was also reported. Increased levels of GC>TA transversions and mutations at A:T base pairs were reported in three clonally amplified BPA-treated cell populations in comparison with one clonally amplified control cell line, which could suggest a mutagenic potential

This study represents a novel approach to study mutations at genome level. Although this is a very powerful method able to identify also very weak mutagens, the novelty of the techniques, the lack of information on the reliability of the methodology and the relatively small number of clonal cell lines analysed do not allow firm conclusions to be reached on the mutagenic potential of BPA.

5e. Changes in gene expression and DNA methylation

Changes in DNA methylation have been investigated in several studies (De Felice et al, 2015 [RefID 1462]; Porreca et al., 2016 [RefID 5892]; Karmakar et al., 2017 [RefID 3388]; Karaman et al., 2019 [RefID 269-G]). No specific discussion on DNA repair or DDR genes is reported in these publications.

None of the information present in these studies is relevant for the clarification of the genotoxic potential of BPA.

5f. Studies in humans

A few studies have investigated the association between environmental or occupational BPA exposure, measured by urinary BPA levels, and makers of oxidative DNA damage and/or oxidative stress in urine (Huang YF et al., 2017b [RefID 2962]; Lv YS et al., 2017 [RefID 4702]; Rocha et al., 2018 [RefID 402-G]), sperm DNA damage (Omran et al., 2018 [RefID 373-G]; Radwan et al., 2018 [RefID 390-G]; Kiwitt-Cárdenas et al., 2021 [RefID 321-G]) and sperm aneuploidy (Radwan et al., 2018 [RefID 390-G]) in humans. Mixed results were reported for BPA and urinary 8-OHdG (weak association in Lv YS et al., 2017 [RefID 4702], no or doubtful association in Huang YF et al., 2017b [RefID 2962] and Rocha et al., 2018 [RefID 402-G]) or sperm DNA damage (positive in Omran et al., 2018 [RefID 373-G], equivocal in Kiwitt-Cárdenas et al., 2021 [RefID 321-G] and negative in Radwan et al., 2018 [RefID 390-G]). However, all studies were considered Inadequate to establish a causal association between the markers analyzed and BPA exposure, because of their observational nature and/or the low number of subjects recruited and/or the Inadequate control of confounders.

Overall, human studies are not considered to provide additional relevant information for the evaluation of BPA genotoxicity.

6. Mode of action

BPA did not induce gene mutations in bacteria. All the available *in vitro* studies on chromosomal damage, classified as of high or limited relevance, reported positive results such as increase of CA or MN frequency, in different cellular systems. The increases of BPA-induced chromatid and chromosome breaks observed in some studies (Xin et al., 2015 [RefID 8150]; Santovito et al., 2018 [RefID 11220]; Di Pietro et al., 2020 [RefID 258-G]) in association with the induction of DNA strand breaks, detected by comet assay (Xin et al., 2015 [RefID 8150]) are consistent with a clastogenic activity. Moreover, the

potential of BPA to affect the spindle integrity and interfere with the chromosome segregation machinery was demonstrated in some reliable studies. Johnson and Parry (2008) reported the formation of aberrant mitotic spindles, with multiple poles, in V79 cells treated with BPA. Altered cytoskeleton organization, with multipolar spindles, failure of microtubule attachment to the kinetochore with the concomitant activation of SAC and chromosome misalignment, were also observed in HeLa cells (Kim et al., 2019 [RefID 319-G]). Studies on spindle morphology of mouse (Yang et al., 2020 [RefID 469-G]) and bovine (Campen et al., 2018 [RefID 240-G]) oocytes during *in vitro* maturation reported a pattern of alterations similar to that observed in permanent cell lines, namely shorter and multipolar spindles, with altered kinetochore-microtubule attachment and chromosome misalignment at M II.

The conclusion, based on these *in vitro* studies, is that BPA may act by both clastogenic and aneugenic mechanisms.

The large majority (11 out of 12) of the *in vitro* studies on comet assay, classified as of limited relevance, reported BPA-induced increases of DNA strand breaks. In some studies, the increase of DNA damage was associated with a parallel increase of ROS and MDA and decrease in antioxidant capacity and in total GSH (Xin et al., 2014 [RefID 8147]; Li XH et al., 2017 [RefID 4176]; Huang FM et al., 2018 [RefID 296-G]; Yuan et al., 2019 [RefID 478-G]; Kose et al., 2020 [RefID 325-G]). A study in macrophages reported also a release of cytochrome *c* from mitochondria along with increased apoptosis with the indication that the DNA strand breaks could be mainly through the oxidative stress-associated mitochondrial apoptotic pathway (Huang FM et al., 2018 [RefID 296-G]). This mechanism was reported also in studies on liver effects (see Chapter 3.1.2.4). In a study on human PBMC, the application of comet assay with the addition of endonuclease III (Nth) and 8-oxoguanine DNA glycosylase (hOGG1) DNA repair enzymes allowed the detection of oxidative damage to DNA bases (Mokra et al., 2018 [RefID 364-G]). Further indication of the role of oxidative damage in induction of DNA strand breaks was provided by the protective effects on DNA damage induced by the pre-treatment with NAC (Xin et al., 2014 [RefID 8147]; Huang FM et al., 2018 [RefID 296-G]).

In conclusion, the evidence of DNA strand breaks *in vitro* is in agreement with the ability of BPA to induce clastogenic damage. In addition, the studies on comet assay provide consistent evidence that BPA induces DNA strand breaks most probably related to the induction of oxidative stress.

The available *in vivo* studies for BPA-induced chromosomal damage in somatic cells reported mixed results. No increase of CA and MN frequency was reported after a single administration of BPA to mice in a range of doses inducing toxicity at the bone marrow level (Naik and Vijayalaxmi, 2009). In contrast, in another study in mice, increased MN frequency was detected in the presence of high bone marrow toxicity (Fawzy et al., 2018 [RefID 270-G]). Positive results were observed in two rat studies (Tiwari et al., 2012; Panpatil et al., 2020 [RefID 379-G]) after repeated dose administration, possibly associated with lipid peroxidation and oxidative stress in the first study. No induction of hyperploidy or polyploidy was observed in these studies.

These results indicate that the *in vivo* induction of chromosomal damage requires specific conditions such as repeated exposure to BPA.

Induction of DNA strand breaks, detected by comet assay *in vivo*, was observed only after repeated exposure for extensive periods of time up to 8 weeks (Tiwari et al., 2012; Zhou YX et al., 2017 [RefID 9083]; Panpatil et al., 2020 [RefID 379-G]). Only one study of high relevance was available on single administration of BPA reporting negative results in multiple mouse organs in a range of doses up to the MTD of 500 mg/kg bw (Sharma et al., 2018 [RefID 662-G]). An indication of a possible role of the oxidative stress in inducing DNA strand breaks by BPA was provided by the results of several studies (Abdel-Rahman et al., 2018 [RefID 199-G]; Fawzy et al., 2018 [RefID 270-G]; Kazmi et al., 2018 [RefID 315-G]; Majid et al., 2019 [RefID 354-G]; Mohammed et al., 2020 [RefID 363-G]) showing the

protective effects of natural extracts with antioxidant properties. However, these studies were evaluated as low relevance.

Finally, studies on germ cells, carried out by four laboratories in the framework of a collaborative project on aneugenic chemicals, did not provide any evidence of increased frequency of aneuploidy in mouse oocytes and zygotes and in sperm cells following exposure to low BPA doses (Pacchierotti et al., 2008).

BPA is genotoxic *in vitro* inducing chromosomal damage and DNA breaks. However, *in vivo* the evidence of genotoxic properties of BPA is contradictory. This might depend on multiple mechanisms of action described or proposed for BPA. A major difficulty in the interpretation of these contradictory results is the lack of knowledge on the role of BPA metabolism that could be operational in genotoxic activity. Indeed, the role of the proposed DNA adducts has not been clarified. Other uncertainties include the role of ER receptors in the oxidative stress induced by BPA.

3.1.9.2. Conclusion on hazard identification for Genotoxicity effects of BPA

In 2015, the CEF Panel concluded that:

> The available data support that BPA is not mutagenic (in bacteria or mammalian cells), or clastogenic (MN and CAs). 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.*

Based on the scientific literature considered in the previous EFSA opinions and published thereafter until 21 July 2021, the CEP Panel concluded that:

- BPA does not induce gene mutations in bacteria;
- BPA induces DNA strand breaks, clastogenic and aneugenic effects in mammalian cells *in vitro*;
- oxidative stress related mechanism(s) are likely to be involved in the DNA damaging and clastogenic activity elicited by BPA *in vitro*;
- there is some evidence for DNA and chromosomal damaging activities of BPA *in vivo* following repeated administrations, but not following single administrations;
- the available studies do not provide evidence of aneugenicity of BPA in germ cells *in vivo*.

In contrast with consistent positive *in vitro* findings, the *in vivo* findings in several studies with high/limited reliability were inconsistent. The CEP Panel concluded that the evidence does not support an *in vivo* genotoxic hazard posed by BPA through direct interaction with DNA.

3.1.9.3 Uncertainty analysis for genotoxicity

The purpose of the uncertainty analysis for genotoxicity was to assess the degree of certainty for the conclusion on whether BPA presents a genotoxic hazard by a direct mechanism (direct interaction with DNA), taking into account the available evidence and also the associated uncertainties. This overall question was divided into two sub-questions, which were assessed by three WG members with specialist expertise in genotoxicity assessment:

Sub-question 1: What is your probability (%) that there is a genotoxic hazard in humans from BPA?

Sub-question 2: If there would be a genotoxic hazard in humans from BPA, what is your probability that its causes include a direct mechanism?

The experts' judgements were elicited by the structured procedure described in Chapter 2.3.4.2. When assessing the two sub-questions, the experts considered all the data they had reviewed for the genotoxicity assessment, including results from *in vitro* studies and animal models, taking into account

their relevance to humans; the available data from human studies were considered not relevant (see Chapter 3.1.9.1, Paragraph 5f).

Table 17 shows the revised judgements provided by the three experts together after sharing and discussing their initial judgements and reasoning. The third row of Table 17 shows their probabilities for the overall question, which were obtained by multiplying each expert's probabilities for the two sub-questions, as explained in Chapter 2.3.4.2. These are their probabilities that BPA does present a genotoxic hazard and that there is a direct mechanism. The bottom row of Table 17 shows the complement of the probabilities in the third row, obtained by subtracting each probability from 100%. These are the experts' probabilities for the opposite outcome: that BPA does *not* present a genotoxic hazard by a direct mechanism. The fifth column of Table 17 shows the 'envelope' of the probabilities for the three experts, obtained by taking the lowest and highest probabilities in each row. These express the range of opinion across the three experts.

Table 17: Results of the uncertainty analysis for the genotoxicity assessment.

	Expert A	Expert B	Expert C	Envelope of the three experts	Assessment (rounded values) *
Experts' probabilities that BPA presents a genotoxic hazard in humans (sub-question 1)	70 – 90%	66 – 90%	70 – 90%	66 – 90%	66 – 90%
Experts' probabilities that, if BPA is genotoxic, there is a direct mechanism (sub-question 2)	10 – 33%	10 – 33%	20 – 30%	10 – 33%	10 – 33%
Calculated probabilities that BPA is genotoxic by a direct mechanism (sub-question 1 x sub-question 2)	7 – 29.7%	6.6 – 29.7%	14 – 27%	6.6 – 29.7%	5 – 30%
Calculated probabilities that BPA is not genotoxic by a direct mechanism (100% minus row above)	70.3 – 93%	70.3 –93.4%	73 – 86%	70.3 – 93.4%	70 – 95%

*The calculated probabilities were rounded to the nearest 5%. The experts' probabilities of 33% and 66% were not changed because they correspond approximately to a 1 in 3 chance and a 2 in 3 chance, respectively.

The results in Table 17 and the reasoning of the three experts were presented and discussed in detail at a facilitated meeting with the full WG. It was agreed to take the envelope of the 3 experts' results as the consensus of the WG, taking account of the available evidence and associated uncertainties. The WG also agreed that their consensus probability that BPA is genotoxic by a direct mechanism should be rounded to 5 – 30%, as shown in the right-hand column of Table 17, to take account that it is based on expert judgement and avoid the implied precision of the calculated values. Similarly, the WG rounded their consensus probability that BPA is not genotoxic by a direct mechanism to 70 – 95%.

The width of the consensus probability range for BPA not being genotoxic by a direct mechanism, reflects the uncertainty of the three experts and the other WG members about the judgements on sub-questions 1 and 2. The WG discussed in more detail which lines of evidence tended to support probabilities in the lower end of this range, and which tended to support the upper end of the range (Table 18).

Table 18: Summary of lines of evidence supporting either lower or higher probabilities that BPA does not present a genotoxic hazard by a direct mechanism, within the range assessed by the WG (70-95%).

Evidence supporting probabilities closer to 95%	• Consistent negative Ames tests

	• Indications of carcinogenic effects of BPA do not indicate direct genotoxic mechanism because only at very low doses and not higher doses (non-monotonic), only after developmental exposure (up to weaning) and only in one target tissue • Reactive non-conjugated metabolites of BPA are observed in animals but not in humans • Effects only from repeated exposures, so might be secondary • Evidence for several indirect mechanisms
Evidence supporting probabilities closer to 70%	• Presence of uncharacterised DNA adducts • Mutational spectrum from whole genome assessment

It was concluded that it is Unlikely to Very Unlikely (5 – 30% probability) that BPA presents a genotoxic hazard, the causes of which include a direct mechanism (combining subquestion 1 and 2, see third row of Table 17). Accordingly, it was concluded that it is Likely to Very Likely (70 - 95% probability) that BPA either presents a genotoxic hazard only through indirect mechanism(s) or is not genotoxic. The likelihood terms used in these conclusions are taken from the approximate probability scale, which is recommended by EFSA (Table 2 in EFSA Scientific Committee, 2018a) for harmonised use in EFSA assessments.

EFSA Scientific Committee (2017c) has advised that, where the overall evaluation of genotoxicity for a substance leaves no concerns for genotoxicity, HBGVs may be established. However, if concerns for genotoxicity remain, establishing a HBGV is not considered appropriate and a Margin of Exposure (MoE) approach should be followed.

Considering the WoE for probabilities closer to either 70% or 95% that BPA does not present a genotoxic hazard by a direct mechanism (Table 18), the CEP Panel concluded that probabilities close to 95% are more strongly supported by the evidence than probabilities close to 70% and, therefore, the balance of evidence allows a HBGV to be established.

3.2. Hazard characterisation

3.2.1 Dose–response modelling

The CEP Panel used BMD analysis for dose-response modelling and performed it in accordance with the updated guidance of the Scientific Committee on BMD modelling (EFSA Scientific Committee, 2017a), using the R-package PROAST as made available in the EFSA BMD online web-tool. The detailed reports of the BMD analyses are presented in Annex I, including the rationale for the selection of the BMRs. A summary of the outcome of the analyses is shown in Table 19.

The approach described in the guidance on BMD modelling includes criteria to evaluate whether a dose-response relationship is present. However, when the data are relatively poor or uninformative, the resulting BMD confidence interval (CI) will tend to be wide, and the BMDL might be much lower than the true BMD. It might happen that the data are so poor that using the associated BMDL as a potential RP appears unwarranted. This might be concluded when the BMD CI is wide or when different models result in widely different BMDL values (EFSA Scientific Committee, 2017a). The current EFSA guidance does not provide guidance on how to deal with poor data sets.

It was noted that a cut-off value of 10 for the ratio of the lowest non-zero dose and the BMDL is used in the BMDS software to discard datasets for BMD analysis (US EPA, 2020). In the absence of EFSA guidance, the CEP Panel decided to apply this cut-off value to bring a study forward for selection of the RP. If this criterion is not fulfilled, the BMDL is extrapolated too far outside the dose range and the study design is considered not adequate to evaluate the relevant effect sizes. However, the study will be considered in the uncertainty analysis.

BMD analysis was performed using the BPA administered doses without conversion to HED. HED converted values will be used subsequently to compare the different modelling outcomes.

3.2.1.1 Immunotoxicity

Using a WoE approach, the CEP Panel assigned a likelihood level of Likely to BPA induced effects on the Th17 cells (Luo et al., 2016 [RefID 4679]), on the neutrophils in epididymis (Ogo et al., 2018 [RefID 11201]) and on eosinophils in the BAL (Tajiki-Nishino et al., 2018 [RefID 13221]) and of Very Likely to BPA-induced effects on serum OVA-specific IgE (O'Brien et al., 2014a [RefID 5462]). Therefore, these endpoints were brought forward for BMD analysis; the CEP Panel noted that the datasets on neutrophils in epididymis (Ogo et al., 2018 [RefID 11201]) and eosinophils in the BAL (Tajiki-Nishino et al., 2018 [RefID 13221]) included only two dose groups and a control group.

Due to a litter effect in the dataset on the effect of BPA on serum OVA-specific IgE (O'Brien et al., 2014a [RefID 5462]) and the lack of information to take the litter effect into account in the BMD analysis, no BMD analysis was performed on this endpoint.

For the effect of BPA on Th17 cells in mice, the lowest $BMDL_{20}$–$BMDU_{20}$ CI (0.06–0.74 µg/kg bw per day) was observed in females at PND21; at PND42 the $BMDL_{20}$–$BMDU_{20}$ CI in females was 0.17–1.79 µg/kg bw per day. In males, similar $BMDL_{20}$–$BMDU_{20}$ CI were observed for both time points of measurement (0.30–3.39 µg/kg bw per day for PND21 and 0.35–3.38 µg/kg bw per day for PND42).

For the effect of BPA on the neutrophils in the epididymis, a $BMDL_{20}$–$BMDU_{20}$ CI of 6.8–90.4 µg/kg bw per day was calculated for the caput/corpus while for the cauda, the $BMDL_{20}$ (0.5 µg/kg bw per day) was too far outside the tested dose-range to be taken forward for identifying the RP. Also the $BMDL_{20}$ (0.00046 µg/kg bw per day) calculated for the effect on eosinophils in the BAL was too far outside the tested dose range to be taken forward for identifying the RP.

3.2.1.2 Metabolic effects

The CEP Panel considered that the available evidence showed a Likely effect of BPA for the endpoint uric acid. Therefore, the endpoints hepatic and serum uric acid concentrations reported by Ma et al. (2018) [RefID 12637] were taken forward for BMD analysis (see Table 19 and Annex I for further details).

For the effect of BPA on serum uric acid in C57BL6 mice, none of the fitted models was better than the null model, indicating that there is no observable trend. For the effect of BPA on serum uric acid in CD1 mice, the $BMDL_{20}$ (0.39 µg/kg bw per day) was too far outside the tested dose-range to be used for identifying the RP. For the effect on hepatic uric acid a $BMDL_{20}$–$BMDU_{20}$ CI of 1.59–399 µg/kg bw per day was calculated; the Panel noted the wide CI. However, as explained above, the EFSA guidance does not provide guidance on how to deal with poor data sets.

3.2.1.3 Neurotoxicity and developmental neurotoxicity

The CEP Panel considered that the available evidence showed a Likely effect of BPA for the following endpoints:

- anxiety/emotionality (Xu XH et al., 2015 [RefID 8232]; Chen Z et al., 2018 [RefID 11734]; Liang et al., 2018 [RefID 12508]; Xin et al., 2018 [RefID 13482]);
- learning and memory (Wang C et al., 2014 [RefID 7579]; Johnson et al., 2016 [RefID 3241]; Wang C et al., 2016 [RefID 7576]; Zhou YX et al., 2017 [RefID 9083]; Chen Z et al., 2018 [RefID 11734]);
- male sexual behaviour (Picot et al., [RefID 5830]);
- salt preference (Nuñez et al., 2018 [RefID 11199]);
- dendritic spine density (Liu ZH et al., 2014 [RefID 10411]; Kimura et al., 2016 [RefID 3566]; Chen Z et al., 2018 [RefID 11734]);
- number of neurons in hippocampus (CA1 and CA3 areas) (Zhou YX et al., 2017 [RefID 9083]);
- AChE activity (Khadrawy et al., 2016 [RefID 3462]).

Therefore, these endpoints were taken forward for BMD analysis; the CEP Panel noted that the datasets on anxiety/emotionality (Xu XH et al., 2015 [RefID 8232]), dendritic spine density (Kimura et al., 2016 [RefID 3566]) and AChE activity (Khadrawy et al., 2016 [RefID 3462]) included only two dose groups and a control group.

Due to lack of information on litter effects in the learning and memory studies (Wang C et al., 2014 [RefID 7579] and 2016 [RefID 7576]), no BMD analyses were performed.

No observable trend was noted for the endpoints number of neurons in hippocampus (CA1 and CA3 areas) (Zhou YX et al., 2017 [RefID 9083]), learning and memory (Zhou YX et al., 2017 [RefID 9083]), salt preference (Nuñez et al., 2018 [RefID 11199]) and anxiety/emotionality (immobility) in females (Xu XH et al., 2015 [RefID 8232]) (see Table 19 and Annex I for further details).

For anxiety/emotionality, the $BMDL_{50}$ calculated from the dataset reported by Xin et al. (2018) [RefID 13482] was not further considered due to the large uncertainty as illustrated by the large $BMDL_{50}$-$BMDU_{50}$ CI interval (6.1 to 1.06e+14 µg/kg bw per day). The $BMDL_{50}$ calculated from the data reported by Chen Z et al. (2018) [RefID 11734] was not further considered based on the ratio of the lowest non-zero dose and the BMDL. Xu XH et al. (2015) [RefID 8232] studied immobility and the time in open arms. A $BMDL_{50}$–$BMDU_{50}$ CI of 497-80400 µg/kg bw per day was calculated for males; the Panel noted the wide CI. The other $BMDL_{50}$ values, calculated from the data reported by Xu XH et al. (2015) [RefID 8232], were too far outside the tested dose-range to be taken forward for identifying the RP.

For the effect of BPA on learning and memory as studied by Johnson et al. (2016) [RefID 3241], a $BMDL_{50}$–$BMDU_{50}$ CI of 10.10–2160 µg/kg bw per day was calculated for females and 1.47–1520 µg/kg bw per day for males; the Panel noted the wide CIs. Chen Z et al. (2018) [RefID 11734] studied several endpoints in relation to this cluster. For the relative expression of NR2 in V1, a $BMDL_{20}$–$BMDU_{20}$ CI of 7.96–842 µg/kg bw per day was calculated and for platform duration a $BMDL_{50}$–$BMDU_{50}$ of 10700–2.4e+7 µg/kg bw per day; the Panel noted the wide CIs. The BMDL values for the relative expression of NR2 and GluR1 in the hippocampus were too far outside the tested dose-range to be taken forward for identifying the RP.

For dendritic spine density a $BMDL_{20}$–$BMDU_{20}$ CI of 4.24–2350 µg/kg bw per day was estimated from the dataset reported by Chen Z et al. (2018) [RefID 11734]; the Panel noted the wide CI. For the same endpoint, a $BMDL_{20}$–$BMDU_{20}$ CI of 16,800–70,100 µg/kg bw per day was calculated from the dataset reported by Liu ZH et al. (2014) [RefID 10411]; the Panel noted that the latter CI was completely outside the dose range. The $BMDL_{20}$ value calculated from the data reported by Kimura et al. (2016) [RefID 3566] was not considered further based on the ratio of the lowest non-zero dose and the BMDL. The same applied to the BMDL values calculated for AChE activity.

3.2.1.4 Reproductive and developmental toxicity

The CEP Panel considered that the available evidence showed a Likely effect of BPA for the following endpoints:

- ovary weight (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]);
- uterus histology (Vigezzi et al., 2015 [RefID 7472]; NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]);
- ovary histology (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370] during the developmental exposure period; NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370] during developmental and adult exposure; Hu et al., 2018 [RefID 11119] during adult exposure);
- decreased implantation incidence (Li et al., 2016 [RefID 4128]);
- epididymis histology (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]);
- testis histology (Gurmeet et al., 2014 [RefID 2502]);
- effects on sperm (Wang HF et al., 2016 [RefID 7618]).

Therefore, these endpoints were taken forward for BMD analysis; the CEP Panel noted that the dataset on uterus histology (Vigezzi et al., 2015 [RefID 7472]) included only two dose groups and a control group.

The dataset on decreased implantation incidence (Li et al., 2016 [RefID 4128]) could not be used for BMD analysis as the actual number of animals per dose group was not reported.

Within the cluster female reproductive toxicity, no observable trend was noted for the endpoints uterus histology during developmental exposure and ovary histology during developmental and adult exposure reported by NTP Clarity Report (2018)/Camacho et al. (2019) [RefID 11370].

A $BMDL_{05}$–$BMDU_{05}$ CI of 0.63–25000 µg/kg bw per day was calculated for ovary weight (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]) and a $BMDL_{10}$–$BMDU_{10}$ CI of 5.53–3680 µg/kg bw per day for ovary histology during developmental exposure period reported by NTP Clarity Report (2018)/Camacho et al. (2019) [RefID 11370]. A $BMDL_{05}$–$BMDU_{05}$ CI of 0.96-349 µg/kg bw per day was calculated for the ratio of primordial and total follicles as reported by Hu et al. (2018) [RefID 11119]. The Panel noted the wide CIs for these data sets. For the other datasets, the BMDL values were too far outside the tested dose-range to be taken forward for identifying the RP.

Within the cluster male reproductive toxicity, no observable trend was noted for the incidence of inflammation in the epididymis as reported by NTP Clarity Report (2018)/Camacho et al. (2019) [RefID 11370] and for testis histology as reported by Gurmeet et al. (2014) [RefID 2502]. For the incidence of exfoliated germ cells as reported by NTP Clarity Report (2018)/Camacho et al. (2019) [RefID 11370], a $BMDL_{10}$–$BMDU_{10}$ CI of 2,260–27,500 µg/kg bw per day was estimated. $BMDL_{20}$–$BMDU_{20}$ CIs of 26.1–2460 and 3.41–74.8 µg/kg bw per day were calculated for sperm viability and motility, respectively (Wang HF et al., 2016 [RefID 7618]). For the effect of BPA on AR, the BMDL value was too far outside the tested dose-range to be taken forward for identifying the RP.

3.2.1.5 Carcinogenicity and mammary gland proliferative effects

Overall, the CEP Panel did not assign a likelihood level of Likely to the pre-neoplastic and neoplastic histological changes. However, the Panel assigned a likelihood level of Likely to the following non-neoplastic uterine histological effects of BPA: gland cell anomalies (Vigezzi et al., 2015 [RefID 7472]) and endometrial cystic hyperplasia (NTP Clarity Report, 2018/Camacho et al., 2019 [RefID 11370]). Therefore, these endpoints were taken forward for BMD analysis. The outcome of the BMD analysis on these endpoints is reported in Chapter 3.2.1.4.

3.2.2 Identification of the reference point

All BMDL values identified for the selection of the RP were converted to HED (Table 20). The CEP Panel selected the lowest BMDL value (expressed as HED) of 0.93 ng/kg bw per day for the effect of BPA on Th17 cells in mice to be used as RP for the risk assessment of BPA.

The BMD analysis from which the RP was selected is repeated in Appendix F.

11979 **Table 19:** Overview of BMD analyses.

Reference	Endpoint	Species	Dose range[(b)] (µg/kg bw per day)	BMR	Group	BMDL (µg/kg bw per day)	BMDU (µg/kg bw per day)	Ratio dose /BMDL[(d)]
Immunotoxicity								
Luo et al. (2016) [RefID 4679]	Th17 cells	Mouse	0.475–47.5	20%	F PND21	0.06	0.74	**7.9**
		Mouse	0.475–47.5	20%	F PND42	0.17	1.79	**2.8**
		Mouse	0.475–47.5	20%	M PND21	0.30	3.39	**1.6**
		Mouse	0.475–47.5	20%	M PND42	0.35	3.38	**1.4**
Ogo et al. (2018) [RefID 11201]	Neutrophils in epididymis: Caput/corpus	Rat	20–200	20%		6.8	90.4	**2.9**
	Neutrophils in epididymis: cauda	Rat	20–200	20%		0.5	4.62	40
Tajiki-Nishino et al. (2018) [RefID 13221]	Eosinophil infiltration in BAL fluid	Mouse	60–200	20%		0.00046	34.5	130434.8
Metabolic effects								
Ma et al. (2018) [RefID 12637]	Hepatic uric acid concentration	Mouse	5–500	20%		1.59	399	**3.1**
	Serum uric acid concentration	Mouse	5–500	20%	CD1 mice	0.39	91.5	12.8
		Mouse	5–500	20%	C57BL6 mice	None of the fitted models is better than the null model[(a)]		
Neurotoxicity- and developmental neurotoxicity								
Xin et al. (2018) [RefID 13482]	Time spent immobile in the forced swim test (Anxiety/emotionality)	Mouse	10–10000	50%		6.1	1.06e+14	1.6
Johnson et al. (2016) [RefID 3241]	Sniffing incorrect holes on day 7 (learning and memory)	Rat	2.5–2500	50%	F	10.10	2160	**0.2**
		Rat	2.5–2500	50%	M	1.47	1520	**1.7**
Chen Z et al. (2018) [RefID 11734]	First entry time in the open field test (Anxiety/emotionality).	Rat	40–4000	50%		0.03	517	1333.3
	Platform duration (learning and memory)	Rat	40–4000	50%		10700	2.4e+7	**0.004**

	Relative expression NR2 in the hippocampus (learning and memory)	Rat	40-4000	20%		2.18	439	18.3
	Relative expression GluR1 in the hippocampus (learning and memory)	Rat	40-4000	20%		0.31	2410	129.0
	Relative expression NR2 in V1 (learning and memory)	Rat	40-4000	20%		7.96	842	**5.0**
Zhou YX et al. (2017) [RefID 9083]	Quantity of hippocampal CA1 neurons	Mouse	0.5–500	20%		None of the fitted models is better than the null model(a)		
	Quantity of hippocampal CA3 neurons	Mouse	0.5–500	20%		None of the fitted models is better than the null model(a)		
	Trials to qualify for the standard (learning and memory)	Mouse	0.5–500	50%		None of the fitted models is better than the null model(a)		
Xu XH et al. (2015) [RefID 8232]	Time in open arms (Anxiety/emotionality)	Mouse	40–40,000	50%	F	1.71	1260	23.4
		Mouse	40–40000	50%	M	497	80,400	**0.1**
	Immobility (Anxiety/emotionality)	Mouse	40–40000	50%	F	None of the fitted models is better than the null model(a)		
		Mouse	40–40000	50%	M	0.03	89.4	1333.3
Nuñez et al. (2018) [RefID 11199]	Salt preference	Rat	10–500	10%		None of the fitted models is better than the null model(a)		
Kimura et al. (2016) [RefID 3566]	Dendritic spine density	Mouse	40–400	20%		2.43	58.5	16.5
Liu ZH et al. (2014) [RefID 10411]	Dendritic spine density	Rats	918–9175	20%		16800	70100	**0.1**
Chen Z et al. (2018) [RefID 11734]	Dendritic spine density	Rat	40–4000	20%		4.24	2350	**9.4**
Khadrawy et al. (2016) [RefID 3462]	AChE activity in the cortex	Rat	10000–25000	20%		570	20700	17.5
	AChE activity in the hippocampus	Rat	10000–25000	20%		2.7	6330	3703.7
Reproductive and developmental toxicity								
Carcinogenicity and mammary gland proliferative effects [c]								

Camacho et al., 2019 [RefID 11370] [(e)]	Ovary weight	Rat	2.5–25000	5%	0.63	25000	**4.0**
Camacho et al., 2019 [RefID 11370] [(c)] [(e)]	Incidence of hyperplasia, cystic, endometrium (uterus histology)	Rat	2.5–25000	10%	None of the fitted models is better than the null model[(a)]		
	Incidence of squamous metaplasia (uterus histology)	Rat	2.5–25000	10%	None of the fitted models is better than the null model[(a)]		
Vigezzi et al. (2015) [RefID 7472] [(c)]	Incidence of glands with cellular anomalies (uterus histology)	Rat	0.5–50	10%	2.8e–05	8.34	17857.1
Camacho et al. (2019) [RefID 11370] [(e)]	Incidence of follicle cysts (ovary histology)	Rat	2.5–25000	10%	5.53	3680	**0.5**
Camacho et al. (2019) [RefID 11370] [(e)]	Incidence of interstitial cell hypertrophy (ovary histology)	Rat	2.5–25000	10%	None of the fitted models is better than the null model[(a)]		
Hu et al. (2018) [RefID 11119]	Ratio of primordial and primary follicles	Mouse	1–10000	5%	0.08	2.34	12.5
	Ratio of primordial and total follicles	Mouse	1–10000	5%	0.96	349	**1.0**
Camacho et al. (2019) [RefID 11370] [(e)]	Incidence of exfoliated germ cells (epididymis histology)	Rat	2.5–25000	10%	2260	27500	**0.001**
	Incidence of inflammation (epididymis histology)	Rat	2.5–25000	10%	None of the fitted models is better than the null model[(a)]		
Gurmeet et al. (2014) [RefID 2502]	Seminiferous tubule diameter (testis histology)	Rat	1000–100000	5%	None of the fitted models is better than the null model[(a)]		
Wang HF et al. (2016) [RefID 7618]	Viability (effects on sperm)	Mouse	10–250	20%	26.1	2460	**0.4**
	Motility (effects on sperm)	Mouse	10–250	20%	3.41	74.8	**2.9**
	AR (effects on sperm)	Mouse	10–250	20%	0.31	1230	32.3

AR: acrosome reaction; AChE: acetylcholinesterase; BMDL: lower confidence limit of the benchmark dose; BMDU: upper confidence limit of the benchmark dose; BMR: benchmark response; F: female; M: male; PND: post-natal day.

(a): All fitted models' Akaike information criterion (AIC) values are larger than null model's AIC – 2.

(b): Dose range of BPA treated animals; the dose of 0 µg/kg bw per day was included in all studies but not included in the presented dose range. All doses are expressed as oral.

(c): Studies with footnote (c) were identified for both HOCs; the other studies for Reproductive and developmental toxicity only.

(d): Ratio of the lowest non-zero dose and the BMDL; values below 10 are shown in bold.

(e): Full reference: NTP Clarity Report (2018)/Camacho et al. (2019).

11987 **Table 20:** Overview of BMD confidence intervals used for the identification of the Reference Point[(c)] to derive a HBGV.

Reference	Endpoint	Species	BMR	Group	Administered doses		Administered doses converted to HED[(b)]	
					BMDL (µg/kg bw per day)	BMDU	BMDL (ng/kg bw per day)	BMDU
Immunotoxicity								
Luo et al. (2016) [RefID 4679]	Th17 cells	Mouse	20%	F PND21	0.06	0.74	**0.93**	11.5
		Mouse	20%	F PND42	0.17	1.79	2.64	27.7
		Mouse	20%	M PND21	0.30	3.39	4.65	52.5
		Mouse	20%	M PND42	0.35	3.38	5.43	52.4
Ogo et al. (2018) [RefID 11201]	Neutrophils in epididymis: Caput/corpus	Rat	20%		6.8	90.4	1126	14970
Metabolic effects								
Ma et al. (2018) [RefID 12637]	Hepatic uric acid	Mouse	20%		1.59	399	24.6	6185
Neurotoxicity and developmental neurotoxicity								
Johnson et al. (2016) [RefID 3241]	Sniffing incorrect holes on day 7 (learning and memory)	Rat	50%	F	10.1	2160	1673	357696
		Rat	50%	M	1.47	1520	243	251712
Chen Z et al. (2018) [RefID 11734]	Platform duration (learning and memory)	Rat	50%		10700	2.4e+7	1.77e+6	4.02e+9
	Relative expression NR2 in V1 (learning and memory)	Rat	20%		7.96	842	1318	139435
Xu XH et al. (2015) [RefID 8232]	Time in open arms (Anxiety/emotionality)	Mouse	50%	M	497	80400	7704	1.25e+06
Liu ZH et al. (2014) [RefID 10411]	Dendritic spine density	Rat	20%		16800	70100	2.78e+06	1.16e+07
Chen Z et al. (2018) [RefID 11734]	Dendritic spine density	Rat	20%		4.24	2350	702	389160
Reproductive and developmental toxicity								
Camacho et al. (2019) [RefID 11370] [(e)]	Ovary weight	Rat	5%		0.63	25,000	104	4.14e+06
Camacho et al. (2019) [RefID 11370] [(e)]	Incidence of follicle cysts (ovary histology)	Rat	10%		5.53	3680	916	6.09e+05
Hu et al. (2018) [RefID 11119]	Ratio of primordial and total follicles	Mouse	5%		0.96	349	14.9	5410
Camacho et al. (2019) [RefID 11370] [(e)]	Incidence of exfoliated germ cells (epididymis histology)	Rat	10%		2260	27500	3.74e+05	4.55e+06
Wang HF et al. (2016) [RefID 7618]	Viability (effects on sperm)	Mouse	20%		26.1	2460	405	38130
	Motility (effects on sperm)	Mouse	20%		3.41	74.8	53	1159

11988 BMDL: lower confidence limit of the benchmark dose; BMDU: upper confidence limit of the benchmark dose; BMR: benchmark response; F: female; M: male; PND: post-natal day.
11989 (a): All fitted models' Akaike information criterion (AIC) values are larger than null model's AIC – 2.
11990 (b) For the calculation of the BMDL/BMDU values expressed as HED, a HEDF of 0.0155 was used for studies in mice and a HEDF of 0.1656 for studies in rats. The lowest BMDL value is shown in bold.
11991 (c) The confidence intervals are shown as administered and as the corresponding human equivalent dose (HED).
11992 (e): Full reference: NTP Clarity Report (2018)/Camacho et al. (2019).

3.2.3 Uncertainty in the hazard characterisation

The purpose of the uncertainty analysis was to assess whether other effects of BPA may potentially occur after exposure to lower doses than the endpoint on which the RP is based and, if so, inform a decision on what size of additional UF would be suitable to take those effects into account. This was carried out in the series of steps described in Chapter 2.3.4, where an overview of the approach is provided in Figure 1. The final outcome of the uncertainty analysis is reported in this section. Detailed results of the steps leading to this outcome are presented in Appendix D.

Five HOCs were considered at this phase of the uncertainty analysis: Immunotoxicity, Metabolic effects, Neurotoxicity and developmental neurotoxicity, Reproductive and developmental toxicity, and Carcinogenicity and mammary gland proliferative effects. Cardiotoxicity was not considered in the uncertainty analysis because the evidence was either judged as Not Likely or as Inadequate. The WG experts noted that no Likely or Very Likely clusters were identified for the HOC General toxicity, and the lowest effect level reported was 8 ng/kg bw per day (HED) for relative liver weight (Ke et al., 2016 [RefID 3447]; single dose study) with an effect size of about 4%, which was considered not adverse. Therefore this HOC was not considered further in the uncertainty analysis.

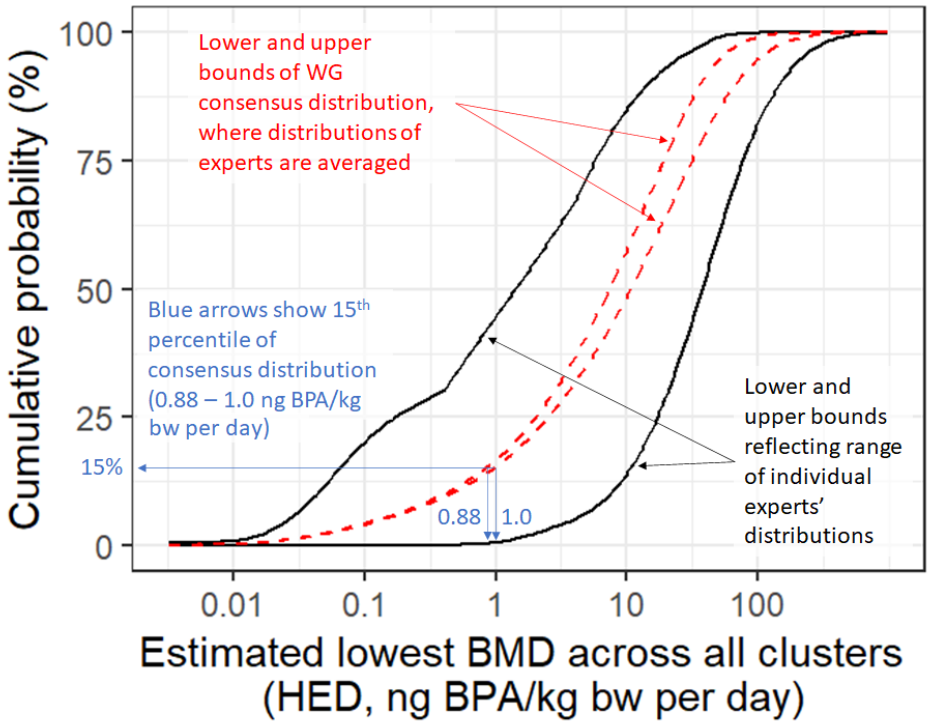
Within the other five HOCs, the uncertainty analysis focused on 21 clusters of endpoints that were rated ALAN, Likely or Very Likely in the WoE assessment. Uncertainty was assessed by quantitative expert judgements for each cluster (see Chapter 2.3.4), elicited from two or three experts per cluster. The expert's judgements were combined to produce a distribution quantifying the experts' uncertainty about the estimated lowest BMD in each cluster. The distributions for the 21 clusters were then combined to produce a distribution quantifying uncertainty about the estimated lowest BMD across all clusters, which is the distribution required to inform consideration of the need for an additional UF for deriving the TDI (illustrated by the pink distribution shown at the top of Figure 1 in Chapter 2.3.4).

Sensitivity analysis showed that the combined distribution was determined mainly by just one of the 21 clusters: allergic lung inflammation. Uncertainties affecting this cluster were therefore subjected to further assessment by the whole WG. After reviewing the judgements and reasoning of the two experts with specialist knowledge of this cluster and considering the opinions of other WG members, the WG agreed on a consensus probability of 66% that at least one of the endpoints in this cluster that occurs in animals, is both relevant and adverse for humans.

Distributions were elicited separately from 14 WG experts for the lowest BMD for an endpoint in the cluster allergic lung inflammation that occurs in animals and is both relevant and adverse for humans. These were then used to produce lower and upper bounds for a revised distribution quantifying uncertainty about the estimated lowest BMD across all clusters, which is shown by the black solid curves in Figure 7. The distance between the lower and upper bounds is mostly due to differences between experts in their assessment of the lowest BMD in each cluster, primarily for the cluster allergic lung inflammation where the experts' individual distributions ranged over five orders of magnitude.

The WG experts identified and discussed a list of additional uncertainties potentially affecting the hazard assessment (see Appendix D). The WG had already considered many of these when making their judgements earlier in the uncertainty analysis and judged that the additional uncertainties would not alter the assessment provided by the wide bounds of the distribution resulting from the judgements of different experts (black curves in Figure 7).

The distance between the lower and upper bounds was reduced when the distributions of different experts were combined by averaging for each cluster, as shown by the red dashed curves in Figure 7. The remaining distance between the red curves is due to the range of responses given by the experts when assessing, for each cluster other than allergic lung inflammation, their probability that at least one endpoint occurs in animals and is both relevant and adverse for humans. The WG experts agreed on the red dashed curves in Figure 7 as their consensus assessment of overall uncertainty for the estimated lowest BMD across all clusters that were rated ALAN, Likely or Very Likely in the WoE assessment (including General toxicity clusters, for the reasons given above).



The solid black curves show the lower and upper bounds of the distribution resulting from the range of judgements between WG experts. The red dashed curves show the lower and upper bounds of the consensus distribution of the WG, where judgements of different experts for each cluster were aggregated by averaging. Selected percentiles of the consensus distribution are shown in Table 21.

Figure 7: Lower and upper bounds for cumulative distributions quantifying uncertainty about the estimated lowest BMD across all 21 clusters considered in the uncertainty analysis.

As the final step in the uncertainty analysis the WG experts considered whether an additional UF is needed when deriving the TDI. To inform this, lower and upper bounds for different percentiles of the consensus distribution for the estimated lowest BMD across all clusters were considered (the red dashed curves in Figure 7), together with the ratios of the RP (BMDL) to each of those percentiles. These results are shown in Table 21.

12055 The WG experts noted from Table 21 that the RP of 0.93 ng BPA/kg bw per day is close to the 15th
12056 percentile of both the lower and upper bounds of the consensus cpf. This implies about 15% probability
12057 that the estimated lowest BMD for all clusters is lower than the RP and therefore 85% probability that the
12058 estimated lowest BMD for all clusters is above the RP.

12059 Accordingly, in the right-hand columns of Table 21, the ratio of the RP to the 15th percentile of both bounds
12060 of the cpf is close to 1. This implies about 85% probability that an additional UF of 1 (i.e. no additional UF)
12061 would be sufficient to cover all clusters of endpoints that were rated ALAN, Likely or Very Likely in the WoE
12062 assessment. When the calculations were repeated with the histograms provided by the experts, rather than
12063 fitted parametric distributions, the probability that the estimated lowest BMD for all clusters is below the
12064 RP increased slightly, to 87%.

12065 The WG experts also noted that the large range of endpoints tested for BPA makes the hazard assessment
12066 for BPA more conservative than for most other chemicals, where only the standard endpoints are tested.

12067 Taking all these considerations together, the CEP Panel concluded that no additional UF is needed.

12068 **Table 21:** Percentiles of the lower and upper bounds of the consensus distribution for the estimated
12069 lowest BMD across all clusters (columns 2 and 3) and ratios of the RP of 0.93 ng BPA/kg bw per day
12070 to each percentile (columns 4 and 5). The row shown in bold is the basis for the CEP Panel's
12071 conclusion that an additional UF is not needed. See text for details and Figure 7 for explanation of
12072 how percentiles were derived from the consensus distribution.

Percentile of consensus cpf	Lower bound for percentile (ng BPA/kg bw per day)	Upper bound for percentile (ng BPA/kg bw per day)	Ratio of reference point to lower bound	Ratio of reference point to upper bound
1%	0.028	0.028	33.8	32.7
2.5%	0.059	0.061	15.7	15.2
5%	0.135	0.141	6.9	6.6
10%	0.412	0.447	2.3	2.1
15%	**0.881**	**1.002**	**1.1**	**0.9**
20%	1.485	1.754	0.6	0.5
25%	2.182	2.673	0.4	0.3
30%	2.989	3.800	0.3	0.2
35%	3.931	5.171	0.2	0.2
40%	5.028	6.797	0.2	0.1
45%	6.293	8.705	0.1	0.1
50%	7.742	10.955	0.1	0.1
55%	9.406	13.591	0.1	0.1
60%	11.323	16.671	0.1	0.1
65%	13.501	20.438	0.1	0.0
70%	15.991	25.137	0.1	0.0

75%	19.054	31.067	0.0	0.0
80%	23.057	38.870	0.0	0.0
85%	28.472	49.854	0.0	0.0
90%	36.534	67.143	0.0	0.0
95%	51.747	101.729	0.0	0.0

12073

12074 ### 3.2.4 Derivation of a health-based guidance value (HBGV)

12075 Of all endpoints considered for the identification of a RP (Table 20), the CEP Panel noted that the effect of
12076 BPA on Th17 cells in mice (Luo et al., 2016 [RefID 4679]) was the most sensitive. A $BMDL_{20}$ corresponding
12077 to a HED of 0.93 ng/kg bw per day was derived from that study and used to establish a TDI. The CEP Panel
12078 did not apply the UF for inter-species variability in toxicokinetics because this was already taken into account
12079 by the conversion into HED. The remaining UF of 25 was applied to derive the HBGV, thus accounting for
12080 inter-species toxicodynamic difference (2.5) and intra-human variability in toxicokinetics and
12081 toxicodynamics (10).

12082 However, as dose-response analyses could not be performed for several endpoints (see Chapter 3.2.1), an
12083 uncertainty analysis was performed, using EKE, to identify if an additional UF would be needed. This was
12084 done by taking into considerations all other endpoints in clusters judged to be ALAN, Likely or Very Likely.
12085 After screening of all such endpoints, the study by O'Brien et al. (2014a) [RefID 5462], reporting effects
12086 on mast cell-mediated production of pro-inflammatory mediators and specific IgE in mice exposed pre-
12087 natally to BPA, was considered the most critical. All other endpoints in the other clusters were much less
12088 sensitive and judged to be of no influence for the uncertainty analysis.

12089 In the O'Brien study, effects on specific IgE were observed at the lowest dose tested (LOAEL) corresponding
12090 to 7.5 ng/kg bw per day, corresponding to a HED of 0.116 ng/kg bw per day. As such it could not be
12091 excluded, *a priori*, that if the O'Brien study had been conducted in such a way that a BMD analysis could
12092 have been performed, a lower RP might have been derived. The uncertainty analysis tried to address this,
12093 first by considering the probability that the findings in the O'Brien study would be adverse in humans and
12094 what the true BMD from the O'Brien study might have been if the study had been without any limitations
12095 or weaknesses. The relevance of the effect observed in the O'Brien study for humans was estimated to be
12096 66%. In addition, individual experts' judgments (EKE) on where the true BMD of that study would have
12097 been were scattered over two orders of magnitude. Based on that assessment, the WG's overall probability
12098 that no additional UF was needed was in the range 85 –87%.

12099 The CEP Panel concluded that no additional UF was needed and that a HBGV based on the identified RP is
12100 justified. Therefore, an UF of 25 was applied to the RP of 0.93 ng/kg bw per day for the effect of BPA on
12101 Th17 cells in mice (Luo et al., 2016 [RefID 4679]), resulting in a TDI of 0.04 ng/kg bw per day.

12102 ## 3.3. Risk characterisation

12103 The CEP Panel had to carry out an assessment of 'the risk to public health related to the presence of BPA
12104 in foodstuffs', in accordance with the Terms of Reference as provided by the requestor, without performing
12105 an updated exposure assessment. Therefore, the CEP Panel compared the newly derived TDI of 0.04 ng/kg
12106 bw per day with the dietary exposure estimates for BPA (see Table 22), taken from Table 20 in the 2015

EFSA opinion (EFSA CEF Panel, 2015), in which the data are provided for different age groups and subpopulations.

Table 22: Summary table on average (=mean) (A) and high (H) (=95th percentile) dietary exposure (µg/kg bw per day) to BPA in the different age groups of the general population (taken from EFSA CEF Panel, 2015).

Age group	Exposure level	Dietary exposure (µg/kg bw per day)	Dietary exposure/TDI (0.04 ng/kg bw)
		Oral (food and beverages)	
Infants 1–5 days (breastfed	A	0.225	5625
	H	0.435	10875
Infants 6 days – 3 months (breastfed)	A	0.165	4125
	H	0.6	15000
Infants 4–6 months (breastfed)	A	0.145	13625
	H	0.528	13200
Infants 0–6 months (formula fed)	A	0.03	**750**
	H	0.08	2000
Infants 6–12 months	A	0.375	9375
	H	0.857	**21425**
Toddlers 1–3 years	A	0.375	9375
	H	0.857	**21425**
Children 3–10 years	A	0.290	7250
	H	0.813	20325
Adolescents 10–18 years	A	0.159	3975
	H	0.381	9525
Women 18–45 years	A	0.132	3300
	H	0.388	9700
Men 18–45 years	A	0.126	3150
	H	0.335	8375
Other adults 45–65 years	A	0.126	3150
	H	0.341	8525
Elderly and very elderly, 65 years and over	A	0.116	2900
	H	0.375	9375

The comparison of the dietary exposure estimates with the TDI showed that both the mean and the 95th percentile dietary exposures in all age groups (including all infants and toddler groups) exceeded the TDI by two to four orders of magnitude.

The CEP Panel noted that a TDI based on the second most sensitive endpoint (ovarian follicle counts, Hu et al., 2018 [RefID 11119]) would also be exceeded by the dietary exposure by two to three orders of magnitude.

The CEP 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: (1) uncertainties in exposure (as discussed in the earlier EFSA opinion, EFSA CEF Panel 2015, in Section 4.7.2 in Part I-Exposure

assessment), and (2) uncertainties in the identification and characterisation of hazard (Chapter 3.2.3 of this scientific opinion). The CEP Panel noted that the use of exposure data presented in the 2015 assessment adds to the uncertainty. Following the 2015 opinion, the SML for the authorisation of BPA in plastic FCMs was revised at the EU level (from 0.6 mg/kg to 0.05 mg/kg), and the same SML of 0.05 mg/kg was introduced for varnishes and coatings. The ban on the use of BPA in the manufacture of PC baby bottles was extended to its use to manufacture sippy cups for infants and young children (= toddlers) and to the migration of BPA from varnishes and coatings applied to FCMs specifically intended to come into contact with foods for babies, infants and toddlers. The CEP Panel is aware that the exposure assessment presented in the 2015 opinion may therefore not fully represent the current dietary exposure.

Even considering this uncertainty, as the TDI was exceeded by two to four orders of magnitude for both mean and 95th percentile estimates of dietary exposures, the CEP Panel concluded that there is a health concern from dietary BPA exposure for all age groups of the general population.

4. Conclusions

4.1 Hazard identification

4.1.1 Toxicokinetics

- The studies in mice and rats did not contribute to a better understanding of toxicokinetic aspects of BPA. The studies in ewes showed that the absolute bioavailability was lower when BPA was given by nasogastric tubing compared with BPA administration via pellets. This finding is most probably explained by the buccal absorption of BPA.
- The human data showed that BPA is absorbed to nearly 100% and pre-systemically metabolised to a great extent to glucuronide and sulfate conjugates. The concentration in the systemic circulation is low (mean C_{max} = 0.43 nM, following 30 µg/kg bw; mean C_{max} = 6.5 nM, following 100 µg/kg bw). The dose-corrected AUCs were clearly different in the two studies. The most probable explanation would be that in the study with dose-corrected higher C_{max} and higher AUC, the contact time with the buccal mucosa could be longer compared with the other study because of the mode of administration (BPA in cookies versus BPA in soup).
- The CEP Panel decided to use the median value of the AUCs from both studies for the calculation of the HEDF, because both modes of administration are realistic for humans. The median value was 15.7 nM × h, which is 4-fold higher than the modelled AUC value used for calculating the HEDF in the 2015 EFSA opinion (EFSA CEF Panel, 2015).
- To calculate the HEDF, the AUC data were used from the 2015 EFSA opinion (EFSA CEF Panel, 2015) opinion for mice, rats, monkeys and dogs. For ewes, the data reported in the current opinion were used.
- The following HEDFs were obtained: 0.0115 for mice, 0.165 for rats, 0.095 for monkeys, 0.1395 for dogs, 0.1197 for ewes (gavage) and 0.4357 for ewes (diet).
- Specific factors were applied to convert the doses from studies in which BPA was given by other routes than the oral one to allow to compare the doses.

4.1.2 General toxicity

- The newly available literature data indicate that in the HOC General toxicity several organs are potential targets of toxicity for BPA and that haematological parameters can be affected by this compound.
- Within the HOC General toxicity, no human studies were available, while 10 clusters with relevant endpoints were identified in animal studies: body weight, liver effects, kidney effects, lung effects, thyroid effects, parathyroid effects, pituitary gland effects, adrenal gland effects, bone marrow effects and effects on haematological parameters.
- Overall, none of the evaluated clusters' effects in the HOC General toxicity was considered Very Likely or Likely. In each of the evaluated clusters, effects were noted at least in one exposure period, but there were less consistent results among the available studies and, therefore, these effects were judged as ALAN in all the clusters.
- MoA studies suggested oxidative stress as a potential pathogenetic mechanism for kidney damage. Similarly, oxidative stress in liver cells may be related to impaired mitochondrial function and liver toxicity. MoA studies also proposed that epigenetic changes via DNA methylation may have an impact on different signalling pathways related to lipid and carbohydrate metabolism. MoA studies in lungs suggested that BPA can delay fetal lung maturation evaluated through reduced alveolar airspace and thickened septa. Both these findings may be related to an increase in the lung weight. MoA studies on thyroid cells suggested mechanisms responsible for an increase in proliferation, supporting the limited evidence of hyperplastic changes observed in the animal studies. Moreover, it was suggested that BPA could enhance the susceptibility to thyroid carcinoma in combination with other endogenous or external factors.

4.1.3 Immunotoxicity

- The newly available data from literature indicate that the immune system is a target of toxicity for BPA.
- Within the HOC Immunotoxicity, one relevant cluster of endpoints was identified in the human studies: asthma/allergy, including data from the exposure periods pregnancy and childhood.
- In the animal studies, five clusters of relevant endpoints were identified: innate immunity, cellular immunity, humoral immunity, inflammation and allergic lung inflammation.
- Based on the human data, a positive association between BPA exposure and asthma/allergy was judged as ALAN.
- Based on the animal data, the clusters cellular immunity and allergic lung inflammation showed effects that were judged as Likely. In the other clusters, effects were also noted, but there were less consistent results, and these effects were judged as ALAN. In the cluster allergic lung inflammation, the effect noted was on the production of specific IgE in response to an allergen, which is deemed as adverse as it is a crucial parameter in inducing allergic reactions in the respiratory tract. Other effects in that cluster supported the likelihood of this effect. The likely effect

in the cluster cellular immunity was supported by the consistency of the different endpoints within that cluster. The most sensitive parameter affected by BPA was the increased number of Th17 cells. Although Th17 cells are T cells, and therefore were put in the cluster cellular immunity, they play a role in allergic responses, and therefore the effect on Th17 cells is consistent with the effect on specific IgE. *In vivo* evidence was supported by MoA studies. *In vitro* studies indicated the ability of BPA to induce immune deregulation, increasing susceptibility to develop inflammatory diseases. Th17 cells are a specific subset of CD4+ T helper cells, which participate in various immune diseases, including asthma and autoimmune diseases. Potential mechanisms by which BPA may contribute to immune-mediated disorders included modulation of ERK1/2 phosphorylation, NF-κB activation, modulation of ERs, GR and AR as well as cytokine/chemokine secretion, and oxidative stress. Effects may be on non-specific cells belonging to the immune system or influencing the immune system (such as APCs and epithelial cells). This will, through presentation of antigens to T-lymphocytes or release of mediators, influence the regulatory homeostasis of the immune system, suppressing T regulatory cells and stimulating Th-17 cells. Thus, BPA appeared to promote multiple interwoven pathways involved in immune deregulation that may play a role in immune-related disorders.

4.1.4 Metabolic effects

- The newly available literature data indicate that BPA may induce adverse metabolic effects.
- Within the HOC Metabolic effects, five clusters of endpoints were identified in the human studies: obesity, cardiometabolic effects, thyroid effects, T2DM and gestational diabetes mellitus, including data from one or more of the exposure periods pregnancy, childhood and adulthood.
- In the animal studies, eight clusters of relevant endpoints were identified: obesity, fat deposition in the liver, glucose regulation, blood lipids, uric acid, T1DM, other metabolic hormones and thyroid hormones. The clusters included data from one or more of the exposure periods developmental until weaning, developmental until adulthood, growth phase, adult exposure and indirect (germline) exposure.
- Based on the human data, none of the metabolic clusters showed effects that were considered Likely or Very Likely. A positive association between BPA exposure and obesity and T2DM was judged as ALAN, while a positive association between BPA exposure and cardiometabolic effects, thyroid effects and gestational diabetes mellitus was judged as Not Likely.
- Based on the animal data, no metabolic clusters were considered Very Likely. The cluster uric acid was considered Likely (in the adult exposure period), as increased levels were observed in the liver of mice and in the serum of mice and rats after BPA exposure. The other metabolic endpoints were considered either ALAN (obesity, fat deposition in the liver, glucose regulation, blood lipids and T1DM) or Not Likely (other metabolic hormones and thyroid hormones), in one or more exposure periods.
- Substantial amounts of supporting evidence for plausible MoAs of BPA were available on obesity, fat deposition in the liver and glucose regulation, mostly from animal and *in vitro* studies. The MoA data in animals showed that BPA could increase the formation of hepatic uric acid by increasing

the activity of the enzyme xanthinoxidase, which catalyses the conversion of purines hypoxanthine and xanthine into uric acid. The MoA data on T1DM were very limited and the results depended on the animal model used.

4.1.5 Neurotoxicity and developmental neurotoxicity

- The newly available literature data indicate that the central nervous system is a target of toxicity for BPA.
- Within the HOC Neurotoxicity and developmental neurotoxicity, the evaluation of the human data considered endpoints from the cluster neurodevelopment. In the animal studies, three clusters of endpoints were identified: neuromorphology, nervous system functionality and behaviour.
- Based on the human data, it was concluded that the evidence for an association between BPA exposure and impaired neurodevelopment was Not Likely.
- Based on the animal data, all three neurotoxicity clusters showed effects that were judged as Likely:
  - In the neuromorphology cluster, Likely effects were found for the endpoints dendritic spine density of pyramidal cells in hippocampus (CA1 and dentate gyrus areas) after developmental exposure and for the endpoints number of neurons in hippocampus (CA1 and CA3 areas), and dendritic spine density in pyramidal cells in the medial part of the PFC after exposure during the growth phase/young age.
  - In the nervous system functionality cluster, a Likely effect on the endpoint AChE activity during the adult exposure period was identified.
  - In the behaviour cluster, Likely effects were noted for the endpoint anxiety/emotionality during all exposure periods (developmental, growth phase/young age, adult and exposure through the male germline). Furthermore, the endpoint learning/memory showed a Likely influence of BPA from developmental and growth phase/young age exposure, and effects on sensory-motor coordination and salt preference were considered Likely in adults.
- The mechanisms of action that link the identified effects of BPA on various endpoints of brain structure, function and development have not been sufficiently explored in the literature to draw conclusions. There is evidence for an involvement of steroid-hormone-dependent pathways (oestrogen, androgens, corticosterone); oxidative stress, mitochondrial function and calcium regulation; gene expression changes through DNA methylation and other signaling pathways (canonical and non-canonical Wnt pathways, kinases).

4.1.6 Reproductive and developmental toxicity

- The newly available literature data indicate that the reproductive system is a target of toxicity for BPA.
- Within the HOC Reproductive and developmental toxicity, five relevant clusters of endpoints were identified in the human studies: fetal and post-natal growth, prematurity, pre-eclampsia, male

fertility and female fertility, including data from one or more of the exposure periods pregnancy, childhood and adulthood.

- In the animal studies, three clusters of relevant endpoints were identified: developmental toxicity, female reproductive toxicity and male reproductive toxicity. The clusters included data from one or more of the exposure periods developmental until weaning, developmental until adulthood, growth phase, adult exposure and indirect (germline) exposure.
- Based on the human data, none of the clusters showed effects that were judged as Likely or Very Likely. An association between maternal BPA exposure and impaired pre- and post-natal growth, shorter duration of gestation or preterm delivery, reduced male fertility and pubertal development when exposed during childhood, was judged as Not Likely. An association between BPA exposure and reduced female fertility and pre-eclampsia during adulthood and pubertal development when exposed during pregnancy was judged as ALAN
- Based on the animal data, both female and male reproductive toxicity clusters showed effects that were judged as Likely:
  - In the female reproductive toxicity cluster, there were Likely effects on ovary weight and histology and uterus histology after developmental exposure, on ovary histology after developmental and adult exposure, on implantation rate after growth phase/young age exposure and on ovary histology (follicle counts) after adult exposure.
  - In the male reproductive toxicity cluster, there were Likely effects on epididymis (exfoliated germ cells and inflammation) after developmental exposure (pre-natal and/or post-natal until adult), on testis histology (decreased seminiferous tubule diameter) after growth phase/young age exposure and on sperm (motility, viability and acrosome reaction) after adult exposure.
- In the developmental toxicity cluster, effects were also noted, but the results were less consistent results, and these effects were judged as ALAN for the endpoints bone development, mammary gland histology, body weight (in the developmental exposure), mammary gland weight and mammary gland histology (in the developmental and adult exposure) as well as body weight and age at first oestrus (in the growth phase/young age exposure).
- Supporting evidence for plausible MoAs of BPA on reproductive toxicity effects was available. They include ER and AR interactions and associated downstream and cross-stream effects, including epigenetic changes. Other possible mechanisms, including BPA-induced generation of oxidative stress, have been less explored.

4.1.7 Cardiotoxicity

- The newly available literature data investigated the cardiovascular system as a target of toxicity for BPA.
- Within the human HOC Cardiotoxicity, no case-control or cohort studies were available. Therefore, the evidence for a positive association between BPA exposure and cardiotoxicity in human was considered Inadequate.

- In the animal studies, five clusters of relevant endpoints were identified: absolute and relative heart weight, incidence of cardiac lesions, cardiac structural changes (as measured by echocardiography), effects on cardiac function (as measured by echocardiography), blood pressure and atherosclerotic lesions.

- Based on the animal studies, the evidence of BPA effects was judged as Not Likely in the majority of the cardiotoxicity clusters, and in few clusters as Inadequate, in one or more exposure periods. Given the functional relationship between the endpoints, the outcome of the WoE was considered biologically plausible.

- Considering the Not Likely evidence for BPA on cardiotoxic effects, the data from *in vitro* and mechanistic studies were not included for this HOC.

4.1.8 Carcinogenicity and mammary gland proliferative effects

- The newly available literature data indicate that, in the HOC Carcinogenicity and mammary gland proliferative effects, the following organs are targets of BPA-induced toxicity: mammary gland, prostate and uterus.

- Within the HOC Carcinogenicity and mammary gland proliferative effects, no human studies were available, while five clusters with relevant endpoints were identified in animal studies: mammary gland weight, mammary gland histology, prostate histology, uterus weight and uterus histology. For histology, four subclusters were considered, if available: non-neoplastic changes, pre-neoplastic lesions, neoplastic lesions, proliferation and apoptosis as evaluated by quantitative immunohistochemistry.

- The cluster mammary gland weight was judged Not Likely. The clusters mammary gland histology, prostate histology and uterus weight showed effects that were not consistently reported in the available studies and, therefore, these effects were judged as ALAN.

- Also, regarding the subclusters linked to lesions in the mammary gland, inconsistencies were noted: in the developmental until weaning exposure period no increase in pre-neoplastic lesions (Not Likely), but a higher incidence in neoplastic lesions (Likely) was observed. In the developmental to adult exposure period an increase in pre-neoplastic lesions (ALAN) was reported but no increase in neoplastic lesions was detected (Not Likely). Therefore, these effects contributed to the overall judgement ALAN in the cluster mammary gland histology.

- In the cluster uterus histology, the non-neoplastic changes gland cellular anomalies, squamous metaplasia and cystic endometrial hyperplasia were considered adverse and judged as Likely based on studies with developmental exposure (pre-natal and/or post-natal until weaning) to BPA.

- MoA studies in mammary gland addressing epigenetic effects, changes in gene expression and changes in hormone receptor levels suggested various MoAs of BPA possibly involved in the induction of proliferative/morphological changes. Some *in vivo* studies indicated that stromal-epithelial interactions may play a crucial role in the BPA-induced developmental changes in the

mammary gland. *In vitro* studies provided some support for the hypothesis that BPA contributes to a higher susceptibility to mammary gland carcinogenesis. MoA studies on prostate cancer indicated that BPA can enhance the susceptibility to tumorigenesis in rodents co-treated with very high levels of E2 and testosterone, while developmental and chronic exposure to BPA without additional sex hormones did not demonstrate a direct tumorigenic effect. *In vitro* MoA studies on uterine cells indicated that BPA increases the proliferative rate. Data from other *in vitro* studies suggested that BPA modulates various mechanisms underlying the onset, growth and invasion of uterine tumours. However, the results of rodent studies did not demonstrate a tumorigenic activity of BPA.

4.1.9 Genotoxicity

- The analysis of the available literature data indicate that BPA does not induce gene mutations in bacteria. BPA induces DNA strand breaks, clastogenic and aneugenic effects in mammalian cells *in vitro*. Oxidative stress-related mechanism(s) are likely to be involved in this DNA damaging and clastogenic activity.
- In contrast with consistent positive *in vitro* findings, the *in vivo* findings in several studies with high/limited reliability were inconsistent. The CEP Panel concluded that the evidence does not support an *in vivo* genotoxic hazard posed by BPA through direct interaction with DNA.
- The CEP Panel concluded that it is unlikely to very unlikely that BPA presents a genotoxic hazard, the causes of which include a direct mechanism, and that the balance of evidence allows a HBGV to be established.

4.2 Hazard characterisation

- The CEP Panel used BMD analysis for dose-response modelling of the endpoints that were assigned a likelihood level of Likely or Very Likely.
- After conversion of the doses to HED, the CEP Panel selected the lowest BMDL value of 0.93 ng/kg bw per day for the effect of BPA on Th17 cells in mice to be used as RP for the risk assessment of BPA.
- Based on an assessment of other endpoints that could require an additional UF, the WG's overall probability that no additional UF was needed was in the range 85-87%. The CEP Panel concluded that no additional UF was needed and that a HBGV based on the identified RP is justified.
- The CEP Panel applied the UFs for inter-species toxicodynamic difference (2.5) and intra-human variability in toxicokinetics and toxicodynamics (10) and established a TDI of 0.04 ng/kg bw per day.

4.3 Risk characterisation

- The comparison of the dietary exposure estimates from the 2015 EFSA opinion with the new TDI showed that both the mean and the 95th percentile dietary exposures in all age groups (including all infants and toddler groups) exceeded the TDI by two to four orders of magnitude.
- The CEP Panel is aware that the exposure assessment presented in the 2015 opinion may not fully represent the current dietary exposure. Even considering this uncertainty, since the exceedance

was so large, the CEP Panel concluded that there is a health concern from dietary BPA exposure for all age groups of the general population.

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15135 # Abbreviations

8-isoPF2α	Isoprostaglandin F2α
8-NO2Gua	8-Nitroguanine
8-OHdG	8-Hydroxy-2′-deoxyguanosine
ACC	Acetyl-CoA carboxylase
AChE	Acetylcholine esterase
ACTH	Adrenocorticotrophic hormone
ADHD	Attention deficit hyperactivity disorder
AGD	Anogenital distance
AGDac	Anogenital distance anus–clitoris
AGDaf	Anogenital distance anus–fourchette
AGDap	Anogenital distance anus–penis
AGDas	Anogenital distance anus–scrotum
AIF	Apoptosis-inducing factor
AIR	Acute insulin response
Akt	Protein kinase B
ALAN	As Likely As Not
ALB/GLO	Albumin/globulin
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AMH	Anti-Müllerian hormone
AMPK	Adenosine monophosphate-activated protein kinase
ANSES	French Agency for Food, Environmental and Occupational Health and Safety
APCs	Antigen-presenting cells
AR	Androgen receptor
ASC	Adipose stromal/stem cells
ASP	Aspartate
AST	Aspartate aminotransferase
ATGL	Adipose triglyceride lipase
ATM	Ataxia-telangiectasia mutated
AU	Arbitrary units
AUC	Area under the curve

AVPV	Anteroventral periventricular nucleus
BAL	Bronchoalveolar lavage
BASC-2	Behaviour Assessment System for Children-2
BAT	Brown adipose tissue
BDNF	Brain-derived neurotrophic factor
BER	Base excision repair
BL	Body length
BMD	Benchmark dose
BMDL	Benchmark dose lower confidence interval
BMDU	Benchmark dose upper confidence interval
BMI	Body mass index
BMR	Benchmark response
$BNST_{dl}$	Dorsolateral bed nucleus of stria terminalis
$BNST_{p}$	Posterior bed nucleus of stria terminalis
BP-1	Sulphonylbis(benzene-4,1-diyloxy)]diethanol
BP-2	4,4′-Sulphanediyldiphenol
BPA	Bisphenol A
BPAF	Bisphenol AF
BPA-G	Bisphenol A-glucuronide
BPA-S	Bisphenol A-sulfate
BPF	Bisphenol F
BPS	Bisphenol S
BPZ	Bisphenol Z
BrdU	5-bromo-2′-deoxyuridine
BRIEF-P	Behaviour Rating Inventory of Executive Function–Preschool
BSID	Bayley Scales of Infant Development
BSID-II	Bayley Scales of Infant Development-II
BTB	Blood-testis barrier
BUN	Blood urea nitrogen
BW	Birth weight
CA	Chromosomal aberrations
CAT	Catalase

CBCL	Child Behaviour Checklist
CBMA	Cytokinesis blocked micronucleus assay
CCCEH	Columbia Center for Children's Environmental Health
Cd	Cadmium
CDIIT	Comprehensive Developmental Inventory for Infants and Toddlers
CDRS	Children's Depression Rating Scale-Revised
CEBS	Chemical Effects in Biological Systems
CEF	Food Contact Materials, Enzymes, Flavourings and Processing Aids
CHO cells	Chinese hamster ovary cells
CI	Confidence interval
cPEN	Caudal periventricular nucleus
Cpf	Cumulative probability function
CPI	C-peptide index
CPT	Camptothecin
CRH	Corticotropin releasing hormone
CRP	C-reactive protein
CT	Computed tomography
DAMP	Damage-associated molecular pattern
DBP	Dibutyl phthalate
DBS	Double base substitutions
DCFH-DA	Dichlorofluorescein diacetate assay
DDR	DNA damage response
DEXA	Dual energy X-ray absorptiometry
DFI	DNA fragmentation index
DHEA	Dehydroepiandrosterone
DHEA-S	Dehydroepiandrosterone sulfate
DHPN	*N*-bis(2-hydroxypropyl)nitrosamine
DLPFC	Dorsolateral prefrontal cortex
DLT	Dark Light Test
DMBPA	bis(4-hydroxy-3-methylphenyl)propane
DMBPS	4,4'-Sulfonylbis(2-methylphenol
DOR	Decreased ovarian reserve

DSBs	DNA double-strand breaks
E2	Oestradiol
ECHA	European Chemicals Agency
ECoMRI	Echo Magnetic Resonance Imaging System
EDC	Endocrine-disrupting chemical
EDSP	Endocrine disruptor screening program
EE	Ethinyl oestradiol
EF	Ejection fraction
eGFR	Epidermal growth factor receptor
EKE	Expert knowledge elicitation
ELISA	Enzyme-linked immunosorbent assay
EMS	Ethyl methanesulfonate
eNOS	Endothelial nitric oxide
EPM	Elevated plus maze
ER	Oestrogen receptor
ERK	Extracellular-signal-regulated kinase
ERR	Oestrogen related receptor
ERRα	Oestrogen related receptor-α
ERRγ	Oestrogen related receptor gamma
ER2	Oestrogen receptor 2
ESR	Erythrocyte sedimentation rate
ESC	Endometrial stromal cells
ESI	Electrospray ionization
eWAT	Epididymal white adipose tissue
EZM	Elevated zero maze
FATP1	Fatty acid transport protein 1
FDA	Food and Drug Administration
FENO	Fraction of exhaled nitric oxide
FEV_1	Forced respiratory volume in 1 s
FFA	Free fatty acids
FGV	Mammary fibroglandular volume
FMI	Fat mass index

Fpg	Formamidopyrimidine-DNA glycosylase
FS	Fractional shortening
FSH	Follicle stimulating hormone
FST	Forced swimming test
FT_3	Free triiodothyronine
FT_4	Free thyroxine
GABA	Gamma amino butyric acid
GD	Gestation day
GDM	Gestational diabetes mellitus
GE	Ginger extract
GGT	Gamma-glutamyl transferase
GLN	Glutamine
GLP	Good laboratory practice
GLU	Glutamate
GLY	Glycine
GPx	Glutathione peroxidase
GR	Glucocorticoid receptor
GSH	Reduced glutathione
γ-GTP	Gamma-glutamyl transpeptidase
GTT	Glucose tolerance test
gWAT	Gonadal white adipose tissue
HBF	High butter fat
HBGV	Health-based guidance value
HDL	High-density lipoprotein
HED	Human equivalent dose
HEDF	Human equivalent dose factor
HFD	High-fat diet
HFLF	Human fetal lung fibroblasts
HNE-MA	4-Hydroxy-2-nonenal-mercapturic acid
HoC	Health outcome category
hOGG1	8-Oxoguanine DNA glycosylase
HOMA	Homeostatic Model Assessment

HOMA-IR	Homeostatic Model Assessment for Insulin Resistance
HOME	Health Outcomes and Measures of the Environment
HOX	Homeobox-containing genes
HPA	Hypothalamic–pituitary–adrenal axis
HPG	Hypothalamic-pituitary-gonadal axis
HPLC	High-performance liquid chromatography
HPT	Hypothalamic–pituitary–thyroid axis
hUM	Human uterine myoma tissue
HUVEC	Human umbilical vascular endothelial cells
HVA	Homovanillic acid
ICD	International Classification of Diseases
IgA	Immunoglobulin A
IgE	Immunoglobulin E
IGF-1	Insulin-like growth factor 1
IgG	Immunoglobulin G
IgM	Immunoglobulin M
InDel	Insertions and deletions
INMA	INfancia y Medio Ambiente (Environment and Childhood) Project
INSL3	Insulin-like peptide 3
IpGTT	Intraperitoneal glucose tolerance test
IpITT	Intraperitoneal insulin tolerance test
ITT	Insulin tolerance test
IvGTT	Intravenous glucose tolerance test
IVS	Interventricular septum
iWAT	Inguinal white adipocytes
JNK	c-Jun N-terminal kinase
KC	Kupffer cells
LBW	Low birth weight
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LDH	Lactate dehydrogenase
LDL	Low-density lipoprotein
LH	Luteinizing hormone

LIFE	Longitudinal Investigation of Fertility and the Environment
LOAEL	Lowest observed adverse effect level
LOD	Limit of detection
LOQ	Limit of quantification
LPL	Lipoprotein lipase
LPS	Lipopolysaccharides
LVIDd	Left ventricular internal dimension, at diastole
LVIDs	Left ventricular internal dimension, at systole
LVPWd	Left ventricle posterior wall thickness, at diastole
LVWT	Left ventricle wall thickness
MAO	Monoamine oxidase
MAPK	Mitogen-activated protein kinase
MCH	Mean corpuscular haemoglobin
MDA	Malondialdehyde
MDR	Monotonic dose–response
MEFs	Mouse embryonic fibroblasts
MEST	Mesoderm specific transcript
MII	Metaphase II
MMP	Mitochondrial transmembrane potential
MMTV	Mouse mammary tumour virus
MN	Micronuclei
MoA	Mode of action
mPFC	Medial prefrontal cortex
MSC	Mesenchymal stem cell
MTOCs	Modifications of the microtubule organizing centres
mTOR	Mammalian target of rapamycin
MTP	Microsomal triglyceride transfer protein
MUFA	Monounsaturated fatty acids
MWM	Morris water maze
NA	Noradrenaline
n.a.	Not available
NAC	*N*-acetylcysteine

NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NCTR	National Centre for Toxicological Research
NDI	Nuclear division index
NHANES	National Health and Nutrition Examination Survey
NHSII	Nurses' Health Study II
NIEHS	National Institute of Environmental Health Science
NMDR	Non monotonic dose–response
NMR	Nuclear magnetic resonance
NOAEL	No observed adverse effect level
NOD	Non-obese diabetic
NPC	Neuroprogenitor cell
NSC	Neural stem cells
NTP	National Toxicology Program
OECD	Organization for European Economic Cooperation and Development
OFT	Open field test
oGTT	Oral glucose tolerance test
OR	Odds Ratio
OTR	Oxytocin receptor
OTM	Olive tail moment
OV	Ovarian volume
OVA	Ovalbumin
OVX	Ovariectomy/ ovariectomised
PA	Premature anaphase
PBG	Phenylbiguanide
PBMC	Peripheral blood mononuclear cells
PBPK	Physiologically-based pharmacokinetic (model)
PC	Polycarbonate
PCBs	Polychlorinated biphenyls
PCE	Polychromatic erythrocytes
PCNA	Proliferating cell nuclear antigen
PCOS	Polycystic ovary syndrome

PDD-NOS	Pervasive developmental disorder-not otherwise specified
PET	Positron emission tomography
PFC	Prefrontal cortex
PFOA	Perfluorooctanoic acid
PFOS	Perfluorooctane sulfonic acid
PIM	Perceived insufficient milk (supply)
PIN	Prostatic intraepithelial neoplasia
PKA	Protein kinase-A
PND	Post-natal day
PNW	Post-natal week
POMC	Pro-opiomelanocortin
PPARϒ	Peroxisome proliferator-activated receptor gamma
PRL	Prolactin
PS	Prostasphere
PSO	Pumpkin seed oil
PVN	Paraventricular nucleus
pWAT	Perigonadal white adipose tissue
PXR	Pregnane X receptor
RAM	Radial arm maze
RCMAS	Revised Children's Manifest Anxiety Scale
ROS	Reactive oxygen species
RP	Reference point
rPen	Rostral periventricular area
RR	Relative Risk
rT3	Reverse triiodothyronine
RWT	Relative wall thickness
RXR	Retinoid-X-receptor
SAC	Spindle assembly checkpoint
SAT	Subcutaneous adipose tissue
SBS	Single base substitutions
SCD	Sperm chromatin dispersion
SCEs	Sister chromatid exchanges

SCSA	Sperm chromatin structure assay
SD	Sprague Dawley
SDF	Sperm DNA fragmentation
SDF1	Stromal cell-derived factor 1
SDQ	Strength and Difficulties Questionnaire
SEM	Standard error mean
SFA	Saturated fatty acid
SGA	Small for gestational age
SHBG	Sex hormone-binding globulin
SML	Specific migration limit
SMS	Social maturity scale
SNP	Single-nucleotide polymorphisms
SO	Sesame oil
SOD	Superoxide dismutase
SREBPs	Sterol regulatory element-binding proteins
SRS-2	Social responsiveness scale-2
StAR	Steroidogenic acute regulatory protein
STZ	Streptozotocin
SULT	Sulfotransferase
SVZ	Subventricular zone
T1DM	Type-1 diabetes mellitus
T2DM	Type-2 diabetes mellitus
T_3	Triiodothyronine
T_4	Thyroxine
TAG	Triacylglycerol
TAU	Taurine
TBARS	Thiobarbituric acid reactive substances
TBBPA	Tetrabromobisphenol A
TCC	T-type calcium channel
TCR	T-cell receptor
TD	Terminal duct
TDI	Tolerable daily intake

TEBs	Terminal end buds
TG	Test guideline
TJ	Tight junction
TLR	Toll-like receptor
Top1	Topoisomerase-I
TSH	Thyroid stimulating hormone
TSLP	Thymic stromal lymphopoietin
TT_3	Total triiodothyronine
TT_4	Total thyroxine
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
UF	Uncertainty factor
UGT	UDP-glucuronyl-transferase
US EPA	United States Environmental Protection Agency
VAT	Visceral adipose tissue
VM	Ventral mesencephalon
VMH	Ventromedial hypothalamus
VMWM	Virtual Morris water maze
WAT	White adipose tissue
WG	Working group
WGS	Whole genome sequencing
WHHL	Watanabe heritable hyper-lipidemic
WISC-IV	Wechsler intelligence scale for children IV
WoE	Weight of evidence
WPPSI-III	Wechsler primary and preschool scale of intelligence–III
XO	Xanthine oxidase
XOR	Xanthine oxidoreductase

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15138 # Appendices

Appendix A – Outcome of the call for data[20]

Call for data relevant to the hazard assessment of Bisphenol A (BPA)

Published: 9 Marzo 2018 *Deadline:* 15 Ottobre 2018 - 23:59 (CEST)

EFSA-Q-number: EFSA-Q-2018-00221

- Published: 09/03/2018
- Deadline for registering interest: 30/06/2018
- Deadline for submission of data: 31/08/2018
- Updated deadline: 15/10/2018

Indice

Documents

Collegamenti tematici

15139

15140 The data submitted though the call and the approach used for their consideration/exclusion are reported
15141 in Table A1.

15142 **Table A1:** Data submitted through the call for data relevant to the hazard assessment of BPA.

Data submitted	Notes (against exclusion and inclusion criteria)	RefID number in Distiller
Data submitted by Joseph Laakso on behalf of Angel Nadal from the Endocrine Society APPENDIX: Selected publications on the effects of BPA:		
Stahlhut RW, Myers JP, Taylor JA, Nadal A, Dyer JA and Vom Saal FS, 2018. Experimental BPA exposure and glucose-stimulated insulin response in adult men and women. Journal of the Endocrine Society, 2(10), 1173–1187. doi:10.1210/js.2018-00151	Included	RefID 13171
Li Q, Lawrence CR, Nowak RA, Flaws JA, Bagchi MK and Bagchi IC, 2018d. Bisphenol A and phthalates modulate peritoneal macrophage function in female mice involving SYMD2-H3K36 dimethylation. Endocrinology, 159(5), 2216–2228. doi:10.1210/en.2017-03000	Included	RefID 12475
Drobná Z, Henriksen AD, Wolstenholme JT, Montiel C, Lambeth PS, Shang S, Harris EP, Zhou C, Flaws JA, Adli M and Rissman EF, 2018. Transgenerational effects of bisphenol A on gene expression and DNA methylation of imprinted	Included	RefID 11853

[20] https://www.efsa.europa.eu/it/consultations/call/180309-0

genes in brain. Endocrinology, 159(1), 132–144. doi:10.1210/en.2017-00730		
Eckstrum KS, Edwards W, Banerjee A, Wang W, Flaws JA, Katzenellenbogen JA, Kim SH and Raetzman LT, 2018. Effects of exposure to the endocrine-disrupting chemical bisphenol A during critical windows of murine pituitary development. Endocrinology, 159(1), 119–131. doi:10.1210/en.2017-00565	Included	RefID 11874
Mahalingam S, Ther L, Gao L, Wang W, Ziv-Gal A and Flaws JA, 2017. The effects of in utero bisphenol A exposure on ovarian follicle numbers and steroidogenesis in the F1 and F2 generations of mice. Reproductive Toxicology, 74, 150–157. doi:10.1016/j.reprotox.2017.09.013	Included	RefID 4779
Olson MR, Su R, Flaws JA and Fazleabas AT, 2017. Bisphenol A impairs decidualization of human uterine stromal fibroblasts. Reproductive Toxicology, 73, 339–344. doi:10.1016/j.reprotox.2017.07.008	Included	RefID 5518
Patel S, Brehm E, Gao L, Rattan S, Ziv-Gal A and Flaws JA, 2017. Bisphenol A exposure, ovarian follicle numbers, and female sex steroid hormone levels: Results from a CLARITY-BPA study. Endocrinology, 158(6), 1727–1738. doi:10.1210/en.2016-1887	Included	RefID 5708
Ziv-Gal A and Flaws JA, 2016. Evidence for bisphenol A-induced female infertility: A review (2007–2016). Fertility and Sterility, 106(4), 827–856. doi:10.1016/j.fertnstert.2016.06.027	Included	RefID 9141
Li Q, Davila J, Kannan A, Flaws JA, Bagchi MK and Bagchi IC, 2016. Chronic exposure to bisphenol A affects uterine function during early pregnancy in mice. Endocrinology, 157(5), 1764–1774. doi:10.1210/en.2015-2031	Included	RefID 4128
Berger A, Ziv-Gal A, Cudiamat J, Wang W, Zhou C and Flaws JA, 2016. The effects of in utero bisphenol A exposure on the ovaries in multiple generations of mice. Reproductive Toxicology, 60, 39–52. doi:10.1016/j.reprotox.2015.12.004	Included	RefID 524
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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(1), 116–127. doi:10.1210/en.2006-0561	Excluded, published in 2007	Not applicable
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Markey CM, Wadia PR, Rubin BS, Sonnenschein C and Soto AM, 2005. Long-term effects of fetal exposure to low doses of the xenoestrogen	Excluded, published in 2005	Not applicable

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Markey CM, Luque EH, Munoz De Toro M, 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(4), 1215–1223. doi:10.1093/biolreprod/65.4.1215	Excluded, published in 2001	Not applicable
Papers on BPA replacement chemicals:		
Kolla S, Morcos M, Martin B and Vandenberg LN, 2018. Low dose bisphenol S or ethinyl estradiol exposures during the perinatal period alter female mouse mammary gland development. Reproductive Toxicology, 78, 50–59. Perinatal exposure to bisphenol-A alters peripubertal mammary gland development in mice. Endocrinology doi:10.1016/j.reprotox.2018.03.003	Excluded, it does not investigate BPA	Not applicable
LaPlante CD, Catanese MC, Bansal R and Vandenberg LN, 2017. Bisphenol S alters the lactating mammary gland and nursing behaviors in mice exposed during pregnancy and lactation. Endocrinology, 158(10), 3448–3461. doi:10.1210/en.2017-00437	Excluded, it does not investigate BPA	Not applicable
Hill CE, Sapouckey SA, Suvorov A and Vandenberg LN, 2017. Developmental exposures to bisphenol S, a BPA replacement, alter estrogen-responsiveness of the female reproductive tract: A pilot study. Cogent Medicine, 4(1. doi:10.1080/2331205X.2017.1317690, PubMed: 31231671	Excluded, it does not investigate BPA	Not applicable
Catanese MC and Vandenberg LN, 2017. Bisphenol S (BPS) alters maternal behavior and brain in mice exposed during pregnancy/lactation and their daughters. Endocrinology, 158(3), 516–530. doi:10.1210/en.2016-1723	Excluded, it does not investigate BPA	Not applicable
Kim B, Colon E, Chawla S, Vandenberg LN and Suvorov A, 2015. Endocrine disruptors alter social behaviours and indirectly influence social hierarchies via changes in body weight. Environmental Health: A Global Access Science Source, 14, 64. doi:10.1186/s12940-015-0051-6	Excluded, it does not investigate BPA	Not applicable

2. Ninja Reineke on behalf of CHEM Trust (report)	**Notes**	**RefID in Distiller**

CHEMTrust (2018).'From BPA to BPZ a toxic soup?: How companies switch from a known hazardous chemical to one with similar properties, and how regulators could stop them.' CHEM Trust Report	Excluded, secondary publication	RefID 13775

3. Submission by Hanane Yasmina Anteur (2 articles)	**Notes**	**Distiller**
Anteur HY, Bendahmane M and Khan NA, 2016. Perinatal exposure to bisphenol A affects body weight and the reproductive function of Wistar rat. Journal of Applied Environmental and Biological Sciences., 6(3), 1–8.	Included	RefID 13773
Anteur HY, Bendahmane M, Mehida H, Beghdadli B, Aboubekr FA, Khan NA and Kandouci BA, 2016. Is bisphenol A exposure associated with uterine leiomyoma in Western Algerian women of childbearing potential? Journal of Disease and Global Health, 8(3), 131–140.	Included	RefID 13774

4. Submission by Plastics Europe (10 submissions)	**Notes**	**Distiller**
2. Comments from the PlasticsEurope EU PC/BPA Group in Response to EFSA's Call for Data on BPA	Excluded, they are just a description of the CLARITY-BPA NTP study design.	Not applicable
3. Final Report 'Analysis of Draft Results for the CLARITY-BPA Core Study' Exponent, August 31, 2018	Excluded, secondary study	RefID 1380
5. Final Report: 'Three-generation reproductive toxicity evaluation of Bisphenol A administered in the feed to CD (Sprague–Dawley) Rats', RTI Identification Number: 65C-07036-000 (full study report 17 pdf files submitted)	Unpublished study report, date of completion May 12, 2000 Excluded: Study report of Tyl et al., 2002; Study already evaluated in the 2006, 2008 and 2015 EFSA BPA opinions	Not applicable
6. Final Report: 'A dietary developmental neurotoxicity study of bisphenol A in rats' Study Number WIL-186056; Donald G. Stump, PhD, DABT Full study report: 20 pdf files submitted Study initiation date: 26 June 2008 Study completion date: 30 September 2009	Excluded, already evaluated in CEF Panel, 2010)	Not applicable
7. Extended abstract: 'Pharmacokinetics of Bisphenol A in humans following dermal administration', Kristina A Thayer, Division of the National Toxicology Program. Kristina A. Thayer, Daniel R. Doerge, Dawn Hunt, Shepherd Schurman, Nathan C. Twaddle, Mona I. Churchwell, Stavros Garantziotis, Grace E. Kissling, Michael R. Easterling, John R. Bucher, Linda S. Birnbaum	Preliminary results: These findings have not been peer-reviewed and should not be considered to represent NTP opinion. Excluded, final data reported in RefID 7183, considered by the CEP Panel	Not applicable
8. Final Report: 'Two-generation reproduction study of Bisphenol A in rats', Study No: SR-98101, Makoto Ema, National Institute of Health Sciences, Osaka	Raw data of: Ema M, Fujii S, Furukawa M, Kiguchi M, Ikka T, Harazono A, 2001. Rat two-generation reproductive toxicity study of	Not applicable

Full study report submitted (7 pdf files 'CCSRI 2000 – Part X') Date of commencement: December 17, 1998 Date of completion: May 12, 2000	bisphenol A. Reproductive Toxicology, 15(5), 505–523: https://www.ncbi.nlm.nih.gov/pubmed/11780958 Excluded, Published in 2001	
9. Draft report: 'Draft NTP Research Report on The CLARITY-BPA Core Study: A Perinatal and Chronic Extended-Dose-Range Study of Bisphenol A in Rats', February 2018	This is the draft CLARITY-BPA report. The final report of 2018 and the Camacho et al., 2019 paper (both assigned to RefID 11370) were considered for the assessment	It corresponds to RefID 11370
10. NCTR GLP/NTP Technical report: 'Evaluation of the toxicity of Bisphenol A (BPA) in male and female Sprague–Dawley rats exposed orally from gestation day 6 through PND 90', PI: Barry Delclos, 2013 Technical report: Finalised July 2013 Date of Original Technical Report: March 4, 2013 Date of Technical Report Amendment #1: May 31, 2013 Date of Amendment #2 to Technical Report: July 11, 2013 Data probably for the study Delclos 2013. 50 pdf files submitted	Excluded, already evaluated in the 2015 BPA EFSA opinion	Not applicable

5. Gail Prins – University of Illinois		
Prins GS., Hu WY, Xie L, Shi GB, Hu DP, Birch L and Bosland MC, 2018. Evaluation of Bisphenol A (BPA) exposures on prostate stem cell homeostasis and prostate cancer risk in the NCTR-Sprague-Dawley rat: an NIEHS/FDA CLARITY-BPA Consortium Study. Environmental Health Perspectives, 126(11), 117001. doi:10.1289/EHP3953	Manuscript Draft The final study is now available online. 'Received 25 May 2018; Revised 4 October 2018; Accepted 11 October 2018; Published 2 November 2018 Supplemental Material is available online (https://doi.org/10.1289/EHP3953).' Included	RefID 13779

6. Submission by Michal Filipiak	**Notes**	**Distiller**
EFSA-Q-2018-00221 Toxicological data derived from human studies [toxicity of BPA on my own example] Concerns: observed and confirmed: reproductive, endocrine system, metabolic systems problems. In response to: www.efsa.europa.eu/en/consultations/call/180309–0	Excluded, self-report of a personal pathological situation, not a scientific study	Not applicable

15143

Appendix B – The Montévil et al. (2020) study: Consideration of low-dose effects and non-monotonic dose–response reported in that study

Examining the relationship between gestational exposure BPA and offspring mammary gland development (different morphological features) in Sprague–Dawley rats, Montévil et al. (2020) [RefID 13788] concluded that the relationship followed a non-monotonic dose–response (NMDR) curve. This conclusion was based on the author´s statistical evaluation by fitting a linear step function with the unit function placed either between the 25 and 250; or the 250 and 2500 µg/kg bw dose per day.

Such functions are commonly used in engineering to explain time-dependent processes where a sudden (binary) shift in response is initiated (e.g. sudden changes in voltage or market conditions, respectively). The use of step functions to describe biological process is neither common nor conventional.

As the developmental effects examined in this study are biologically relevant and potentially adverse, the WG evaluated the findings from this study using a statistical approach that is more conventional for assessing the dose–response in toxicological studies.

The outcomes evaluated were those reported in Figures 8 and Figure 9 of the study (i.e. weight, gland density, Dimension 3D and angles of branches between beginning and end, thickness of epithelium, variation of ductal thickness and aspect ratio). Raw data for Figure 8, Panel E, could not be assessed as they were not included in the publicly available dataset for the study.

In the WG assessment the presence of any dose–response information in the data was first evaluated by using a simple F-test under null hypothesis that the mean response in all groups is the same. For transparency the test results, including total sum of squares (SSQ), model sum of squares, the corresponding degrees of freedom (DF) and the resulting p-value (F-test) are shown in Table B1.

Table B1: Examining the presence of dose–response in figures 8 and 9 in the Montévil et al. (2020) [RefID 13788] study under the NULL hypothesis that all groups are equal.

	Total		**Model**		**p-value[1]**
Figure 8	**SSQ**	**DF**	**SSQ**	**DF**	
A – SD with 3D	2098	58	420	5	0.033
B – Thickness mm	23941	58	4263	5	0.058
C – Dimension 3D	0.4886	58	0.0545	5	0.266
D – Angle between beginning and end	149.4	58	12.8	5	0.431
E – Dim3	Data not available				
F – Aspect Ratio	14.92	58	2.36	5	0.095
Figure 9	**SSQ**	**DF**	**SSQ**	**DF**	
A – Gland weight, PND90, stop dose	4.990	55	0.751	5	0.136
B – Density analysis PND90, continuous dose	14250	58	1580	5	0.269

C – Density analysis, 6 months, continuous dose	11975	59	1235	5	0.303
D – Density analysis, 9 months, stop dose	9358	59	1208	5	0.176

15165

15166 Only the mean variation of ductal thickness (SD with 3D, figure 8A) reached formal significance ($p < 0.05$)
15167 meaning that the mean response across all groups was significantly different. This significance was driven
15168 by higher response in the 25 μg/kg bw per day dose group compared with controls. For figure 8B there
15169 was a borderline significant effect ($p = 0.058$) for the mean thickness of the epithelium, again driven by
15170 higher response in the 25 μg/kg bw dose group. For the aspect ratio (figure 8F), the 250 μg/kg bw per day
15171 dose groups was significantly different from the controls ($p = 0.02$, t-test) but the F-test for differences
15172 across dose groups did not reach formal significance. Visual inspection of model residuals confirmed that
15173 the use of F-test was appropriate.

15174 Taking figure 8A (the mean variation of ductal thickness) as an example of an outcome showing a potential
15175 dose–response, the shape of the dose–response was evaluated by modelling the data in PROAST using the
15176 following set of models (Table B2).

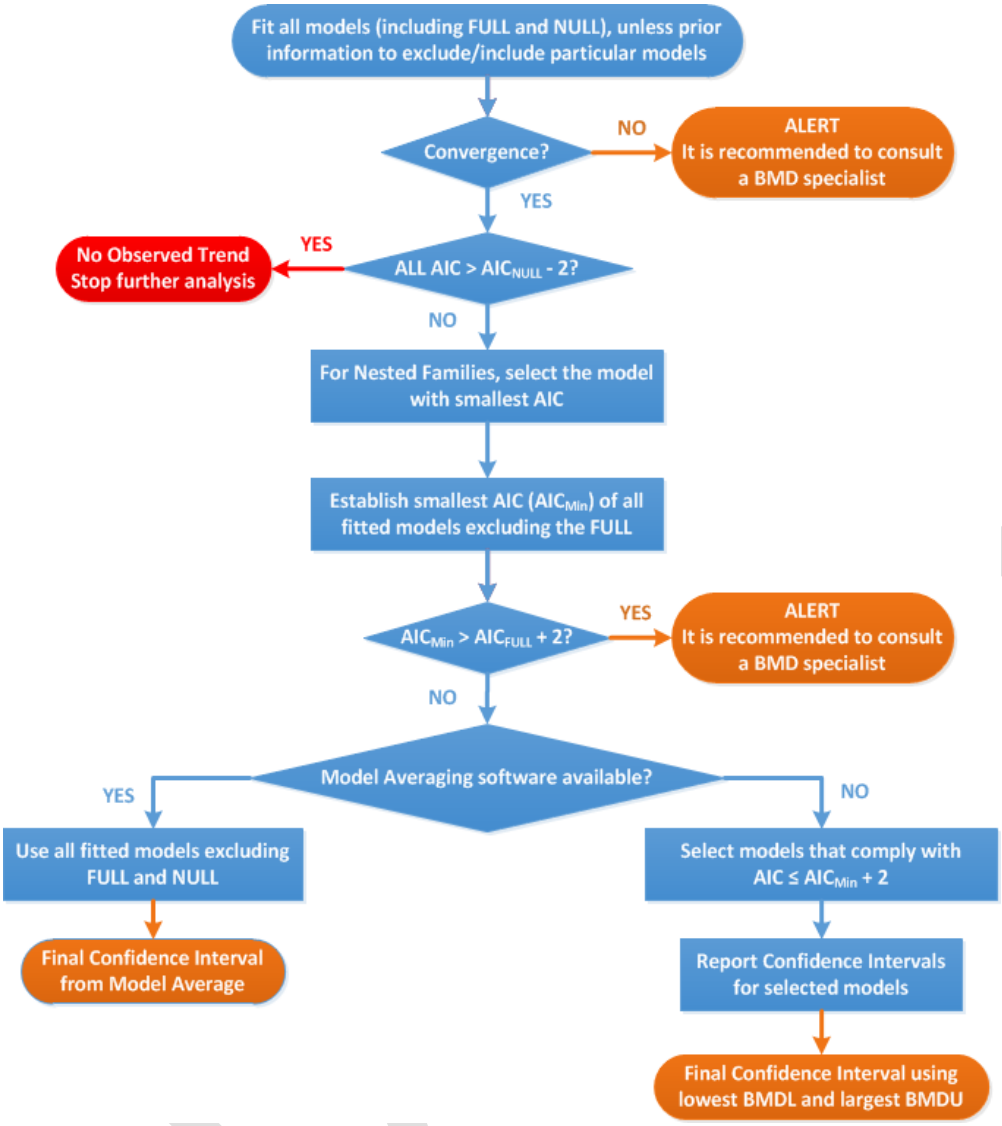
15177 **Table B2:** Modelling of the data in PROAST.

Model	Number of parameters	Formula
Null	1	$y = a$
Full	no. of groups	$y = \text{group mean}$
Exp model 3	3	$y = a \cdot \exp(bx^d)$
Exp model 4	4	$y = a \cdot (c - (c-1)\exp(-bx^d))$
Hill model 3	3	$y = a \cdot \left(1 - \frac{x^d}{b^d + x^d}\right)$
Hill model 4	4	$y = a \cdot \left(1 - \frac{(c-1) \cdot x^d}{b^d + x^d}\right)$
Inverse exponential	4	$y = a \cdot (1 + (c-1)\exp(-bx^{-d}))$
Log-normal family	4	$y = a \cdot (1 + (c-1)\Phi(\ln b + d\ln x))$

15178

15179 These functions are sufficiently flexible to capture presence of non-monotonicity and have been used
15180 previously for that purpose (Beausoleil et al., 2016; Badding et al., 2019).

15181 The criteria for evaluating if there is a significant dose–response is then as follows (Figure B1).



15182
15183

15184 **Figure B1:** Criteria for evaluating if there is a significant dose–response.

15185 In short, none of the models deviated significantly form the NULL model. The PROAST output is shown in
15186 Table B3 below (based on the model log likelihood the p-value for the model fit (Chi-square test), can for
15187 example be derived).

15188 **Table B3:** PROAST output based on the model log likelihood the p-value for the model fit (chi-
15189 squared test).

model	converged	loglik	npar	AIC
full model	Yes	10.09	9	−2.18

null model	Yes	2.23	2	−0.46
Expon. m3-	Yes	3.39	4	1.22
Expon. m5-	Yes	3.52	5	2.96
Hill m3-	Yes	3.39	4	1.22
Hill m5-	Yes	3.67	5	2.66
Inv.Expon. m3-	Yes	2.37	4	3.26
Inv.Expon. m5-	Yes	4.00	5	2.00
LN m3-	Yes	3.45	4	1.10
LN m5-	Yes	3.77	5	2.46

Similar conclusions were also reached for all other outcomes in figures 8 and 9 (data not shown).

Additional analyses using different models, including splines, did not reveal any significant dose–response in the data in figures 8 and 9. Significant dose–response could only be generated for figure 8B by overfitting the data using a restricting cubic spline as implemented in 'proc glmselect' in SAS (v9.2). This example is shown in Figure B2 below where a non-significant fit is obtained when fitting a spline function using 3 knots (p = 0.48). However, when fitting a spline function using 4 knots, which follows changes in response between every dose groups, a significant model fit was obtained (p = 0.03). This is however a clear example of overfitting the data and cannot be considered as a reasonable reflection of dose–response that could be replicated in different experimental setting.

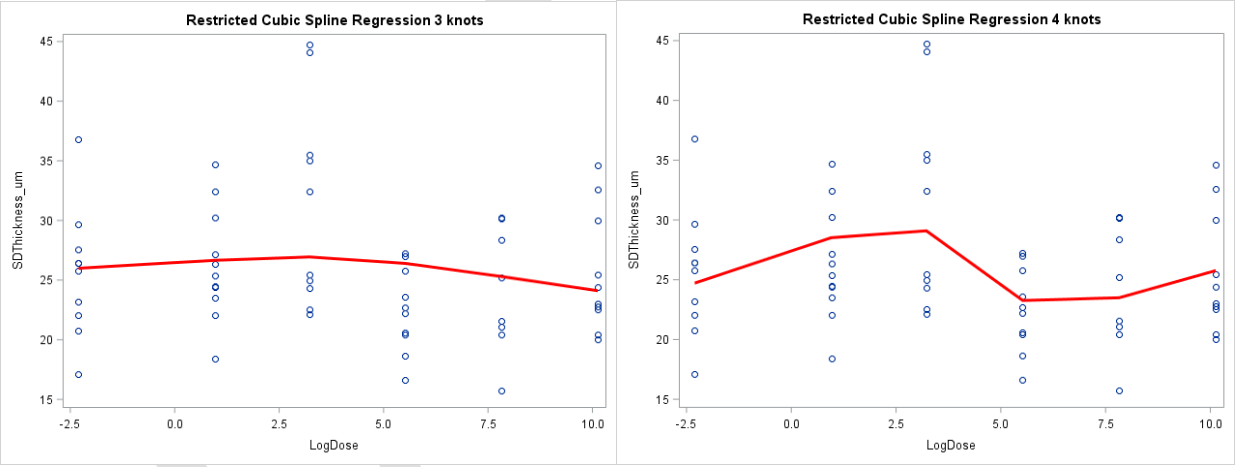


Figure B2: Results from fitting a restricted cubic spline using 3 and 4 knots to the data from figure 8A (Montévil et al., 2020 [RefID 13788]).

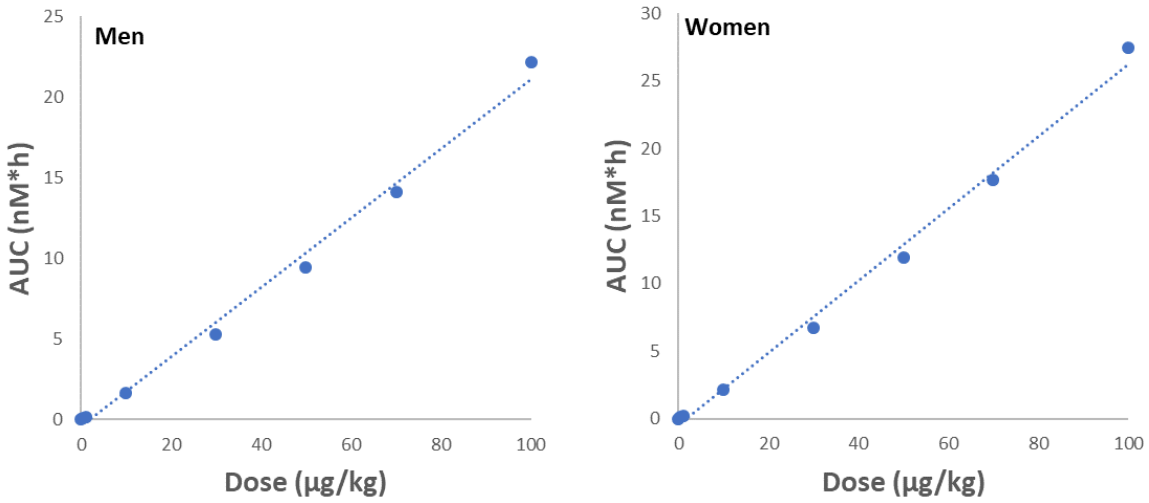
For the left figure (3 knots) the model sum of square was 2098 (DF = 58) and model sum of square was 55 (DF = 2). The resulting p-value was 0.48 (F-test). For the right-hand figure (4 knots) the model sum of square was 307 (DF = 2) and the resulting p-value was 0.032. As can be seen in the 4 knots result in exact fit between each pair of dose groups result in clear overfitting of the data21.

[21] Overfitting is the term used for model fit that corresponds too closely or exactly to a particular set of data, which may therefore fail to fit additional data or predict future observations reliably. Such models usually contain parameters than can be justified by the data.

15207 In summary, based on a simple F-test, only the results from figure 8A show any indication of significant
15208 dose–response. However, when fitting flexible biologically based non-linear models though the data (in
15209 PROAST) or spline functions (without overfitting the data) no indication of any meaningful dose–response
15210 is observed. A significant NMDR could only be generated when overfitting the data using splines or by using
15211 a step function designed around the dose where pairwise significance was reached. Based on the WG
15212 analyses the significant NMDR that the authors (Montévil et al., 2020 [RefID 13788]) generated appears,
15213 not to be very robust; one may equally assume that the significant difference for the 25 µg/kg bw per day
15214 dose group is a chance finding as no clear biologically recognisable pattern is observed in the data. In
15215 biostatistical terms, the evidence for NMDR in the Montévil et al. study appears weak and inconclusive.

Appendix C – Application of PBPK model of Karrer et al. (2018) to check linearity

15216 The PBPK model of Karrer et al. (2018) [RefID 12289] was used to check the linearity of the AUC with the
15217 administered dose. A bolus oral exposure was considered, and the AUC was computed 24 h after the
15218 administration. Few modifications were made to the PBPK model (model code in R given in the
15219 supplementary materials of the paper by Karrer et al. (2018) [RefID 12289]): the flow values were changed
15220 to correspond to plasma flows and not to blood flows by means of the haematocrit, the intake scheme was
15221 adapted to our simulation (a unique oral dose) and the computation of the AUC of BPA in plasma was
15222 added (Figure C1). Several doses were tested: 0.5, 1, 10, 30, 50, 70 and 100 µg/kg bw. Simulations were
15223 run for both men (bw = 73 kg) and women (bw = 60 kg).



15224

15225 **Figure C1:** Application of PBPK model of Karrer et al. (2018) [RefID 12289] to check linearity.

15226

Appendix D – Uncertainty analysis outcome

15227

15228 The purpose of the uncertainty analysis was to assess whether other effects of BPA may potentially occur
15229 after exposure to lower doses than the endpoint on which the reference point (RP) is based and, if so,
15230 inform a decision on what size of additional UF would be suitable to take those effects into account. This
15231 was carried out in the series of steps described in Chapter 2.3.4, and the results are presented below.

15232 D.1. Assessment of clusters by expert judgement

15233 Five HOCs were considered in the main part of the uncertainty analysis: Immunotoxicity, Metabolic effects,
15234 Neurotoxicity and developmental neurotoxicity, Reproductive and developmental toxicity, and
15235 Carcinogenicity and mammary gland proliferative effects (the HOC for General toxicity was considered later
15236 and cardiotoxicity was not considered in the uncertainty analysis because the evidence was either judged
15237 as Not Likely or as Inadequate). Within these five HOCs, the uncertainty analysis focused on 21 clusters of
15238 endpoints that were rated ALAN, Likely or Very Likely in the WoE assessment. Uncertainty was assessed
15239 by quantitative expert judgements of two questions for each cluster, elicited from two or three experts per
15240 cluster, who were chosen for their expertise on the endpoints in that cluster (11 experts in total). Experts
15241 were invited to revise their judgements, if they wished, after discussing them with the other expert(s)
15242 assessing the same cluster.

15243 Question 1 was 'What is your probability that there is at least one endpoint in the WoE table for this cluster
15244 that occurs in animals tested with BPA and is relevant and adverse in humans?' In effect, this quantifies
15245 uncertainty in hazard identification for each cluster. The experts gave their judgement on this in the form
15246 of approximate probabilities, i.e. ranges of probabilities, and their responses are presented graphically in
15247 Figure D1.

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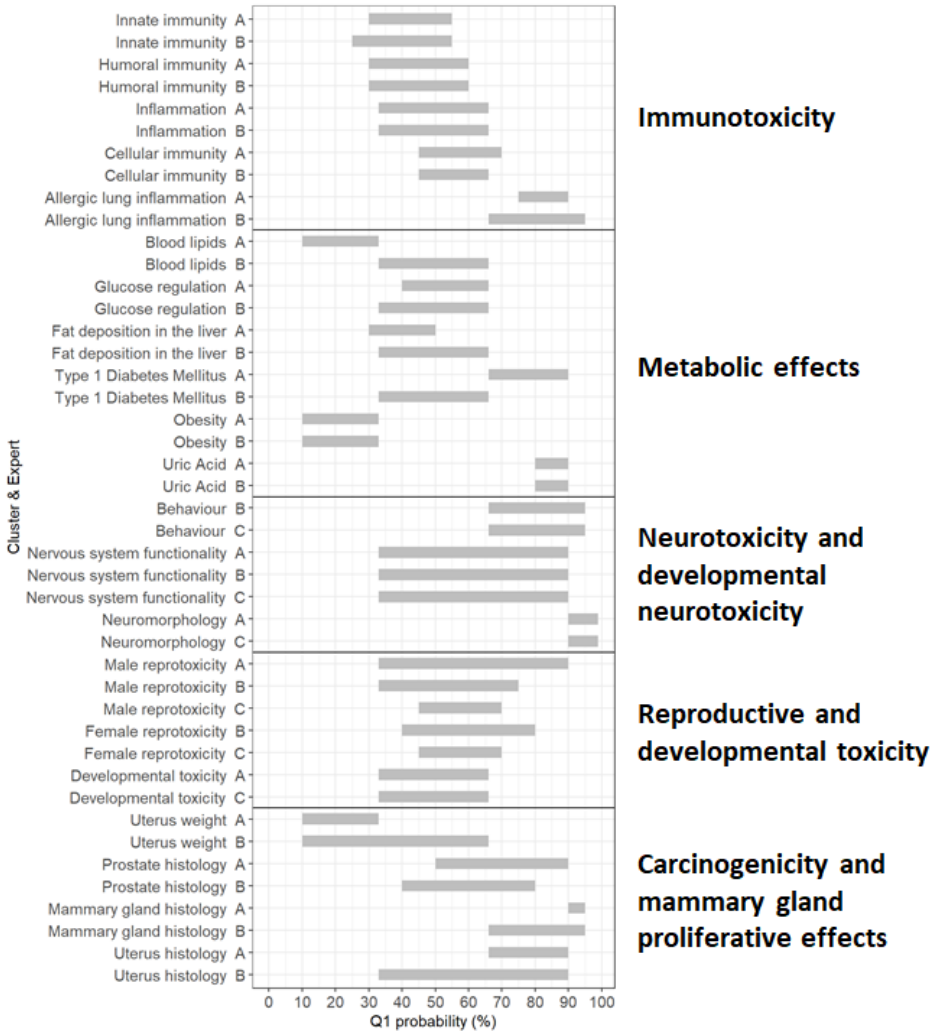
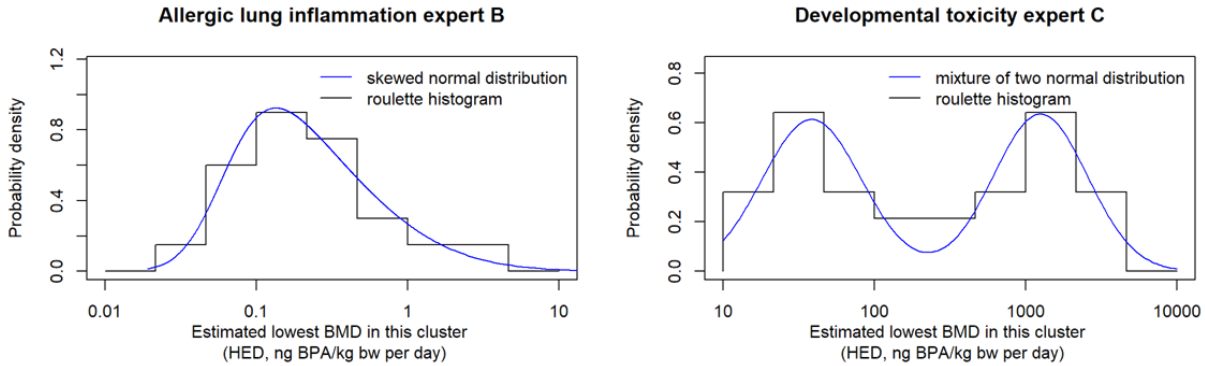


Each bar represents the range of % probability for the specified cluster and expert. Experts were identified as A, B, C for each cluster.

Figure D1: Experts' approximate probabilities, for each cluster, that there is at least one endpoint in the WoE table for the cluster that occurs in animals tested with BPA and is relevant and adverse in humans.

Question 2 was 'If one or more endpoints in the WoE table for this cluster occurs in animals tested with BPA and is both relevant and adverse for humans, what is your prediction for the lowest BMD of those endpoints, expressed as HED?'. In effect, this quantifies uncertainty in hazard characterisation (dose-response) for each cluster. The experts gave their judgement on this in the form of a probability distribution for the estimated lowest BMD in each cluster. Two examples of these distributions are shown in Figure D2, together with the parametric distributions that were fitted to them. The examples in Figure D2 were chosen to illustrate two different types of distributions that were considered to find a good fit for each cluster and expert (a single unskewed or skewed distribution, or a mixture of two unskewed distributions).

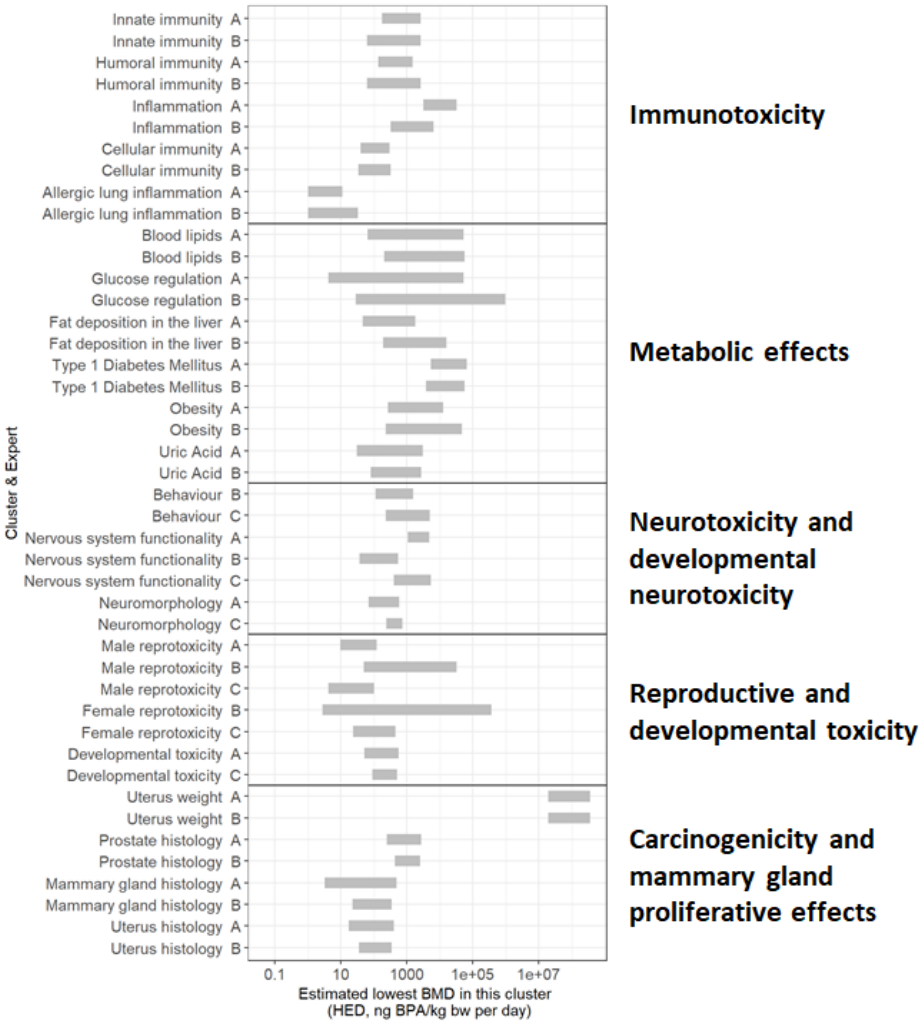
15267 Histograms and fitted distributions for all the clusters and experts are presented in Annex K. A summary of
15268 the fitted distributions is shown in Figure D3, to facilitate comparisons between clusters and between
15269 experts for the same cluster.



15270

15271 Each graph shows the histogram provided by the experts, and the parametric distribution that was subsequently fitted to their
15272 judgements.
15273

15274 **Figure D2:** Two examples, for different clusters, of probability distributions elicited from experts
15275 for the estimated lowest BMD of those endpoints in the cluster that occur in animals tested with BPA
15276 and is both relevant and adverse for humans.



Each bar represents the 90% probability interval of the distribution for the specified cluster and expert. Experts were identified as A, B, C for each cluster.

Figure D3: Summary of distributions fitted to each experts' judgements, for each cluster, for the estimated lowest BMD in the cluster for endpoints that occur in animals tested with BPA and is relevant and adverse in humans.

D.2. Combining judgements for Questions 1 and 2

For each cluster and expert, the WG expert's probability from Question 1 was combined with their distribution from Question 2 by multiplication. This provides, for each cluster and expert, a Cpf for the estimated lowest BMD of effects in that cluster that occur in animals and are relevant and adverse for humans.

Figure D4 shows examples of these cpfs for one expert's judgements on five clusters in the HOC Immunotoxicity. As their probability for Question 1 was expressed as a range, each cpf has a lower and

upper bound, shown as two curves of the same colour. The lower and upper bound of each cpf resulted from multiplying the distribution for Question 2 by, respectively, the lower and upper bound of the probability for Question 1. The lower and upper bounds of the cpf show, on the vertical axis, a range for expert's judgement of the probability that the cluster contains an endpoint that occurs in animals *and* is relevant and adverse in humans *and* has a BMD that is *lower* than the dose shown on the horizontal axis.

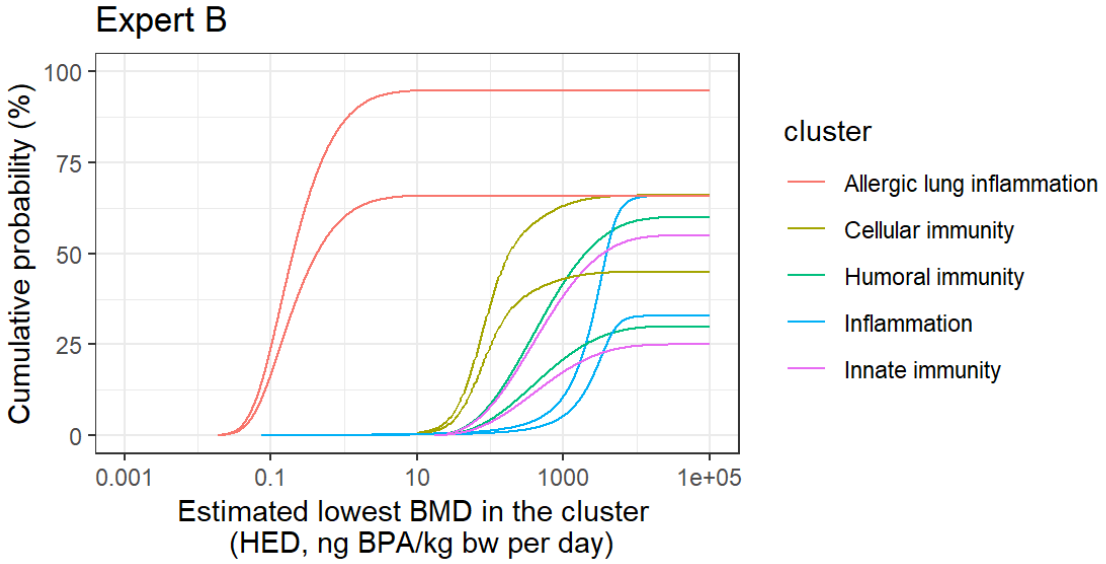


Figure D4: Example of the results of combining one expert's judgements on Questions 1 and 2 for five immunotoxicity clusters; see text for explanation.

D.3. Combining judgements of different experts for the same cluster

The cpfs for different experts were combined by enveloping, i.e., taking the minimum and maximum cumulative probability at each dose. This reduces the 2 or 3 cpfs for the different experts assessing the cluster to a single cpf with a wider gap between its lower and upper bounds, reflecting the combined uncertainty arising from (a) the approximate nature of the Question 1 probabilities and (b) the range of opinion between the experts for both Questions 1 and 2.

Figure D5 shows an example of this process for one immunotoxicity cluster: allergic lung inflammation. The left-hand graph shows the separate cpfs for the two experts who assessed this cluster, and the right-hand graph is the same but with the addition of the lower and upper bounds for the combined cpf (solid black curves).

Interpretation of Figure D5 is similar to Figure D4, but for multiple experts. The lower and upper bound of the enveloped cpf shows, on the vertical axis, the range of probabilities (combined across experts) that the cluster contains an endpoint that occurs in animals *and* is relevant and adverse in humans *and* has a BMD that is *lower* than the dose shown on the horizontal axis.

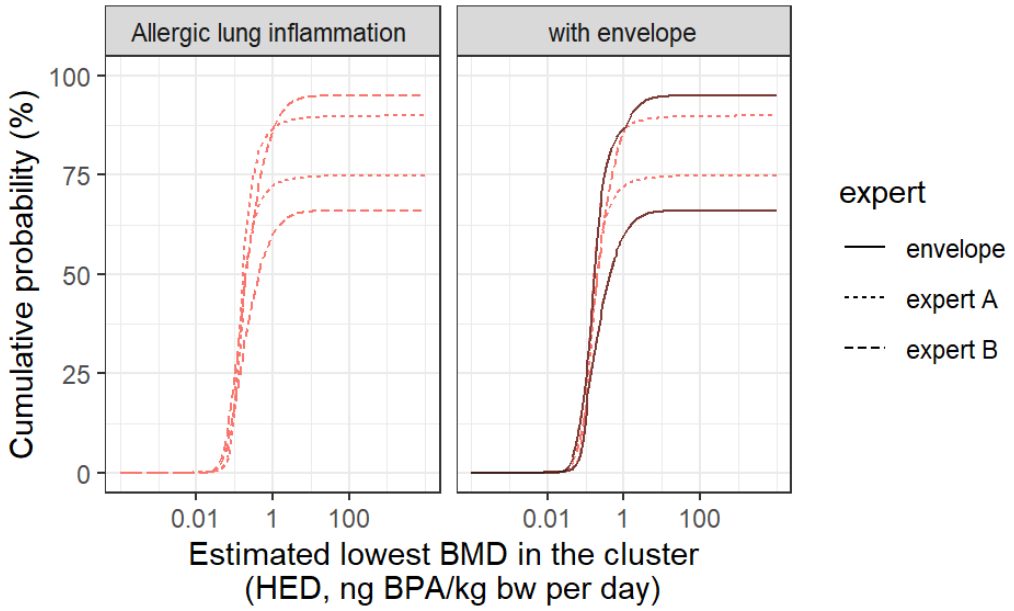


Figure D5: Example illustrating how different experts' judgements for the same cluster were combined by enveloping; see text for explanation.

D.4. Combining judgements for different clusters

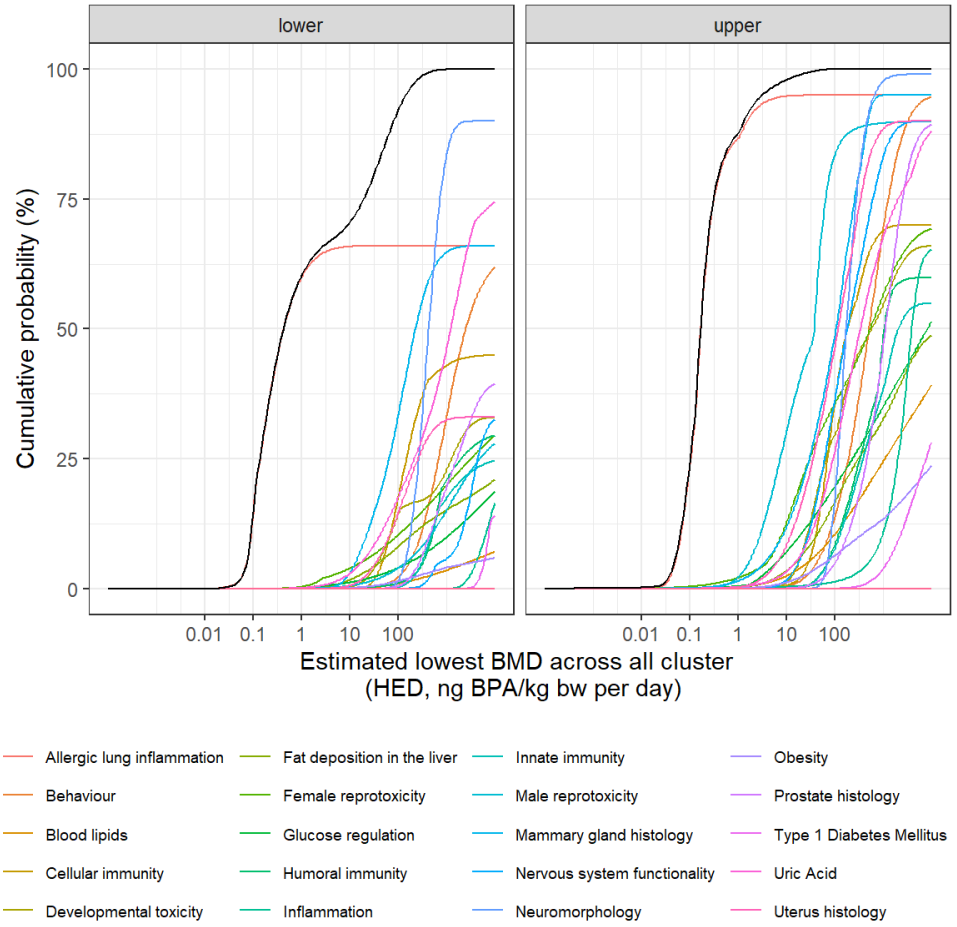
The cpfs for different clusters were combined by the probability calculation described in Annex K to produce a cpf for the estimated lowest BMD across all 21 clusters for endpoints that occur in animals and are relevant and adverse for humans, assuming that the judgements for different clusters are independent. As the cpf for each cluster had a lower and upper bound, the cpf for all 21 clusters also has a lower and upper bound.

Figure 6 shows the inputs and outputs of this calculation. The coloured curves are the lower (left-hand graph) or upper (right-hand graph) bounds of the cpfs for the 21 different clusters, and the black curves are the lower and upper bounds of the cpf for the estimated lowest BMD across all clusters.

Interpretation of Figure D6 is similar to Figures D4 and D5 but combining all the clusters as well as the different experts. The lower and upper bounds for the combined cpf show, on the vertical axis, the range of probabilities (combined across experts) that *one or more* of the 21 clusters contains an endpoint that occurs in animals *and* is relevant and adverse in humans *and* has a BMD that is *lower* than the dose shown on the horizontal axis. This is relevant because the purpose of the uncertainty analysis is to assess whether the probability of one or more effects in the 21 clusters occurring after exposure to lower doses than the endpoint used for the RP is high enough to warrant an additional UF and, if so, determine how large the UF should be to reduce that probability of such effects to an appropriately low level.

It can be seen from Figure 6 that both the lower and upper bounds of the combined cpf are determined mainly by the cluster for allergic lung inflammation. The lower part of each black curve follows the coloured

curve for allergic lung inflammation, because there is virtually no probability of effects in other clusters in that dose range. The cpfs for other clusters are at higher doses (further to the right) so, at those doses, the combined cpf rises above the cpf for allergic lung inflammation due to the added probability of effects from one or more of those clusters.



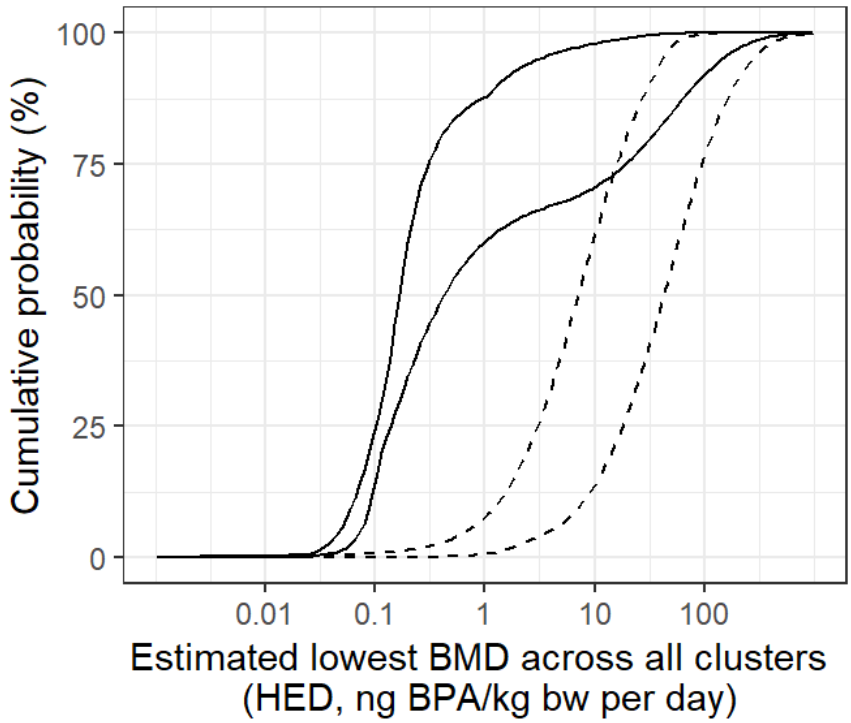
The coloured curves show the lower and upper bounds of the cpfs for the individual clusters, from which the combined cpf is calculated.

Figure D6: The black curves show the lower and upper bounds of the cpf for the estimated lowest BMD across all clusters for endpoints that occur in animals and are relevant and adverse for humans, assuming that the experts' judgements for different clusters are independent. The coloured curves show the lower and upper bounds of the cpfs for the individual clusters, from which the combined cpf is calculated.

D.5. Initial sensitivity analysis

A series of calculations was done to explore the sensitivity of the combined cpf to different factors. First, the calculations were repeated including and excluding the cluster for allergic lung inflammation, to confirm

its influence on the combined cpf. Figure D7 shows that, when allergic lung inflammation is excluded, the combined cpf shifts to the right by about two orders of magnitude. In other words, when allergic lung inflammation is excluded, the doses at which any effects are expected increase by about two orders of magnitude.



Lower and upper bound curves are shown for each cpf. See text for explanation.

Figure D7: Comparison of cpfs for the estimated lowest BMD combining all the assessed clusters including (solid curves) and excluding (dashed curves) allergic lung inflammation.

A second sensitivity analysis showed very little change in the combined cpfs when the parametric distributions fitted to each expert's judgements for Question 2 were replaced by the histograms provided by each expert.

D.6. Expert review and revision of calculation results

As allergic lung inflammation had much more influence on the combined cpf than other clusters, the assessment of this cluster was reviewed by the whole WG. There was a detailed discussion of the quality and results of the studies considered for allergic lung inflammation and of its relevance to an adversity in humans. In the light of this discussion, the two experts responsible for assessing this cluster both revised their judgements for Question 1 to 50–90% probability but retained their original judgements for Question 2. When the revision for Question 1 was shared with the rest of the WG, three other experts proposed a

lower bound probability of 33% rather than 50%, and it was agreed to explore the effect of this difference of opinion when revising the calculations.

In the same meeting, the WG experts considered whether it was necessary to elicit judgements for the General toxicity clusters, which were excluded from the earlier assessment. It was noted that no Likely or Very Likely clusters were identified for this HOC, and the lowest effect level reported was 8 ng/kg bw per day (HED) for relative liver weight (Ke et al., 2016 [RefID 3447]; single dose study) with an effect size of about 4% which was considered non-adverse. Therefore elicitation was not needed for any of the General toxicity clusters.

D.7. Elicitation of additional judgements for the most influential cluster

In the light of the range of opinion expressed when the WG experts discussed allergic lung inflammation, judgements on Question 2 for this cluster were elicited from all the experts in the WG. Before eliciting the judgements, the key endpoints from the dose-response studies for this cluster were reviewed (Table D1) and uncertainties affecting these studies were discussed in detail. These included issues relating to litter control, missing animals for some endpoints, and lack of information about cross-contamination precautions for O'Brien et al., 2014a [RefID 5462]; a single control litter with only three mice and only males or females in two of the dose groups and differing proportions of male and female animals in control and treated groups for O'Brien et al., 2014b [RefID 5463]; no details of analysis of diet for BPA in either RefID 5462 or 5463; unclear group sizes and insufficient information on blinding in Tajiki-Nishino et al., 2018 [RefID 13211].

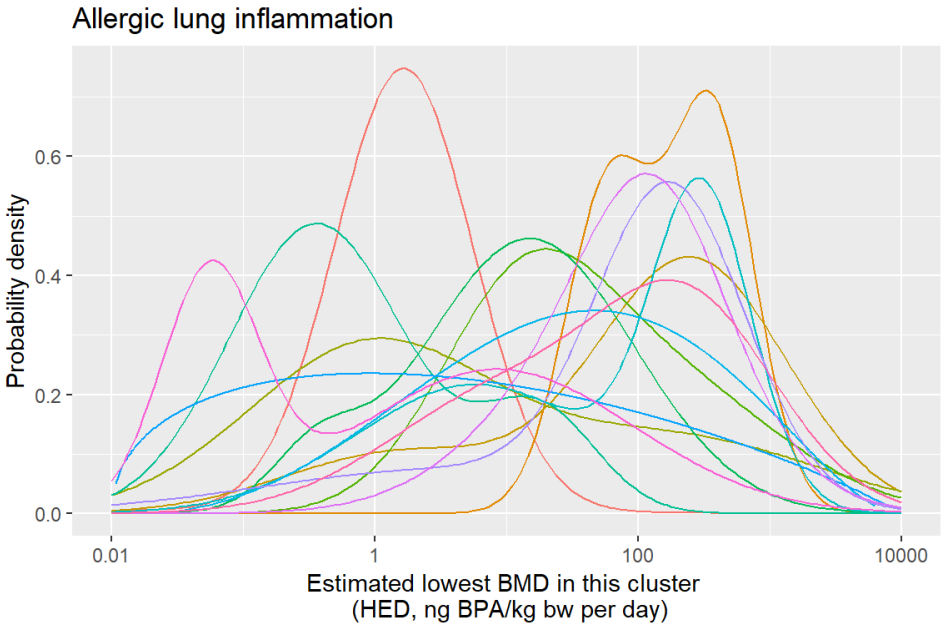
Table D1: Summary of key endpoints considered for elicitation of judgements on Question 2 for allergic lung inflammation.

RefID	Endpoint	NOAEL (ng/kg bw per day) HED	LOAEL (ng/kg bw per day) HED	Effect size compared with the control
5462 O'Brien et al., 2014a	anti-OVA IgE in females	n.a.	0.116	39% (at the LOAEL)
5462 O'Brien et al., 2014a	anti-OVA IgE in males	0.116	116	271% (at the NOAEL)
5463 O'Brien et al., 2014b	cysteinyl leukotrienes	n.a.	0.116	1693% (at the LOAEL)
5463 O'Brien et al., 2014b	prostaglandin D2	116	116000	89% (at the NOAEL)
13211 Tajiki-Nishino et al., 2018	score of red colour	n.a.	930	140% (at the LOAEL)
13211 Tajiki-Nishino et al., 2018	eosinophil infiltration in BALF	930	3100	67% (at the NOAEL)

LOAEL: Lowest observed adverse effect level
NOAEL : No observed adverse effect level
n.a: not available

The experts were then asked to consider individually their plausible limits for Question 2 for this cluster, and it was established that all of these lay within the range from 0.01 ng/kg bw per day HED to 3000 ng/kg bw per day HED. Distributions within this range were then elicited from 14 WG experts including the two experts that made the original assessment for this cluster, who revised their earlier judgements in the light

of the discussion. The resulting judgements are presented in Table 28 in Annex K and the fitted distributions are shown in Figure D8.



The raw judgements are shown in Table 1 of Annex K.

Figure D8: Parametric distributions fitted to the judgements of 14 WG experts for Question 2 for the cluster allergic lung inflammation.

Reasons for the wide range of opinion shown in Figure D8 were explored by discussing and listing the evidence and reasoning for the lower and upper ends of the range covered by the experts' individual distributions. The outcome of this is summarised in Table D2.

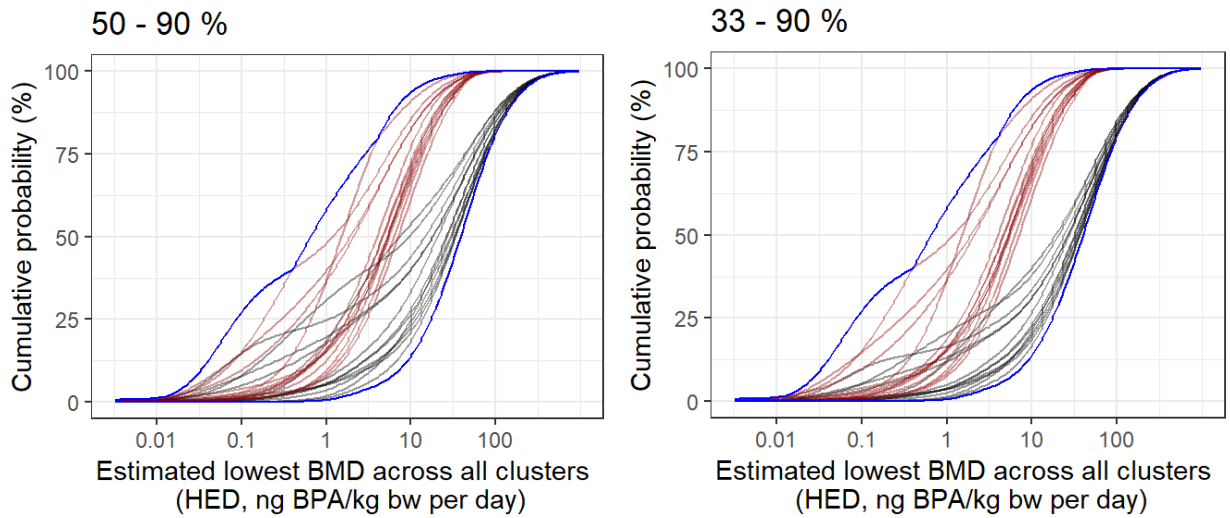
Table D2: Key considerations identified by the WG experts as influencing the lower and upper ends of their distributions for Question 2 for allergic lung inflammation (shown in Table D1 and Figure D8).

Key considerations influencing the *lower* ends of the experts' distributions (lower BMDs)	**Key considerations influencing the *upper* ends of the experts' distributions (higher BMDs)**
• Anti-OVA IgE in females, anti-OVA IgE in males, cysteinyl leukotrienes seen in this dose range and are likely to be adverse • More downstream effects, e.g. eosinophil infiltration in BALF more apical and increase probability that IgE effect is adverse, although appearing at higher doses • If you are sensitised to one or multiple allergens, BPA increases the IgE levels on your mast cells, which increases the potential for adverse/apical effects to follow from subsequent exposure to the same allergens	• Effect on eosinophil infiltration in BALF in better-rated studies than the effects at lower doses • Sex difference in IgE effects may raise uncertainty about those → more weight to effects seen at mid-range (but there are some reasons for expecting greater sensitivity in females) • Small effect in females significant but larger effect in males is not significant – may be due to higher variability in males

• Several parameters in these studies show effects, some with clear dose-response, adding to the weight for the cluster as a whole • 30% of people in EU population are sensitised so significant group at potentially increased risk	• Doubts about doses in studies at very low range (not measured, very difficult to achieve homogeneously), lack of explanation of different group sizes, small groups and litter effect, very large (×1000) dose intervals, only single study so reproducibility is unknown – this all leads to doubt whether the effect at the lowest dose is real or chance result of multiple testing and/or publication bias • Effects at higher doses are more clearly adverse

15411

15412 The probability calculations to obtain a combined cpf for the estimated lowest BMD across all clusters was
15413 performed separately with each expert's fitted distribution for Question 2 for allergic lung inflammation.
15414 Figure D9 shows the resulting upper (red curves) and lower bounds (black curves) of the cpfs for each
15415 expert, and the lower and upper bound of a combined cpf (blue curves), obtained by enveloping the
15416 individual cpfs. Comparing the left and right-hand graphs shows the effect of the alternative lower bound
15417 probability for Question 1 on allergic lung inflammation, moving the lower bounds of the cpfs downwards
15418 and to the right.



15419

15420 The red and black curves show the upper and lower boundaries for the individual experts' cpfs, from which the lower and upper
15421 bounds for combined cpf are obtained. Question 1 approximate probability for allergic lung inflammation is 50–90% in the left-hand
15422 graph and 33–90% in the right-hand graph.

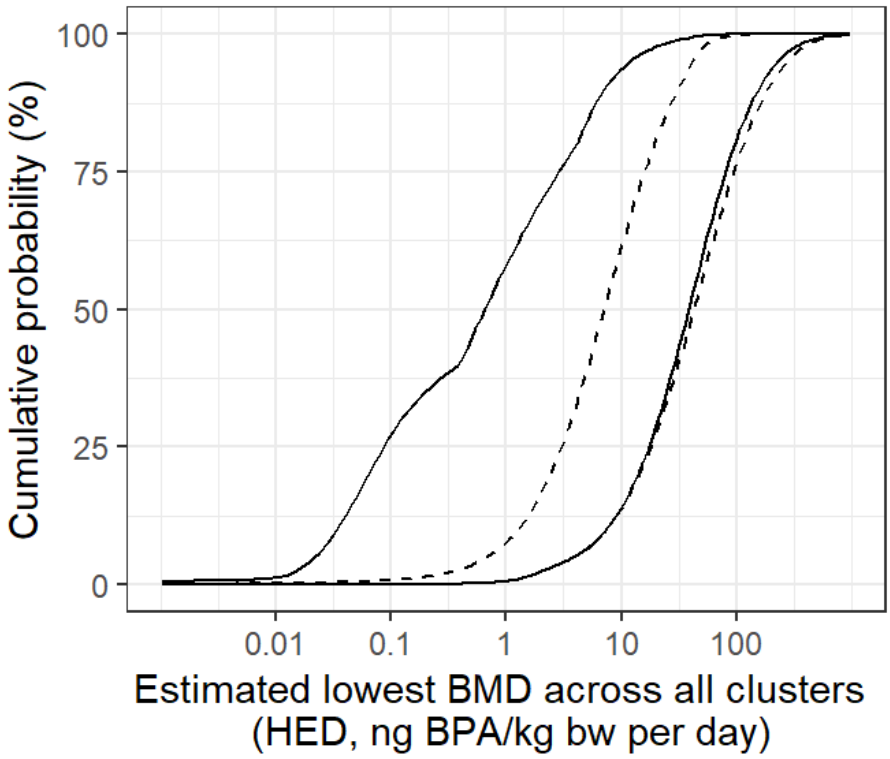
15423 **Figure D9:** The blue curves show the lower and upper bounds for the cpf for the estimated lowest
15424 BMD across all clusters and all experts for endpoints that occur in animals and are relevant and
15425 adverse for humans, assuming that the experts' judgements for different clusters are independent.

15426 ## D.8. Revised sensitivity analysis

15427 More extensive sensitivity analysis was conducted at this point, to inform the WG experts' assessment of
15428 additional uncertainties and dependencies. Comparison of Figure D10 with Figure D7 shows that when the
15429 original judgements for allergic lung inflammation are replaced with the distributions shown in Figure D8,

15430 allergic lung inflammation still has a very large influence on the upper bound of the combined cpf but much
15431 less on the lower bound.

15432

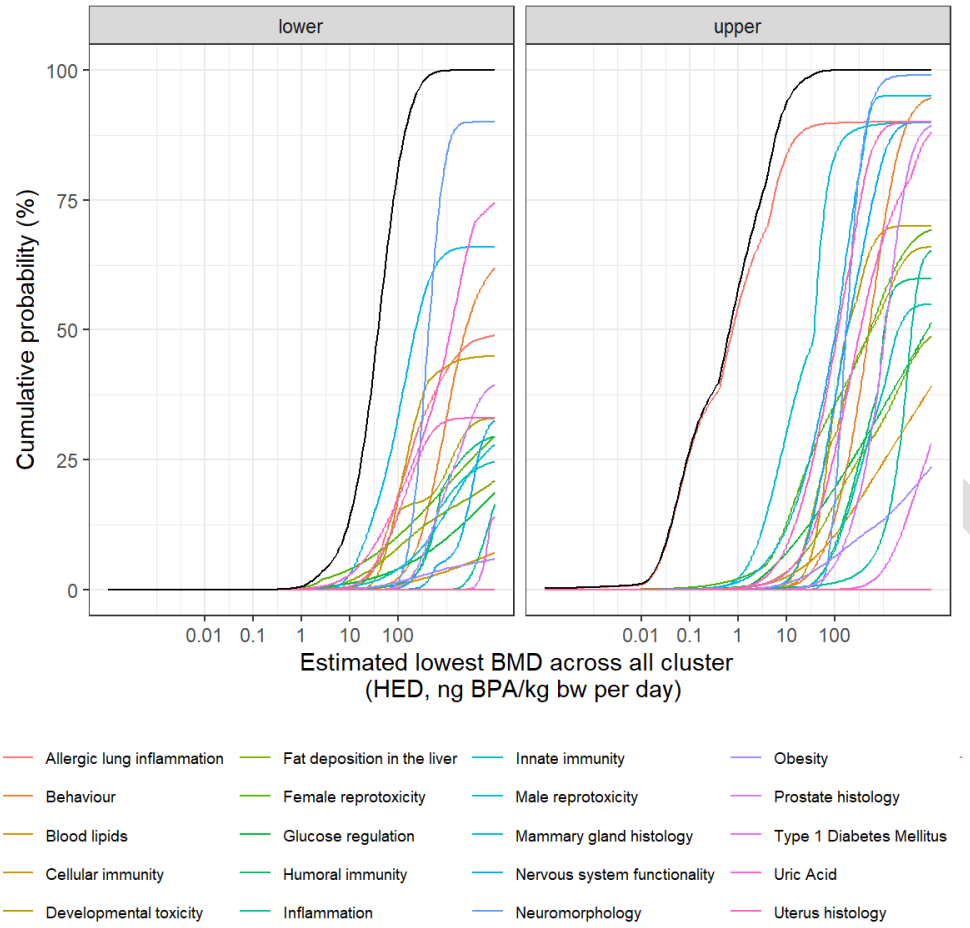


15433

15434 The solid curves are lower and upper boundaries for the combined cpf including allergic lung inflammation, based on the expert
15435 judgements shown in Table D2, while the dashed curves exclude this cluster.

15436 **Figure D10:** Revised comparison of cpfs combining all the assessed clusters, including and excluding
15437 allergic lung inflammation.

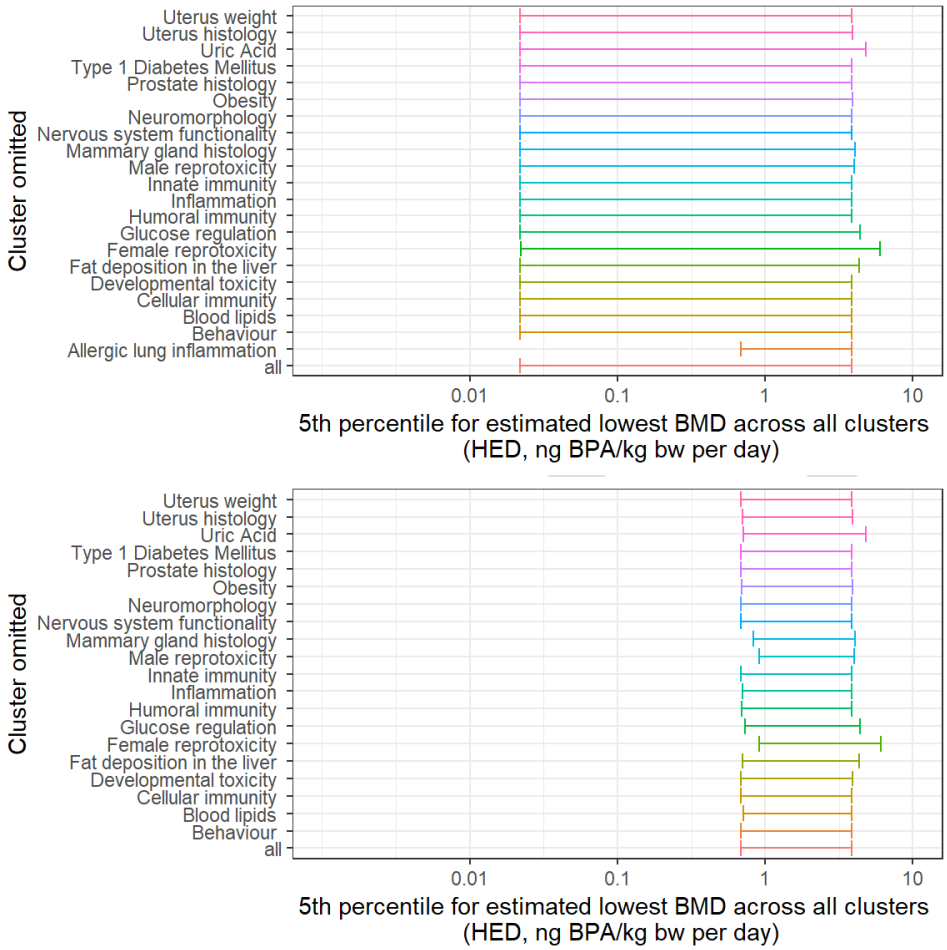
15438 The contributions of different clusters to the combined cpf including allergic lung inflammation are
15439 examined further in Figures D11 and D12. In Figure D11, the upper bound of the combined cpf (black
15440 curve in right-hand graph) is driven entirely by allergic lung inflammation (red) at the lower end, with a
15441 small but increasing contribution from other clusters at the upper end. In contrast with this, the lower
15442 bound for the combined cpf (black, left-hand graph) is entirely a combination of multiple clusters (various
15443 colours) with only a minor contribution from allergic lung inflammation.



The black curves in the left and right-hand graphs show respectively the lower and upper bounds of the combined cpf. The coloured curves show the lower and upper bounds of the cpfs for the individual clusters, from which the combined cpf is calculated. Comparing these graphs with Figure D6 shows the effect of the revised distributions for Question 2 for allergic lung inflammation, shown in Figure D8.

Figure D11: Graph indicating the contributions of different clusters to the combined cpf for the estimated lowest BMD across all clusters.

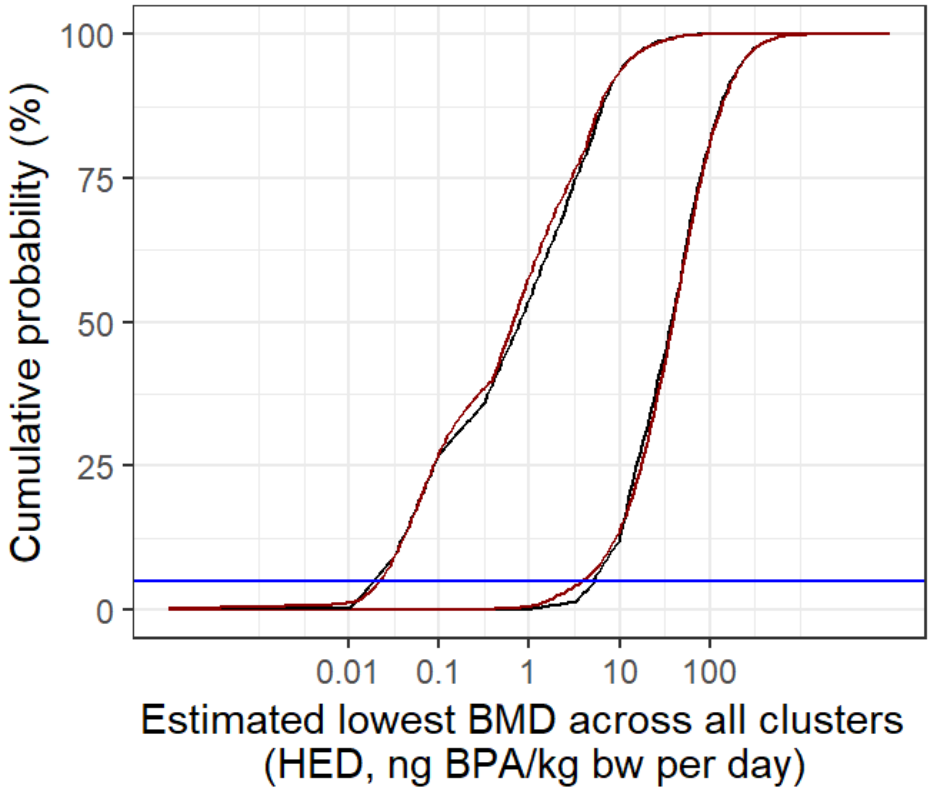
Figure D12 shows two sensitivity analyses in which different clusters were excluded from the calculations in turn to assess their contribution to the fifth percentile of the lower and upper bounds of the combined cpf. The left-hand graph shows that the lower bound for the fifth percentile increases markedly when allergic lung inflammation is excluded, while the upper bound fifth percentile is affected similarly by all clusters, although slightly more for the clusters uric acid, mammary gland histology, glucose regulation, female reproductive toxicity and fat deposition in the liver. The right-hand graph shows that when both allergic lung inflammation and a second cluster are excluded, the choice of second cluster to exclude has much less effect, although slightly larger for the clusters uric acid, mammary gland histology, male reproductive toxicity, glucose regulation, female reproductive toxicity and fat deposition in the liver.



Each bar shows the lower and upper bound for the fifth percentile when a different cluster is omitted. In the upper graph a single cluster is omitted while, in the lower graph, allergic lung inflammation plus a second cluster is omitted.

Figure D12: Impact of omitting different clusters on the fifth percentile of the combined cpf for the estimated lowest BMD across clusters.

Figure D13 shows that, with the revised judgements, there was still little change in the combined cpf when the parametric distributions fitted to each expert's judgements for Question 2 were replaced with the histograms provided by each expert. The difference is larger below the fifth percentile, but as this is below the probability that is conventionally used for the BMDL it is less likely to be important for deciding on whether an additional UF is needed.



The blue horizontal line marks the fifth percentile of cumulative probability.

Figure D13: Sensitivity analysis assessing the difference between using fitted parametric distributions or the histograms provided by the experts for Question 2.

The calculations performed to combine clusters assumed that the distributions for different clusters were independent of one another, so it was important to consider whether dependencies might be present and take account of this when assessing overall uncertainty. Distributions quantifying uncertainty for two different clusters would be dependent if obtaining more information (e.g. new studies) for one cluster might alter the experts' judgements and distributions for the other cluster. As such dependencies could not be ruled out for all combinations of clusters, a sensitivity analysis was performed to explore the potential impact of hypothetical dependencies between allergic lung inflammation and either male or female reproductive toxicity. These three clusters were chosen not because a biological relationship between them was assumed but because they had more influence on the assessment than other clusters and therefore more potential to show an impact of dependency. Figure D14 shows that hypothetical strong positive dependencies (correlation coefficients 0.5 and 0.9) or negative dependency (correlation coefficient −0.9) between allergic lung inflammation and male reproductive toxicity would cause only small changes in the upper third of the cpf. There was negligible change in the lower part of the cpf, which is more relevant for deciding whether an additional UF is needed. Hypothetical dependencies between allergic lung inflammation and female reproductive toxicity also had little impact. It was provisionally concluded that

using parametric distributions and assuming independence between clusters are not important sources of uncertainty.

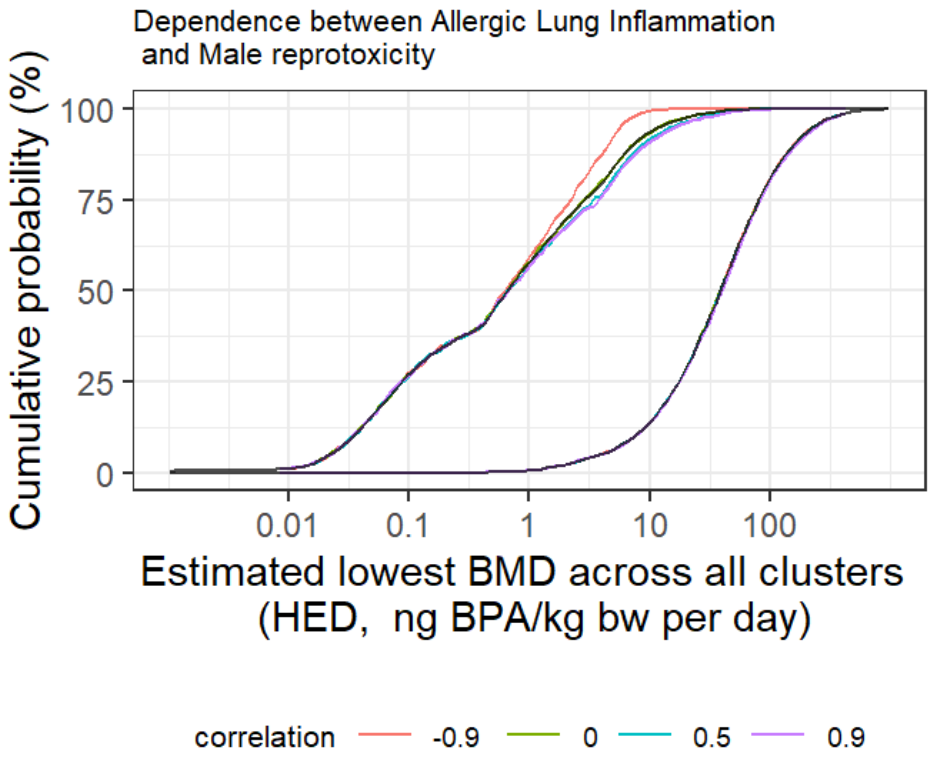


Figure D14: Sensitivity analysis assessing the impact of hypothetical dependencies between allergic lung inflammation and male reproductive toxicity. See text for explanation.

D.9. Consideration of additional uncertainties and dependencies

As preparation for assessing overall uncertainty (EFSA Scientific Committee, 2018a), the WG experts reviewed the results of the revised calculations and sensitivity analysis and held a structured discussion to elicit a list of additional sources of uncertainty potentially affecting the hazard assessment. The WG experts also identified which of these uncertainties had lower or higher potential to contribute to the overall uncertainty of the assessment. The resulting list is presented in Table D3.

15503 **Table D3:** Sources of uncertainty considered as potentially affecting the hazard assessment (excluding the genotoxicity assessment). The
15504 two parts of the table distinguish uncertainties affecting different aspects of the hazard and uncertainty assessments.

Sections of the opinion	Factors of uncertainty	Potential contribution to overall uncertainty
Uncertainty analysis	**Differences in judgement between experts** because the dose range spanned several orders of magnitude (see figure X)	high
	Number of experts per cluster: The number of experts per cluster was considered as a possible source of uncertainty. The EKE for allergic lung inflammation was addressed by the whole WG (14 experts) but for the other clusters (two or three experts), this factor might have more effect. However, each cluster was assessed by WG members with special expertise on the endpoints discussed	low
	Difficulty in the EKE judgements: The EKE judgements (including translation into probabilities) were considered difficult to perform. This was taken into account for Q1 by using approximate probabilities. For Q2, the experts addressed uncertainty in their judgements by expanding the width of the distributions	low
	Calculations: Dependencies between clusters and the set of distributions considered and the rules used for selecting the best fit have much smaller impact than the expert differences	low
Methodology, inclusion criteria, choices and decisions in different parts of the assessment	**Literature search**: The literature search was done according to the protocol; the impact of the language (only English) was investigated (see Appendix D of Annex A) and considered minor. The journals and search strategy used were considered to provide a high coverage. The time span was done according to the terms of references	low
	Publication bias in animal studies: more likely to not publish negative results	high
	Publication bias in epidemiological studies: some negative findings on BPA may not have been published. For longitudinal studies less impact is expected	low
	Screening of title and abstract and full text: The criteria for the screening were well defined and the screening was double-checked	low
	Internal validity: Three key questions were defined in the assessment of the internal validity in animal studies; if one of them was scored negatively a study would be allocated directly to Tier 3 (low internal validity and high risk of bias (see Annex A, Section 6)) Some variation in answering to these key questions in different clusters may be possible. This variation represents a possible source of uncertainty because it would imply a variation in the tier allocation of the study, which, in turn, could have an impact on the selection of the studies reporting relevant endpoints and, therefore, to be considered in the WoE.	high
	Lack of consistent terminology in the publications: A source of uncertainty could be linked to the choice of relevant endpoints for each HOC by the experts, for which there was lack of consistent terminology of the endpoints in different published studies (e.g. for cardiotoxicity, where 'cardiomyopathy' comprises several endpoints, hampering their individual assessments, and for Carcinogenicity and mammary gland proliferative effects, for which e.g. ductal growth has not a unique definition)	low
	Grouping of endpoints into clusters: the experts' choices on how to group the relevant endpoints into clusters may have affected the outcome of the WoE (for example, neoplastic lesions in mammary gland that was considered a likely endpoint in a overall ALAN cluster). However, the impact of this is low, because the framing of the elicitation questions (assessing the estimated lowest BMD for any endpoint in each cluster) ensured that all endpoints were considered regardless of to which cluster they were assigned	low
	Statistical analysis: the data of three Grantee studies (Greenberg, 2018 [RefID 13785]; Ben-Jonathan, 2018, [RefID 13786]; Gonzalez-Cadavid, 2018 [RefID 13787]) of the Clarity FDA program were not yet published in peer-reviewed journals when this opinion was	low

	completed. The raw data were available on the FDA website and were considered by EFSA in conformity to the ToR. A statistical analysis was conducted by EFSA following the material and methods provided on the FDA website	
	Statistical analysis: in one case (Montévil et al. 2020 [RefID 13788]), the study authors used an unconventional methodology to assess dose-response and the CEP Panel made its own analysis	low
	Knowledge of the endpoints: limited knowledge on how certain intermediate endpoints are related to apical endpoints and how much is known about their MoA is a source of uncertainty for some clusters but was taken into account in Q1 in the UA	low
	Choice of BMRs (for BMD analysis and uncertainty analysis): the choice of BMRs was based on expert judgement; for some endpoints there was little specific evidence available on this, so default values were used. This aspect was taken into account in Q2 of the UA	low
	BMD analysis: If the BMDL was more than 10 times lower than the lowest non-zero dose, the study was not considered for the selection of the RP. In addition, for some endpoints a BMD analysis was not possible. In the uncertainty analysis all Tier 1 and Tier 2 studies in the WoE table were considered, taking into account the NOAELs, LOAELs and effect sizes and also BMDL and BMDU values, when available	low
	Exposure factors: data for conversion from BPA in feed/drinking water to mg/kg bw per day during gestation/lactation period were not available for few animal species (Rhesus Monkey and Marmoset monkey, see Chapter 3.1.1), therefore, some papers could not be considered in the UA	low
	HED factors: toxicokinetic data on BPA in mice were based on a study (Doerge et al., 2011) where some of the measured values were below the level of detection. The CEP Panel decided, as in 2015, to make assumptions on where the AUC level could be, based on this study and the interspecies comparison (Doerge et al., 2011) TKs data on BPA in humans were available in two experimental studies with different estimates of the BPA AUCs; the CEP Panel decided to use the median of the values from the two studies, in which BPA was administered in two different foods (soup vs. cookies)	high for mice, low for the other species
	AUC for liver: no data were available on the proportion of the pre-systemic elimination of BPA from enterocytes in the gut wall and from the liver cells, which would be needed for parameterising a PBPK model that would allow prediction of the concentration in the liver and the related AUC. In the absence of such data, the AUC in the peripheral blood was used also for the liver, to substitute the toxicokinetic part of the UF by an HEDF (see more detail in the Chapter 3.1.1.4). However, as the liver effects were not identified as critical endpoints, this uncertainty was considered low	low
	MoA studies: the MoA studies were not subjected to the same formal evaluation processes applied to the other studies so variation in their judgments may be possible in different HOCs. However, these studies were not considered for the assessment of the likelihood of the effects	low
	WoE assessment: Some endpoints were excluded from the WoE because occurring only in tier 3 studies (e.g. fat pad weights and liver fat % in HOC Metabolic effects or bronchial reactivity to methacholine in lung inflammation studies)	high
	WoE assessment: in clusters with a small number of studies (e.g. for allergic lung inflammation in Immunotoxicity, uric acid in Metabolic effects) there is a smaller opportunity to detect inconsistent results and, therefore, there is a higher chance to consider them as Likely or Not Likely. In contrast, clusters with more studies (e.g. obesity in Metabolic effects) are more prone to be considered as ALAN. This was considered as a potential source of high uncertainty but was taken into account in the uncertainty analysis for the most influential endpoint, allergic lung inflammation	low

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Reconsideration of previous publications already assessed by EFSA, and the additional literature produced between the ending date of the literature search (15 October 2018) and the publication date of this opinion, were not considered additional sources of uncertainty because they are outside the scope of this opinion as defined by the Terms of Reference.

Reviewing the uncertainties listed in Table D3 and the results of the sensitivity analyses conducted earlier, the WG experts judged that they would not alter the assessment of overall uncertainty provided already by the wide range of distributions (shown in Figure D9), resulting from the judgements of different experts for the most influential cluster. The WG experts also noted that they had already considered many of the listed uncertainties when making their personal judgements earlier in the uncertainty analysis, and they judged that the additional uncertainties would not have altered those judgements. The WG experts therefore agreed to use the range of distributions in Figure D9 as the basis for assessing overall uncertainty about the estimated lowest BMD across all clusters, and to explore options for integrating or averaging individual judgements to assist in developing consensus conclusions on the overall uncertainty.

D.10. Averaging cpfs of different experts for the same cluster

The dashed red curves in the left-hand graph in Figure D15 show the results of revised calculations in which the cpfs of different experts for each cluster were averaged by taking the linear pool. Comparing them with the previous cpfs (black solid curves), for which the experts were aggregated by enveloping, it is evident that averaging over experts greatly reduces the distance between the lower and upper bounds. This confirms that differences between experts (especially for allergic lung inflammation) are the largest contributor to the quantified uncertainty. The remaining distance between the upper and lower bounds of the averaged cpfs reflects the combined effect of the differences between lower and upper bounds of the approximate probabilities for Question 1 for all the clusters and experts.

A sensitivity analysis (not shown) demonstrated that the remaining gap between the lower and upper bounds of the cpfs would be significantly reduced if the WG experts were to agree on a single consensus probability for Question 1 for allergic lung inflammation, replacing the range by a single probability value. The reasoning of the two experts with specialist knowledge of this cluster for their judgements on Question 1 was displayed. After a short discussion, the WG experts agreed on a consensus of 66% probability. The effect of this on the gap between the lower and upper bounds of the cpf for the estimated lowest BMD across all clusters is shown in the right-hand graph in Figure 25.

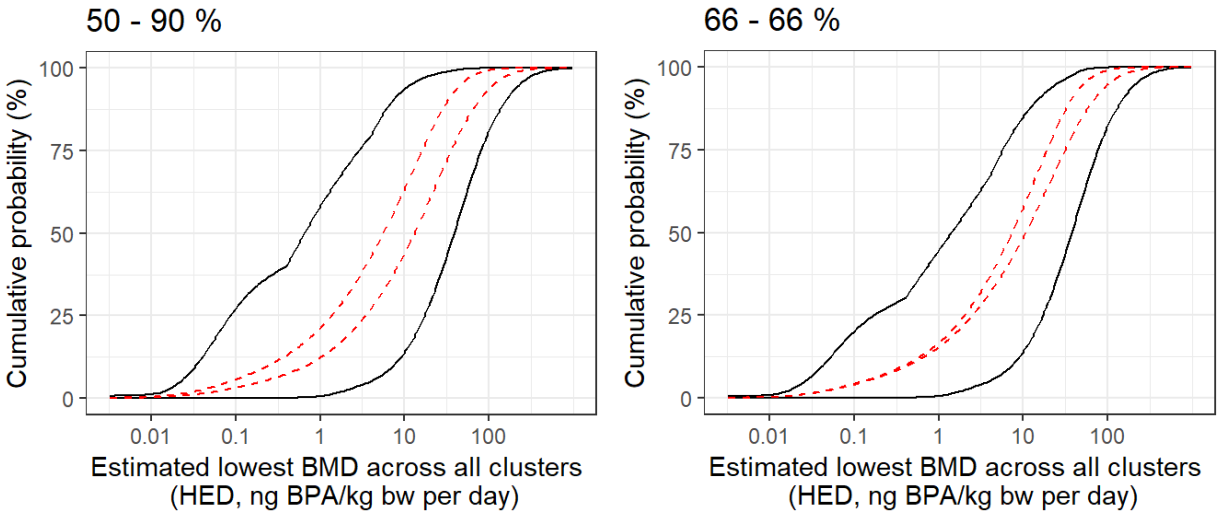


Figure D15: Effect on the lower and upper bounds of the cpf for the estimated lowest BMD across all clusters when the cpfs of different experts for each cluster were aggregated by averaging (dashed red curves) instead of being enveloped (solid black curves) and when the approximate probability of 50–90% for Question 1 for allergic lung inflammation (left-hand graph) was replaced with the WG consensus of 66% (right-hand graph).

The WG experts agreed to take the results of averaging cpfs across experts in each cluster (red dashed curves in right-hand graph of Figure 25) as their consensus assessment of overall uncertainty (including the uncertainties listed in Table 30) for the estimated lowest BMD across all clusters including General toxicity clusters (for the reasons given earlier) and to use this as the basis for considering whether an additional UF is needed when deriving the TDI. They agreed to present also the lower and upper bounds of the combined cpf obtained by enveloping the individual distributions (black solid curves in right-hand graph of Figure D15), to make clear the range of opinion between experts.

D.11. Sensitivity analysis for consensus distribution

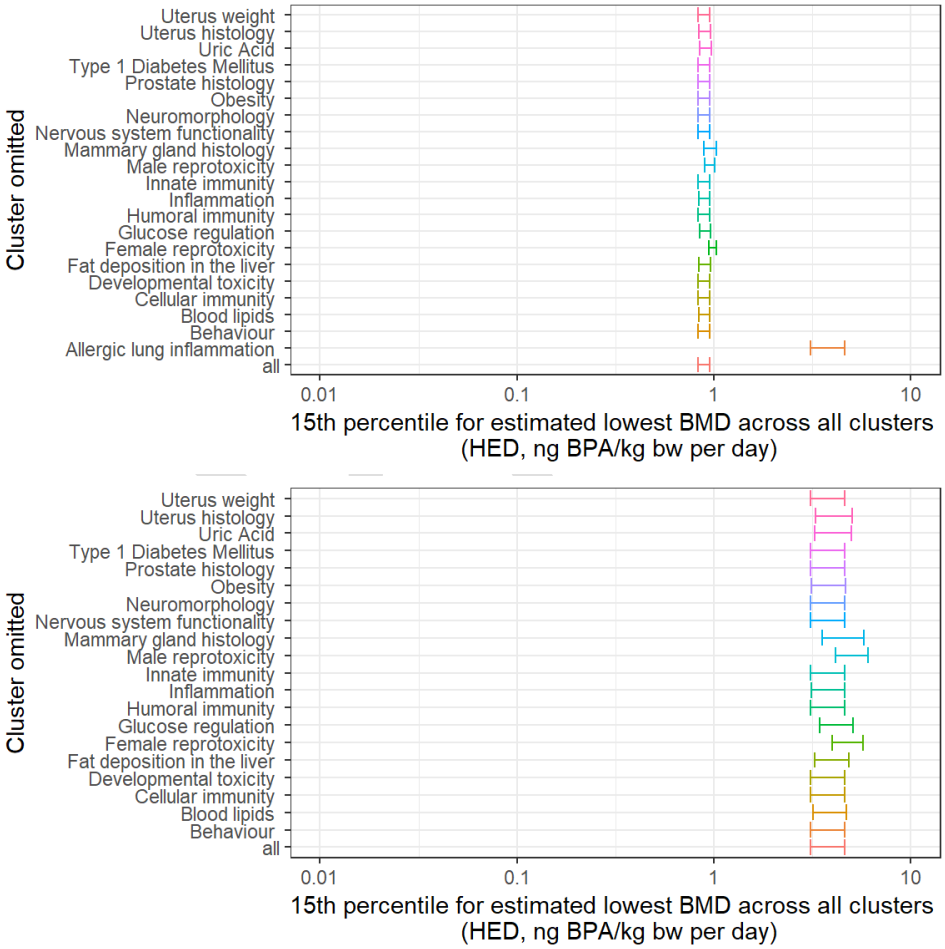
The sensitivity analyses were repeated using the consensus probability of 66% for Question 1 on allergic lung inflammation and the averaged individual cpfs for each cluster. The purpose of this was to check which clusters had most influence on the final consensus cpf for the estimated lowest BMD across all clusters and to check whether the consensus cpf was more sensitive to the method of distribution fitting and hypothetical dependencies between clusters than was the case in earlier sensitivity analysis. The revised sensitivity analyses focused on the 15th percentile of the consensus cpf, as this was close to the RP of 0.93 ng BPA/kg bw per day.

Figure D16 shows that, as in the earlier sensitivity analysis, the consensus cpf is determined mainly by allergic lung inflammation. When both allergic lung inflammation and a second cluster are excluded, the choice of second cluster to exclude has much less effect, although the clusters mammary gland histology, male reproductive toxicity and female reproductive toxicity have more influence than other clusters.

Figure D17 shows that using the histograms provided by the experts for Question 2 shifts the lower and upper bounds of the consensus cpf slightly to the right when compared with those obtained with fitted

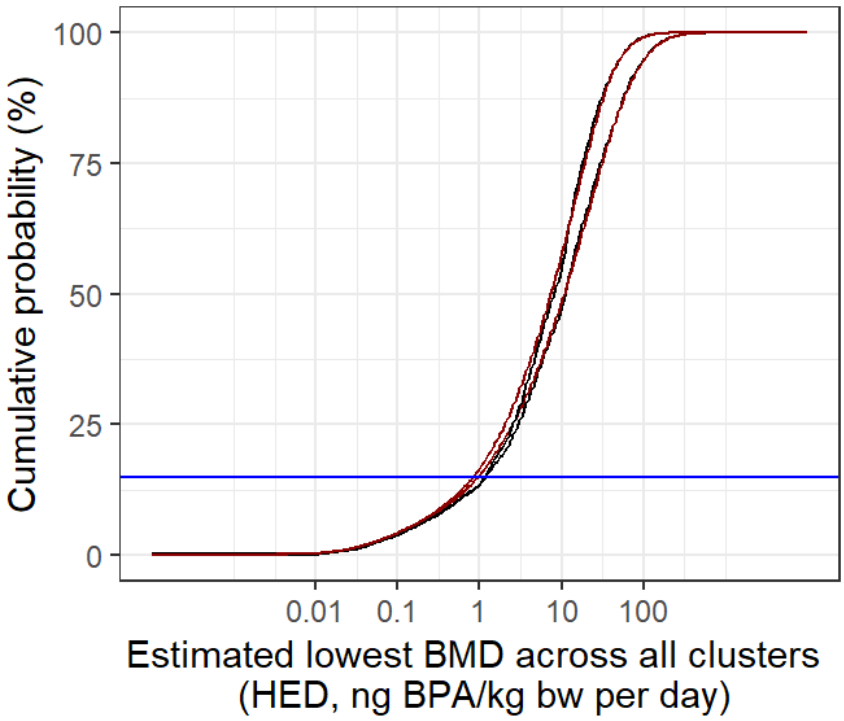
parametric distributions, including in the region of the 15th percentile. As the histograms more directly reflect the experts' judgements, it was agreed to take account of both cpfs in Figure D17 when considering whether an additional UF is needed.

The need to consider potential dependencies and the method used for this are explained in the text preceding Figure D14. Figure D18 shows that hypothetically large positive and negative dependencies between allergic lung inflammation and male reproductive toxicity have little impact on the lower part of the consensus cpf, including at the 15th percentile. The same result was obtained for hypothetical dependencies between allergic lung inflammation and female reproductive toxicity, and between male and female reproductive toxicity. As other clusters have less influence on the consensus cpf, any dependency between them would also be negligible. The CEP Panel concluded that, if there were dependencies between clusters, they would have negligible impact on the consensus cpf.



Each bar shows the lower and upper bound for the 15th percentile when a different cluster is omitted. In the upper graph a single cluster is omitted while, in the lower graph, allergic lung inflammation plus a second cluster is omitted.

Figure D16: Impact of omitting different clusters on the 15th percentile of the consensus cpf for the estimated lowest BMD across clusters.



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15580 The blue horizontal line marks the 15th percentile of cumulative probability.

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15582 **Figure D17:** Sensitivity analysis assessing the difference between using fitted parametric
15583 distributions or the histograms provided by the experts for Question 2.

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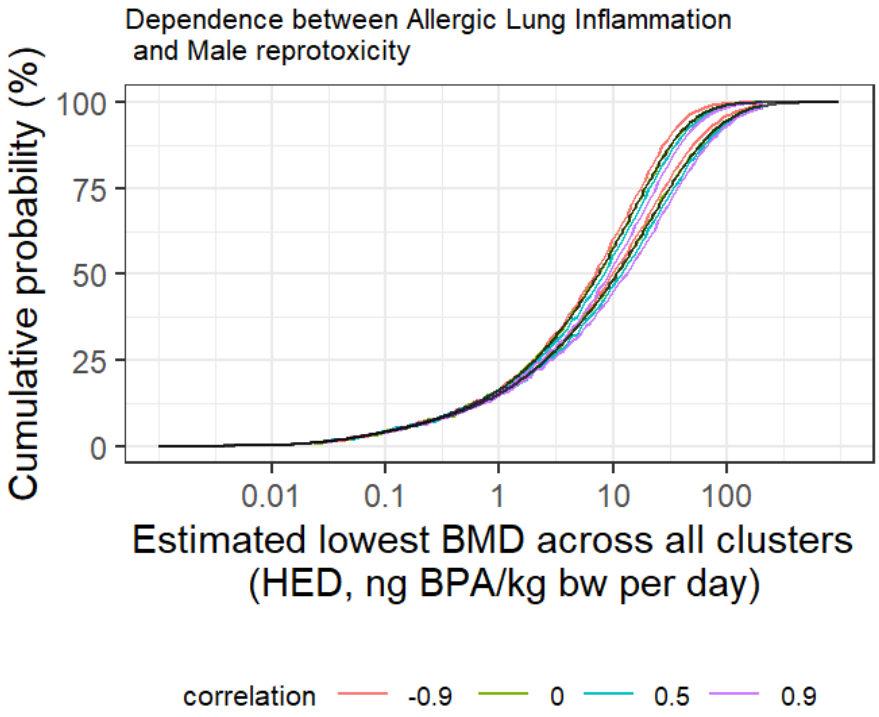

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15587 See text above Figure D14 for explanation of hypothetical dependencies.

15588 **Figure D18:** Sensitivity analysis assessing the impact of hypothetical dependencies between
15589 allergic lung inflammation and male reproductive toxicity.

15590 ## D.12. Additional uncertainty factor for deriving the TDI

15591 As the final step in the uncertainty analysis, the WG experts considered whether an additional UF is needed
15592 when deriving the TDI. To inform this discussion, tabulated percentiles of the cpf for the estimated lowest
15593 BMD across all clusters were displayed, based on the lower and upper bounds of the consensus cpf that
15594 resulted from the decisions made by the WG experts in the preceding steps (red dashed curves in right-
15595 hand graph of Figure D15). The ratios of the RP (BMDL) to each percentile of the lower and upper bounds
15596 of the consensus cpf were also displayed. These results are shown in Table D4.

15597 The WG experts noted from Table D3 that the RP of 0.93 ng BPA/kg bw per day is close to the 15th
15598 percentile of both the lower and upper bounds of the consensus cpf. This implies about 15%
15599 probability that the estimated lowest BMD for all clusters is lower than the RP and, therefore, 85%
15600 probability that the estimated lowest BMD for all clusters is above the RP.

15601 Accordingly, the ratio of the RP to the 15th percentile of both bounds of the cpf is close to 1. This implies
15602 about 85% probability that an additional UF of 1 (i.e. no additional UF) would be sufficient to cover all 21
15603 of the clusters assessed in the HOCs for Immunotoxicity, Metabolic effects, Neurotoxicity and
15604 developmental neurotoxicity, Reproductive and developmental toxicity, and Carcinogenicity and mammary
15605 gland proliferative effects. The WG had concluded earlier (see above) that assessment of these clusters is

also protective for clusters in the HOC General toxicity, so they also do not require an additional UF. When the calculations were repeated with the histograms provided by the experts rather than fitted parametric distributions (as shown in Figure D17), the probability that the estimated lowest BMD for all clusters is below the RP increased slightly, to 87%. The WG also noted that the large range of endpoints tested for BPA makes the risk assessment for BPA more conservative than for most other chemicals, for which only the standard endpoints are tested. Taking all these considerations together, the WG concluded that no additional UF is needed.

Table D4: Percentiles of the lower and upper bounds of the consensus cpf for the estimated lowest BMD across all clusters (columns 2 and 3) and ratios of the RP of 0.93 ng BPA/kg bw per day to each percentile (columns 4 and 5). The row shown in bold is the basis for the CEP Panel conclusion that an additional UF is not needed. See text for details.

Percentile of consensus cpf	Lower bound for percentile (ng BPA/kg bw per day)	Upper bound for percentile (ng BPA/kg bw per day)	Ratio of reference point to lower bound	Ratio of reference point to upper bound
1%	0.028	0.028	33.8	32.7
2.5%	0.059	0.061	15.7	15.2
5%	0.135	0.141	6.9	6.6
10%	0.412	0.447	2.3	2.1
15%	**0.881**	**1.002**	**1.1**	**0.9**
20%	1.485	1.754	0.6	0.5
25%	2.182	2.673	0.4	0.3
30%	2.989	3.800	0.3	0.2
35%	3.931	5.171	0.2	0.2
40%	5.028	6.797	0.2	0.1
45%	6.293	8.705	0.1	0.1
50%	7.742	10.955	0.1	0.1
55%	9.406	13.591	0.1	0.1
60%	11.323	16.671	0.1	0.1
65%	13.501	20.438	0.1	0.0
70%	15.991	25.137	0.1	0.0
75%	19.054	31.067	0.0	0.0
80%	23.057	38.870	0.0	0.0
85%	28.472	49.854	0.0	0.0
90%	36.534	67.143	0.0	0.0
95%	51.747	101.729	0.0	0.0

Appendix E – Summaries of genotoxicity studies

This Appendix reports summaries of genotoxicity studies on BPA identified in the literature (2013 - 2021) and studies considered in the Scientific opinion on the risks to public health related to the presence of bisphenol A (BPA) in foodstuffs (EFSA CEF Panel, 2015).
The evaluation of the studies on the main genetic endpoint (gene mutations, structural and numerical chromosomal alterations) is reported in tabular format in Annex L.

E.1. *In vitro* studies

E.1.1. Gene mutation

Masuda et al., 2005
The study evaluated the mutagenicity of BPA in Ames test in the presence or absence of S9-mix. BPA (Tokyo Kasei Kogyo Co., Ltd) was tested on S. Typhimurium strains TA98 and TA100 at the single dose of 0.1 µmole/plate (100 µL of 1 mM solution). No mutagenic effect was observed.

Tiwari et al., 2012
The study evaluated the mutagenicity of BPA in Ames test. BPA (purity 99%) was tested at concentrations from 6.25 to 200 µg/plate on different strains of *S.* Typhimurium (TA 98, TA 100 and TA 102). The mutagenic response was not observed in any of the tester strains at the various concentration of BPA in absence of S9 fractions. A slight increase in the numbers of revertants was observed in the presence of S9 fractions from the 6.25 - 25 µg/plate of BPA in each strain, but the increase was statistically significant only in strain TA 102 at 25 µg/plate.

Fic et al., 2013
In this study the mutagenic and genotoxic potential of eight BPA (purity >99%) structural analogues [BPF, BPAF, bisphenol Z (BPZ), BPS, bis(4-hydroxy-3-methylphenyl)propane (DMBPA), 4,4'-sulfonylbis(2-methylphenol) (DMBPS), [sulphonylbis(benzene-4,1-diyloxy)]diethanol (BP-1), and 4,4'-sulphanediyldiphenol (BP-2)] were investigated using the Ames and comet assay. None of these bisphenols were mutagenic in *Salmonella* Typhimurium strains TA98 and TA100 either in the presence or absence of external S9-mediated metabolic activation (Aroclor 1254-induced male rat liver). Potential genotoxicity of bisphenols was determined in the HepG2 human hepatoma cell line following 4-h and 24-h exposure to non-cytotoxic concentrations 0.1 µmol/L to 10 µmol/L. In the comet assay, BPA and its analogue BPS induced significant DNA damage only after the 24-h exposure, while analogues DMBPS, BP-1, and BP-2 induced a transient increase in DNA strand breaks observed only after the 4-h exposure. BPF, BPAF, BPZ, and DMBPA did not induce DNA damage.

Xin et al., 2015 [RefID 8150]
The study evaluated the cytotoxic, genotoxic and clastogenic activity of BPA (purity 99%)[22] in CHO cells and its mutagenicity in the Ames test. The battery of assays applied in CHO cells included the MTT assay for the evaluation of cytotoxicity, and the comet, micronucleus and chromosome aberration tests. In the Ames test BPA (10-5000 µg/plate) was uniformly negative in all *Salmonella* Typhimurium strains (TA1535, TA97, TA98, TA100 and TA102), with and without metabolic activation. Exposure of CHO cells to four BPA doses (40, 80, 100 and 120 µM) for 12 and 24 h resulted in a significant decrease in cell viability (at 80 µM and above), which however remained above 50% in all cases; a concentration-related increase of DNA damage was observed in comet assay [increased Olive tail moment (OTM), tail length and % tail DNA, statistically significant at all doses] after 12 and 24 h exposure to BPA; after 24 h treatment, an increase in micronuclei (MN) (statistically significant at 100 and 120 µM) and structural chromosomal aberrations (chromatid breaks and chromosome fragments, statistically significant at 80 µM and above) was also observed.

Zemheri and Uguz, 2016 [RefID 9535]

[22] Information on BPA purity provided by the study authors on 11 October 2021, upon EFSA request

The study evaluated the mutagenicity of BPA (Merck) in a limited Ames test, using two tester strains (TA98 and TA100) and four dose levels (0.1, 1, 10 and 100 µg/plate). The results were negative, with and without metabolic activation.

Balabanič et al., 2021 [RefID 224-G]

The study evaluated cytotoxic and genotoxic effects of some endocrine disrupting chemicals (EDCs), including BPA, which have been previously identified in effluents from two paper mills. BPA (Sigma-Aldrich) tested at concentrations of 1, 10, 100, 1000 µg/L with the bacterial SOS/umuC assay in *S.* Typhimurium TA1535/pSK1002 strain did not induce toxic nor genotoxic effects in the presence or absence of S9 metabolic activation. The compound was also assessed in HepG2 cells with MTT assay for cell viability and with comet assay at 1, 10, 100 and 1000 µg/L for 4 and 24 h. No significant reduction of the viability. A statistically significant concentration-dependent increase of DNA damage, expressed as percent of DNA in tail, was reported starting from 10 µg/L.

Hu X et al., 2021 [RefID 295-G]

This study shows that BPA (purity ≥ 99%) and styrene-7,8-oxide (SO) causes DNA damage and mutations in HEK293T human embryonic immortalized cells. A concentration-dependent increase in the formation of γH2AX foci was induced by a 24 h exposure to BPA (0.1, 1, 100 µM). Multiple clonally amplified cell populations, derived from HEK 293T cells treated with 100 µM BPA for 24 h, were subjected to whole genome sequencing (WGS). To identify mutations that were associated with BPA exposure, genomic changes occurring in BPA-treated cell populations were compared with those occurring in the parental cell line. The frequency of acquired single base substitutions (SBS), double base substitutions (DBS), and small insertions and deletions (InDel) were all increased in BPA-treated cell populations in comparison with untreated cell populations. Analyses of genome-wide point mutations and genomic rearrangements associated with BPA exposure indicate that a subset of SBS- and DBS substitutions occur near or at guanines. The authors conclude that these locations are consistent with BPA preference to form guanosine adducts. The occurrence of other mutational signatures suggests additional mutagenic events occurring at A:T base pairs (TA>CG transitions and TA>GC transversions). Analysis of data from 19 cancer cohorts suggest that tumours of digestive and urinary organs show a relatively high similarity in mutational profiles, with the burden of mutations increasing with age. The authors conclude that BPA (and SO) are relatively mild mutagens and other environmental agents might also potentially generate similar, complex mutational patterns in cancer genomes.

E.1.2. Chromosomal damage

Johnson and Parry, 2008

In this mechanistic study the aneugenicity of two known spindle poisons model compounds, namely rotenone and 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 centres (MTOCs) of the mitotic spindles. For induction of MN BPA (Sigma-Aldrich) was added over a range of 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 h dependent upon any cell cycle delay) in the presence of cytochalasin-B. A minimum of five separate experiments were performed. A concentration-related and statistically significant increase of binucleate-micronucleated cells from 12.3 µg/mL was reported with a clear threshold for induction of MN (NOEL at 10.80 µg/mL and LOEL at 12.3 µg/mL). A NOEL and LOEL for percentage of binucleate cells was also observed at 9.2 µg/mL and 10.8 µg/mL BPA respectively. 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. 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 h (i.e. one cell cycle for V79). 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 were detected and it was suggested by the study authors to be the mechanism for the production of chromosome loss into MN.

Tayama et al., 2008

In this study, the authors evaluated the genotoxicity of some environmental estrogen-like compounds including BPA using sister chromatid exchanges (SCEs), CA and DNA strand breaks (comet) assays in CHO-K1 cell line *in vitro*. For CA and SCEs six concentrations of BPA (purity > 99%) ranging from 0.1 to 0.6 mM were added to CHO-K1 cells for 3 h. Following treatments cells were further incubated for 27 h in the presence of 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 h and following washes of test compound cells were processed for comet assay using a silver-staining method. The method applied for comet analysis was not clearly reported. Statistically significant increase for both SCE and chromosome aberrations were reported at the highest concentrations tested (0.4 and 0.5 mM and 0.5, 0.55 and 0.6 mM respectively). Significant increases of c-mitotic effects were detected at highest concentrations 0.3-0.6 mM. The positive results were observed only in presence of severe cytotoxicity. For comet assay significant increases of DNA breakage were only reported at the highest concentration (0.7 mM) for which the cytotoxicity was not adequately evaluated.

Ribeiro-Varandas et al., 2013 [RefID 6189]

The study investigated micronucleus formation in human umbilical vascular endothelial cells (HUVEC) and human colon adenocarcinoma (HT29) cell lines cultured in the presence of BPA (44 nM and 4.4 µM, i.e. 10 ng/mL and 1 µg/mL) for 24, 48 or 72 h. Effects on DNA stability and DNA damage repair were concurrently evaluated by means of the TUNEL assay and by the analysis of histone-H3 acetylation on lysine 56 (H3K56ac). At the concentrations applied, BPA did not affect cell viability and proliferation of both cell lines. The TUNEL assay did not detect DSBs or apoptotic cells associated with exposure to BPA. Micronucleus frequency was significantly increased in HUVEC cells (1.1% vs 1.6% and 2.2 % in control, low and high BPA dose, respectively), but not affected in HT29 cells (4.45% vs 4.6% and 4.4%). The transcriptional analysis of expression of genes encoding for proteins involved in chromosome segregation (CDCA8, SGOL2, Aurora A, α-tubulin and γ-tubulin), performed by quantitative real-time PCR, showed small (less than two-fold) increases in the expression of CDCA8 (both cell lines) and SGOL2 (HUVEC cells only). The immunofluorescence analysis of cytoskeleton organization of HUVEC cells with anti-α-tubulin and anti-γ-tubulin showed several aberrations, such as multipolar spindles and microtubule misalignment, associated with BPA exposure.

Šutiaková et al., 2014 [RefID 7026]

The study evaluated the genotoxic and cytotoxic effects of BPA (Sigma-Aldrich) on bovine peripheral lymphocytes *in vitro*. Lymphocyte cultures from two animals were exposed to four different concentrations of BPA (1×10^{-4}, 1×10^{-5}, 1×10^{-6} and 1×10^{-7} mol. L^{-1}) 24 h after stimulation by L-phytohemagglutinin, and incubated for total 72 h. Micronucleus frequency was determined using the cytokinesis block method, adding 6 µg/mL cytochalasin B at 44 h. A significant increase in the number of MN ($p= 0.018$) was observed at the highest concentration of BPA; at lower concentrations micronucleus frequency was not significantly different from vehicle (DMSO) control. The nuclear division index (NDI) was not affected by BPA treatment at any concentration level.

Aghajanpour-Mir et al., 2016 [RefID 57]

The study evaluated the clastogenic activity of BPA in the MCF-7 human breast cancer cell line and male and female human amniocytes. Cells were exposed 48 h to 0 (control), 0.4, 1, 4, 40 and 100 µg BPA/mL in the absence of exogenous metabolic activation. Giemsa staining was applied for the identification of structural chromosome aberrations in treated cells (200 per dose). Cytotoxicity was evaluated using the MTT assay. In a preliminary evaluation of cytotoxicity, the IC50 of BPA was 100, 40 and 4 µg/mL in MCF-7 and ER-negative (male) and ER-positive (female) amniocytes, respectively. Following treatment with BPA a significant increase of cells with chromosome aberrations was observed at 1 µg/mL (and above) with all cell types, with no clear association with ER expression. The aberrations recorded were almost exclusively fragments in amniocytes (both female and male), and fragments and chromatid breaks and gaps in MCF-7 cells. The increase in cells with aberrations was not clearly concentration related and decreased at the highest doses, possible due to cytotoxicity that was not concurrently evaluated. A few numerical aberrations (both hypoploidy and hyperploidy) were also

recorded in amniocytes metaphases; in female amniocytes the decrease of cells with normal chromosome number reached statistical significance at the highest not toxic concentration (4 µg/mL).

Huang FM et al., 2018 [RefID 296-G]

The study reported positive results for induction of DNA strand breaks (evaluated by comet assay) and MN frequency in murine macrophage RAW264.7 cells. Cell cultures were treated at 0, 3, 10, 30, and 50 µM of BPA (Sigma-Aldrich) dissolved in DMSO for 24 h. Concentration-dependent increase of tail length, based on the analysis of 50 cells/slide, and of MN frequency by the evaluation of 1000 binucleated cells per concentration were observed. No positive controls were used. The genotoxic effects were observed starting from 10 µM and were associated with an increase of reactive oxygen species (ROS), measured by Dichlorofluorescein Diacetate Assay (DCFH-DA) and a decrease of antioxidant enzymes, including GPx, SOD and CAT. Concomitant phosphorylation of P53 and release of cyto C from mitochondria into cytosol were reported. A reduced expression of antiapoptotic proteins BCL2 and BCL-XL significant from 10 and 3 µM respectively and an increase of the expression of proapoptotic proteins BAX, BID, and BAD beginning at 10, 10 and 30 µM respectively were observed in a concentration-dependent manner. Increased level of the apoptosis-inducing factor (AIF) in the nucleus and a decrease in the mitochondria was detected. Expression of pro-caspase-3 and pro-caspase-9 is reduced by BPA in a concentration-dependent manner and PARP-1 cleavage was induced by BPA. Pre-treatment of the cell cultures with N-acetylcysteine (NAC), a cysteine precursor of the antioxidant glutathione, at the concentration of 10 µM for 30 min reduced BPA-induced cytotoxicity, apoptosis, and genotoxicity. The results of this study indicates that the toxic effects induced by BPA in macrophages was mainly through oxidative stress-associated mitochondrial apoptotic pathway.

Sonavane et al., 2018 [RefID 419-G]

The study investigated BPA's ability to confer resistance to the chemotherapeutic agent camptothecin (CPT). The study reports the results of an experimental study on the high-dose BPA (Sigma-Aldrich) co-exposure effects with the chemotherapeutic agent CPT in mouse embryonic fibroblasts (MEFs). A 24 h and a 48 h treatment of MEFs with a concentration of BPA (150 µM) did not result in an increase of DNA strand breaks detected by the comet assay and by the analysis of phosphorylated H2AX (γH2AX) and of chromosomal aberrations. The BPA treated cells showed a reduction in the nuclear volume associated with a DNA compaction detected by 3-D imaging using the nucleic acid fluorescent stain. This effect reduces the accessibility of DNA to topoisomerase-I (Top1). The mechanism of action of CPT is the formation of Top1 covalent complexes inducing DNA-protein cross-links, that result in single-strand and double-strand DNA breaks and chromosomal aberrations in treated cells. A co-exposure of MEFs with CPT and BPA for 24 and 48 h showed a significative reduction of Top1-DNA adducts decreasing the DNA strand breaks and chromosomal damage induced by CPT alone.

Santovito et al., 2018 [RefID 11220]

In this study the possible induction of chromosomal damage by BPA (Sigma-Aldrich) was tested in human peripheral blood lymphocytes cultures applying the CA assay and the micronucleus test (MN). Cell cultures were exposed to a range of concentrations from 0.01 to 0.20 µg/mL, (including the reference dose established by United States Environmental Protection Agency (US EPA) (0.05 µg/mL), the tolerable daily intake established by European Union (0.01 µg/mL) and the highest concentration of unconjugated BPA found in human serum (0.02 µg/mL)) for 24 h for the chromosomal aberration test and for 48 for the micronucleus test. A statistically significant increase of cells with structural chromosomal aberrations, with a prevalence of chromatid breaks, was reported starting from 0.05 µg/mL; no numerical aberration was observed. A concentration related increase in MN frequency was detected starting from 0.02 µg/mL in which a four-fold increase with respect to the control level was observed.

Ramos et al., 2019 [RefID 396-G]

The study evaluated the cytotoxic and genotoxic effects of BPA in human laryngeal carcinoma cells (Hep-2) and human lung fibroblasts (MRC-5). BPA (Sigma) was freshly diluted in ethanol and added to the culture media at the concentrations of 0.44 nM, 4.4 nM and 4.4 µM (0.1 ng/mL, 1 ng/mL, 1µg/mL) for 48 h. Cell viability after treatment was evaluated using the resazurin assay. DNA damage and oxidative DNA damage were assessed in cryopreserved cell samples by comet assay (100 cells per

slide), with and without the formamidopyrimidine-DNA glycosylase (Fpg). Mitotic abnormalities and MN were evaluated in DAPI stained cells (1000 cells per concentration). Treatments had no effect on cell viability in either cell lines. In comet assays with Hep-2 cells, DNA damage, measured as % DNA in the tail, was significantly increased at the lowest concentration (0.44 nM) and decreased at 4.4 nM and 4.4 µM; oxidative DNA damage (in presence of Fpg) was significantly decreased at all concentrations. In MRC-5 cells, there was no effect on DNA damage and an increase in oxidative DNA damage at the two highest concentrations. The microscopic analysis of DAPI stained cells showed a significant increased mitotic index (percent of cells undergoing mitotic division) in MRC-5 cells at all BPA concentrations and in Hep-2 cells at the highest concentration. The percentage of MN were slightly (up to 2.5-fold) but significantly increased in both cell lines at the two highest concentrations.

Di Pietro et al., 2020 [RefID 258-G]

The study investigated the effects of BPA exposure on cell proliferation, cell cycle progression and DNA damage in human peripheral blood mononuclear cells (PBMC) and the BPA-induced neurotoxicity in rats exposed to environmental relevant doses of BPA during development. Human PBMC from five unrelated healthy donors (adult males and females) were cultured and treated with BPA (Merck) from 5 nM to 200 µM. The treatment with BPA of unstimulated resting PBMC did not affect cell proliferation (determined by the colorimetric MTT) at all the concentrations tested except for 200 µM for which a marked inhibition of cell proliferation was observed at 24 and 48 h after the treatment. By contrast, in PHA-stimulated cells, BPA caused a pronounced increase of cell growth starting from 10 nM to 100 nM and a concentration-dependent decrease of cell proliferation from 25 to 200 µM. The cell cycle was analyzed by flow cytometry. BPA at 50 nM increased the percentage of cells in S phase of the cell cycle at 24 h and this effect was higher at 48 h with an increase of about 17% of cells in the S phase compared with the control. At 100 µM, BPA induced a significant increase of the percentage of cells in the G0/G1 phase, suggesting that BPA affected cell growth in a non-monotonic way. BPA-treatment at 25, 50 and 100 nM for 48 h induced a significant increase ($p < 0.001$) of both the percentage of aberrant cells (about 20% at 100 nM) and structural aberrations (about 27% at 100 nM) including chromatid and chromosome breaks, rings and fragments. BPA also increased significantly the percentage of highly fragmented metaphases (shattered cells). In PHA-stimulated PBMC treated with BPA (50 nM) for 24 h, γH2AX was significantly increased in CD3+ T lymphocytes and was also detected in a higher proportion of CD8+ T lymphocytes than the CD4+ T lymphocytes and a slight percentage of γH2AX was reported among the B cells. The treatment of PHA-stimulated PBMC with BPA (50 nM) induced p21/Waf1 and PARP1 protein expressions approximately within the same time interval. These findings suggest that BPA could affect the p53-p21/ Waf1 checkpoint and PARP1 levels resulting in DNA damage repair defects. BPA (50 nM) for 24 h modulated the expression of ER-α and ER-β in both sexes inducing or inhibiting its expression in males and in females with effects similar to the variations induced by pharmacological concentrations of E2 (100 nM). The study investigated also the BPA-induced neurotoxicity in terms of DNA damage. After the coupling period, three females/group received BPA (0.1 mg/L), or vehicle (ethanol 0.1 mL/L) in the drinking water during gestation, lactation and weaning of their offspring. Five females and three males pups from BPA-exposed mothers and five females and three males newborns from vehicle-treated dams were then sacrificed at PND 17. BPA was shown to induce γH2AX phosphorylation in cells possessing immune function in the CNS, such as microglia and astrocytes of rat hippocampus. In BPA-exposed rats a marked decreasing trend of ERα expression was found therefore proposing a role for this receptor in the effects induced by BPA.

Yu et al., 2020 [RefID 475-G]

In this study, induction of MN and double-strand DNA breaks by BPA, BPF, and BPS were investigated in Chinese hamster V79-derived cell lines expressing various human CYP enzymes and a human hepatoma (C3A) (metabolism-proficient) cell line. In a first step a prediction of BPA, BPF, and BPS as potential substrates for several human CYP enzymes, which are commonly involved in the metabolic activation of compounds, was conducted by molecular docking. The results of the analysis showed a similar affinity of the compound with all the enzymes tested: CYP1A1, 1A2, 1B1, 2B6, 2E1, and 3A4. BPA (99.6% analytical purity) tested at 40, 80 and 160 µM for 9 h, followed by 15 h of recovery induced a concentration related increase of MN frequency in V79-hCYP1A1. In V79-hCYP1B1 cells MN were observed only at the two highest concentrations. No induction of MN was reported in V79-Mz, V79-hCYP1A2, V79-hCYP2E1, or V79-hCYP3A4-hOR cells. A consistency with the results of the molecular

docking was observed only for CYP1A1 and CYP1B1. Following extended exposure (two cell cycles, i.e., 24 h in V79-derived cells) without recovery period, BPA reduced the cell viability at 80 µM in V79-Mz and V79-hCYP1A1 cells; at lower concentrations, the cell growth was even enhanced (compared with the control). BPA induced MN in V79-hCYP1A1 (24 h treatment) and C3A cells (72 h treatment) from 5 and 2.5 to 80 µM in a concentration-dependent manner. The increase of MN was completely blocked by 1-aminobenzotriazole (1-ABT CYP inhibitor) and by 7-hydroxyflavone (7-HF selective CYP1A1 inhibitor). Co-exposure of C3A cells to pentachlorophenol (sulfotransferase 1 inhibitor) or ketoconazole (UDP-glucuronosyltransferase 1A inhibitor) potentiated MN induction with thresholds lowered to 0.6 µM. The use of Centromere Protein B-staining immunofluorescent assay indicated clastogenic effects. BPA tested for 9 h from 10 to 160 µM in V79-Mz, V79-hCYP1A1, and C3A cells induced a concentration-dependent increase of γH2AX foci which were blocked in presence of 1-ABT and 7-HF.

Özgur et al., 2021 [RefID 631-G]
In this study BPA was tested in a chromosomal aberration assay in cultured human peripheral blood lymphocytes. Cells were exposed for 24 h to BPA, dissolved in DMSO, at the final concentrations of 5, 10 and 20 µg/mL. No data on chromosome aberrations are reported as, under the conditions of this study, BPA (source and purity not defined) was completely cytotoxic.

E.1.3. DNA damage (by comet assay)

Iso et al., 2006
In this study the effects of BPA and 17β-estradiol (E2) on DNA damage was analysed in ER-positive MCF-7 cells by comet assay. One thousand higher concentrations of BPA (Wako Pure Chemicals Industries, Ltd.) were needed to induce the same levels of effects of E2. Levels of γH2AX foci measured by immunofluorescence microscopy were increased after treatment with E2 or BPA. Foci of γH2AX co-localized with the Bloom helicase, an enzyme involved in the repair of DSBs. In comparison with MCF-7 cells, DNA damage was not as severe in the ER-negative MDA-MB-231 cells. In addition, the ER antagonist ICI182780 blocked E2 and BPA genotoxic effects on MCF-7 cells. These results together suggest that BPA causes genotoxicity ER dependently in the same way as E2.

Xin et al., 2014 [RefID 8147]
The aim of this study was to assess how BPA can influence the function of pancreatic islets. To measure DNA damage, rat INS-1 insulinoma cells were exposed to different concentrations of BPA (Sigma-Aldrich, 99% purity) (0, 25, 50, 100 µM for 24 h) and analysed by the single-cell gel electrophoresis (comet assay). To investigate the possible mechanism of DNA damage induced by BPA, p53 and p-Chk2 levels were also analysed by western blotting together with measurements of intracellular ROS and glutathione (GSH). The results show that BPA caused an increase in DNA strand-breaks at 50 and 100 µM (as measured by tail moment, tail length and tail DNA %). The authors state that these experimental conditions did not cause any significant toxicity (90% survival; no data provided). Pre-treatment with NAC decreased to half the number of DNA strand breaks induced at the highest dose. A significant increase in intracellular ROS, which was decreased by NAC pre-treatment, was also observed. A significant reduction in the level of GSH levels was observed at all BPA concentrations. Finally, expression of DNA damage-associated proteins (p53 and p-Chk2) was significantly increased by BPA exposure (all concentrations).

Chen et al., 2016 [RefID 1130]
The study investigated the cytotoxic and genotoxic effects induced by BPA alone and in combination with cadmium (Cd) *in vitro* in mouse embryonic fibroblast cell line (NIH3T3). The treatment of the cell cultures with BPA (Sigma-Aldrich) at 2, 10 and 50 µM was shown to induce, only at the highest concentration tested, a decrease in the cell viability and an increase of the oxidative damage as reactive oxygen species (ROS), measured by DCFH-DA and as 8-OHdG. Significant increase of DNA strand breaks was also detected as tail DNA% and tail moment by comet assay. Higher number of γH2AX foci detected through the use of immunofluorescence and increased γH2AX expression evaluated by western blot in BPA treated cells are indicative of DNA double strand breaks. In addition, 50 µM BPA treatment did significantly decrease the percentage of cells in G1 phase and increased the percentage of cells in G2 phase but not in S phase. Pre-treatment of cells with Cd was observed to aggravate BPA-

induced cytotoxicity, and increase ROS production, DNA damage, G2 phase arrest, total TUNEL positive cells and cleaved-PARP expression levels.

Porreca et al., 2016 [RefID 5892]
This study reports on the thyroid-disrupting activity of low-dose BPA (Sigma-Aldrich) on the rat immortalized FRTL-5 thyrocytes cell line using microarray experiments. Exposures to 10^{-9} M BPA for 1 and 3 days resulted in increased ROS production. Exposures for 3 and 7 days lead to gene expression changes with transcriptome alterations being quantitatively and qualitatively dependent on the duration of exposure. Cell survival (decreased), cell death (increased), cell cycle (decreased), and cancer (increased) were among the most significant functions predicted deregulated. The response involved many genes belonging to specific pathways mainly related to cell death/proliferation and DNA repair (genes involved in "DNA replication, recombination and repair" including "checkpoint control" functions). Although gene expression levels are only slightly altered, a long-term exposure to BPA (28 days, 10^{-9} M) impaired the cellular response to other stressors (mainly COP9 signalosome). A delayed reduction of UV-C-induced DNA damage was identified by comet assay. This was due to the impairment of p21-Tp53 axis, with BPA-treated cells being more prone to cell death (increased apoptosis and decreased proliferation following UVC damage). The authors propose a possible mechanism by which BPA does not cause direct genetic damage but may exert an indirect genotoxic activity.

Lei et al., 2017 [RefID 3960]
In this study changes in cytotoxicity, genotoxicity, intracellular ROS formation, and Ca^{2+} levels induced by BPA were evaluated in MCF-7 cells in comparison with other BPs. The range of BPA (98% purity, Tokyo Chemical Industry) concentrations tested was 0.01-100 µM (24 h exposure). An increase in cell viability was observed at 1 µM, while a significant drop in cell survival (to approximately 30%) was observed at higher concentrations (10–100 µM). In parallel, a significantly increase in LDH release was observed (10–100 µM). BPA (1–25 µM) significantly increased ROS levels in a concentration-dependent manner. At 50 µM however BPA caused death of over 90% cells. BPA exposure resulted in a significantly increase in DNA-damaging effect on MCF-7 cells in the range 10–50 µM. In addition BPA at 0.0001–1 µM significantly increased intracellular Ca^{2+} level. Finally, the estrogenic and thyroidal hormone effects induced by BPA were also evaluated using the yeast two-hybrid bioassay.

Li XH et al., 2017 [RefID 4176]
The study investigated the cytotoxic effects and oxidative stress induced by BPA (Sigma-Aldrich) alone and in combination with dibutyl phthalate (DBP) or cadmium (Cd) *in vitro* in HepG2 cells. The cell cultures were exposed for a period of 6 h to a range of concentrations of the single substances ensuring a cell viability above 50%. BPA tested from 10^{-8} to 10^{-4} mol/L for 6 hours induced a concentration dependent increase of reactive oxygen species (ROS), measured by DCFH-DA, and malondialdehyde (MDA) level and a decreased activity of SOD. An increase of DNA strand breaks (up to eight- fold with respect to the control value) applying the comet assay, was detected after BPA treatment at 10^{-8}, 10^{-7}, 10^{-6} mol/L for 24 h without a clear concentration response. The co-exposure treatments (BPA and DBP or BPA and Cd) showed higher ROS and MDA levels and lower SOD activity than the mono-exposure treatments. The combined treatments with BPA and Cd had stronger DNA damage effect.

Mokra et al., 2017 [RefID 5170]
The study reported concentration-related induction of DNA single and double strand breaks (detected with alkaline and neutral comet assay) by BPA (Sigma-Aldrich) and its analogues, BPS, BPF and BPAF in human peripheral blood mononuclear cells (PBMC) treated in the concentrations ranging from 0.01 to 10 µg/mL after 1 and 4 h treatment. No significant decrease of cell viability, evaluated using calcein-AM/PI stains, was observed at the concentrations tested for DNA damage. After 1 h incubation, BPA caused statistically significant increase in DNA strand breaks at 0.1 mg/mL. The highest effects were induced by BPA and BPAF, which produced single strand breaks starting from 0.01 µg/mL, while BPS caused the lowest effect at 10 µg/mL after 4 h of exposure. Statistically significant increases of DNA double strand breaks were induced by BPA at concentrations of 1 µg/mL and 10 µg/mL after 1 h incubation and at 0.1 µg/mL and 1 µg/mL after 4 h incubation. The strongest effect was observed with BPAF. DNA repair was also evaluated at different times (30, 60 and 120 min) after the treatment with

BPA at 10 µg/mL. A significant decrease of the DNA damage was observed at 60 min, but the repair was not complete after 120 min.

Durovcova et al., 2018 [RefID 262-G]

The study evaluated by alkaline comet assay the induction of DNA strand breaks by BPA (Sigma-Aldrich) in cultures of peripheral blood lymphocytes at concentrations of 0.001, 0.1, 2.5 mM. The results show a statistically significant increase only at the first two concentrations tested. The presence of oxidative DNA damage was also shown using modified comet assay with Fpg enzyme: the extent of DNA single strand breaks is higher at the lower concentration. The study reports also a protective effect of BPA on plasmid DNA against iron ions induced single-strand DNA breaks as showed by a test measuring the electrophoretic mobility of pDNA topoisomers (DNA-topology assay).

George and Rupasinghe, 2018 [RefID 277-G]

This study investigated the relative toxicity of BPA (Sigma-Aldrich) and BPS on human bronchial epithelial cells (BEAS-2B). The tested endpoints included cytotoxicity, induction of ROS, DNA fragmentation, γH2AX foci and DNA tail damage. To evaluate mechanism of cell death the DDR and activation of caspase-3 were also investigated. In all the assays a single concentration and a single time of exposure were used (200 µM BPA for 24 h). According to the authors this concentration caused 50% of cell viability loss (IC_{50}). However, the data reported indicate high levels of toxicity (90%), with all the results being unreliable at this level of toxicity.

Goncalves et al., 2018 [RefID 11104]

The aim of this study was to evaluate the effects of BPA (Sigma-Aldrich) on the growth, viability, and testosterone production of TM3 murine Leydig cells after exposure to BPA for 24 or 48 h (1, 10, 100 µM). BPA reduced testosterone production, cell viability and cell growth in a concentration-dependent manner. The highest tested concentration of BPA (100 µM) increased cellular death, as indicated by an increased sub-G1 phase population and a larger number of cells labelled with Hoechst 3342. This concentration of BPA also decreased the number of metabolically active mitochondria as revealed by rhodamine staining. No DNA strand breaks evaluated by alkaline comet assay was detected in the cells after 3 h of BPA exposure at any concentration tested. The authors conclude that BPA is toxic to Leydig TM3 cells and impairs their steroidogenic function.

Mokra et al., 2018 [RefID 364-G]

The study reported that BPA (Sigma-Aldrich) and its analogues, BPS, BPF and BPAF caused oxidative DNA damage to purine and pyrimidines in human peripheral blood mononuclear cells (PBMC) treated at concentrations of 0.01, 0.1 and 1 µg/mL for 4 h and 0.001, 0.01 and 0.1 µg/mL for 48 h. BPA was dissolved in ethanol. No significant decrease of cell viability, evaluated using calcein-AM/PI stains, was observed at the concentrations tested. DNA damage was detected with alkaline comet assay coupled with repair enzyme endonuclease III (Nth) and 8-oxoguanine DNA glycosylase (hOGG1). Statistically significant and concentration related oxidative damage to purines (from 0.01 µg/mL) and to pyrimidines (from 0.1 µg/mL) was reported after 4 h treatment. After 48 h treatment significant damage to purine was observed from 0.001 µg/mL and to pyrimidines from 0.01 µg/mL. Statistically significant differences for DNA damage between 4 h and 48 h exposure at the highest concentrations tested (0.01 and 0.1 µg/mL)

Yuan et al., 2019 [RefID 478-G]

In this study, markers of oxidative stress and DNA damage were evaluated in Marc-145 rhesus monkey embryo renal epithelial cells exposed to BPA (Sigma-Aldrich, purity > 99%) in the range 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} M (24 hr exposure). The results showed that BPA induced a concentration-dependent decrease in cell viability (from 20% at the lowest concentration up to almost 80% at the highest concentration), in SOD activity and GSH level. Concomitant concentration-dependent increases in apoptosis, lactate dehydrogenase (LDH) activity, ROS and thiobarbituric acid reactive substances content were observed. BPA also induced a concentration-dependent increase in DNA strand breaks by comet assay in the range of concentrations measured (10^{-3} -to 10^{-6} M).

Kose et al., 2020 [RefID 325-G]

This study investigated the relative toxicity, potential oxidative stress and genotoxicity induced by BPA (>99% purity), BPS and BPF on the RWPE-1 non-tumorigenic prostatic cell line. RWPE-1 cells were incubated with BPA at concentrations of 50–600 µM for 24 h exposure. The IC_{20} and IC_{50} values, concentrations that causes 20 and 50% of cell viability loss, after a 24 exposure to BPA were 45 and 113.7 µM. BPA induced significant decreases in the activities of glutathione peroxidase (GPx1) and SOD, an increase in glutathione reductase and total GSH and a decrease in total antioxidant capacity. At a single concentration (IC_{20}), BPA produced significantly higher levels of DNA damage vs the control both in the standard (2.5-fold increase) and Fpg-modified comet assays. No changes in the mRNA levels of p53 and the OGG1, Ape-1, DNA polymerase β, base excision repair (BER) proteins were induced by BPA. The single exception was a small decrease in the expression levels of MYH expression.

E.2. Other studies

E.2.1. DNA adducts

De Flora et al., 2011

The ability of BPA to form DNA adducts was investigated in two human prostatic cell lines: PNT1a non tumorigenic epithelial cells and PC3 cells androgen-independent prostate cancer cells originated from bone metastasis of prostatic carcinoma. PNT1a and PC3 cells were treated with BPA (Sigma-Aldrich), dissolved in ethanol at a concentration corresponding to the IC_{50} (200 µM for PNT1a and 250 µM for PC3) for 24 h. PNT1a cells were also treated at a concentration of 1 nM, for 2 months. Significant levels of DNA adducts were detected by ^{32}P-postlabeling technique in prostate cell lines treated with high-concentration of BPA for 24 h (4.2-fold increase over controls) in PNT1a cells and a 2.7-fold increase over controls in PC3 cells) and in a lower extent in PNT1a cells treated at low-concentration for 2 months.

E.2.2. DNA repair

Yin et al., 2014 [RefID 8519]

The study analyzed gene expression profiling in ER-negative human embryonic kidney cells (HEK293) following 48 h exposure to 10^{-6} M BPA. The comparison of gene expression profiles of BPA (no information on purity or the supplier company) treated and control (DMSO) samples identified 15 differentially expressed genes after BPA treatment, eight upregulated and seven downregulated (while more than 300 genes are differentially expressed in ER-positive cells). A computer-assisted functional analysis indicated the BPA exposure perturbated many unrelated metabolic pathways. BAX, a proapoptotic regulator, was downregulated by BPA treatment, suggesting that low-dose BPA may reduce apoptosis of HEK293 cells. Differentially expressed genes of BPA exposure include upregulation of ERCC5 encoding a DNA endonuclease involved in nucleotide-excision repair. Using an electrochemical method based on the preferential electrochemical oxidation of chemically damaged DNA, no DNA damage was identified in HEK293 cells treated with 10^{-6} M BPA for 48 h, while a peak indicating DNA damage was observed in the ER-positive MCF-7 cell line.

Gassman et al., 2015 [RefID 2214]

In this study, DNA damaging effects of BPA were shown to be induced indirectly *in vitro* in mouse fibroblasts through the generation of ROS. A statistically significant increase of intracellular ROS production, measured by DCFH-DA, was observed in mouse fibroblasts (Ku70-deficient cell line) treated *in vitro* with BPA (Sigma-Aldrich) at 150 µM for 1 h. Treatment with BPA increased also the levels of DNA modified bases (5-OH-Cyt and ThyGly) consistent with the increase in ROS. Ku70 cell line, deficient in strand break repair by non-homologous end joining (NHEJ), a back-up repair pathway for BER, was used to investigate the effect of BPA on the BER response to oxidative stress. Ku70-deficient cells were very sensitive to the oxidative damaging agents: treatment with $KBrO_3$ induced a significant dose-response reduction of cell viability. Co-treatment with BPA partially reversed the $KBrO_3$-induced cytotoxicity in these cells, associated with the increase in oxidatively induced DNA base lesions, suggesting that BPA may prevent initiation of repair of oxidized base lesions. Examination of γH2AX foci revealed also a significant reduction in DNA damage signaling induced by BPA confirming the possible inhibition of BER repair.

Tran et al., 2020 [RefID 438-G]
The aim of the study was to investigate the effects of BPA in PBMC by using freshly isolated T cell subpopulations of healthy donors for short and long-term BPA treatment. Long term cultures of CD4+ or CD8+ T lymphocytes isolated from blood of male donors were established. Cell cultures were treated with BPA (purity >99%) from 0.3 to 30 nM for 24 h. A non-monotonic telomerase enzyme inhibition in CD8+T-cells, but not in CD4+T-cells of male donors was observed. The maximum inhibition (30% compared with control) was seen at 0.3 nM BPA. At higher concentrations the effect subsequently weakened. An impact of BPA on DNA repair capacity of CD8+T-cells upon exposure to oxidative stress (treatment with hydrogen peroxide) was also reported. A decreased DNA repair (maximum 45% inhibition at 0.3 nM), measured by comet assay, after BPA treatment compared with the control was observed. DNA repair gene pathway analysis was carried out by using a human DNA repair RT2 Profiler PCR array: no regulation on the expression of 84 DNA repair genes upon BPA exposure could be observed. Long-term treatment (after 35 days of culture) with BPA significantly reduced cell proliferation only at concentration <30 nM. The maximum inhibition was seen at 3 nM, with a reduction of 25% in comparison with the solvent control. Long-term treatment (49 days of culture) of CD8+T cells with BPA impaired T cell response upon antigen stimulation, as shown by decrease of telomere length and mitochondrial DNA (mtDNA) copy number by 35% and 25%, respectively. At a 10-fold higher or lower concentration, the effect was marginally evident for both parameters. A strong inhibitory effect of intracellular anti-viral interferon-γ expression was evident on day 42 of BPA exposure, compared with the solvent control.

E.2.3. Spindle stability

Campen et al., 2018 [RefID 240-G]
The aim of the study was to compare the effects of *in vitro* exposure to either BPA (Sigma-Aldrich) or BPS on meiotic progression, spindle morphology and chromosome alignment in the bovine oocyte. Bovine ovaries were sourced from an abattoir. Groups of 5–20 cumulus–oocyte complexes (COCs) extracted from the bovine ovaries were treated with BPA or BPS at 10 concentrations between 1 fM and 50 µM and underwent to *in vitro* maturation for 24 h, then the oocytes were extracted. For BPA experiments, a total of 939 oocytes were analyzed for meiotic stage (including 250 vehicle-only control oocytes), of which a total of 767 were at metaphase II (MII) (including 211 MII oocytes in the control) and were included for analysis of spindle and chromosome configuration. Immunocytochemistry was used to label the chromatin, actin and microtubules in the fixed oocytes. The meiotic stage was assessed using immunofluorescence, and the MII oocytes were further assessed for spindle morphology and chromosome alignment (in all MII oocytes regardless of spindle morphology). No difference in the proportion of bovine oocytes that reached MII was observed for BPA treatment. Significant effect on spindle morphology ($p < 0.0001$) was induced by BPA treatment at very low concentration (1 fM). Fewer oocytes with bipolar spindles were seen following exposure to BPA at concentrations of 1 fM, 10 fM, 100 fM, 10 pM, 1 nM, 10 nM, 100 nM and 50 µM, compared with the control. There was no effect of BPA on spindle morphology at concentrations of 1 or 100 pM. Increased chromosome misalignments were observed at BPA concentrations of 10 fM, 10 nM and 50 µM of BPA, no effect was detected at any other concentration. The study presents limitations: in the ovaries the effects were evaluated in a specific period of development (namely, the 24 h window of oocyte maturation), without considering potential prior historical exposures *in vivo*.

Kim et al., 2019 [RefID 319-G]
In vitro effects of BPA (Sigma-Aldrich) on mitotic progression were examined in HeLa cells exposed to 100 nM BPA for 5 h. Proteins involved in mitotic processes were detected by western blot, live cell imaging, and immunofluorescence staining. Under the applied treatment conditions, BPA was shown to disturb spindle microtubule attachment to the kinetochore, with the concomitant activation of spindle assembly checkpoint (SAC). Spindle attachment failure was attributed to BPA interference with proper localization of microtubule associated proteins, such as HURP to the proximal ends of spindle microtubules, Kif2a to the minus ends of spindle microtubules, and TPX2 on the mitotic spindle. BPA also caused centriole overduplication, with the formation of multipolar spindle.

Yang et al., 2020 [RefID 469-G]
The effect(s) of exposure to BPA (Sigma-Aldrich) on assembled spindle stability in ovulated oocytes were studied. Mature M II oocytes, recovered from the oviducts of superovulated B6D2F1 mice, were cultured for 4 h in the presence of increasing concentrations (5, 25, and 50 µg/mL) of BPA. After treatment oocytes were analyzed by immunofluorescence and live cell imaging to investigate the effect of BPA on spindle dynamics. BPA disrupted spindle organization in a dose-dependent manner, resulting in significantly shorter spindles with unfocused poles and chromosomes congressed in an abnormally elongated metaphase-like configuration, with increased erroneous kinetochore-microtubule interactions.

E.2.4. γH2AX

Audebert et al., 2011
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 BPA and BPF. The potential genotoxicity of BPA and BPF was assessed by the examination of γH2AX foci. BPA (radiochemical purity: 95%, specific activity: 3.6 GBq/mmol) was purchased from Moravek Biochemicals and Radiochemicals (Brea, CA) 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). Conversely, ACHN cell line was not able to metabolize BPA. Relevant metabolites were separated and quantified by radio-HPLC. Following treatment with BPA (Sigma-Aldrich, purity >99%) at concentration-levels of 1, 5, 10, 50 and 100 µM for 24 h no increases of γH2AX were observed at any concentration tested in HepG2 and in LS174T cell lines. A concentration related increase of γH2AX was observed in ACHN cells.

Pfeifer et al., 2015 [RefID 5815]
The objective of this study was to investigate the effects of low-dose BPA (Sigma-Aldrich) in mammary gland cells. The human cell lines used in the study are the ERα-negative immortalized benign and normal breast epithelial cell lines (MCF10A and 184A1, respectively) and the ERα-positive MCF7 and MDA-MB-231 cell lines originate from human breast epithelial adenocarcinomas. Low concentrations BPA (10 and 100 nM) induced double strand breaks (DSBs) as measured by γH2AX foci in all cell lines. Both MCF10A and MCF7 cells had also a greater number of ATM-pS1981–positive nuclei after 24 h treatment compared with the control. Low-concentration BPA significantly increased the level of c-Myc protein and other cell-cycle regulatory proteins (cyclin D1, cyclin E and E2F1) and induced proliferation in parallel in ERα-negative 184A1 mammary cells. Silencing c-Myc reduced BPA-mediated increase of γH2AX suggesting that c-Myc plays an essential role in BPA-induced DNA damage.
The increased level of DNA double strand breaks induced by BPA exposure in 184A1 cells was also confirmed in a neutral comet assay and was found to be reduced by c-Myc silencing. Similarly, silencing c-Myc abolished BPA-mediated ROS production, which was localized to mitochondria. The authors concluded that low-concentration BPA exerted a c-Myc–dependent genotoxic and mitogenic effects on ERα-negative mammary cells (results reported as tail moment only and a single BPA concentration analysed).

Ganesan and Keating, 2016 [RefID 2141]
The study investigated ovarian DNA damage induced by BPA exposure using an *in vitro* model system. At PND 4, F344 rat ovaries were cultured in medium containing 1% DMSO ± BPA (from Sigma-Aldrich, 440 µM) for 1-4 days. Increased expression of γH2AX foci was observed by immunofluorescence staining in the ovaries evaluated (three ovaries/treatment/time point) at days 1, 2 and 4 of treatment, with a time-related increasing trend. Increased abundance of phosphorylated H2AX and ataxia-telangiectasia mutated (ATM), markers of DNA double-strand breaks, were also detected by western blotting in total ovarian protein homogenates. Expression of DNA repair genes (ATM, Prkdc, Xrcc6, Brca1, Mre11a, Rad50, and Smc1a) assessed by qRT-PCR was also increased ($p < 0.05$) 1 and 2 days after BPA exposure (the 4 day time point was not analysed). Overall, the results of this study indicate that the *in vitro* exposure to BPA can induce DNA double strand breaks that activate a DDR in ovarian cells.

Quesnot et al., 2016 [RefID 6033]
The study authors applied an automated in situ γH2AX detection system in metabolically competent HepaRG cells to evaluate the genotoxicity of 10 environmental contaminants, including BPA, after single or long-term repeated *in vitro* exposure (1, 7 or 14 days). Cytotoxicity was determined using the MTT assay. BPA (Sigma-Aldrich) was tested at four concentrations (20, 40, 50 and 60 µg/mL), selected to avoid excessive toxicity (i.e. with viability > 60%). A significant increase in the H2AX positive cell index (fold increase over control) was observed after 24h at 40 µg/mL and above, but not after 7 days or 14 days treatments. The incidence of Hoechst-stained MN was also quantified in HepaRG cells exposed to BPA for 1 or 7 days before mitogenic stimulation by EGF for 3 days. Under the test conditions, no significant increase of MN was observed in BPA treated cells, while positive results were obtained in parallel experiments with known genotoxic contaminants (benzo(a)pyrene, AFB1, DMBA).

Liang SX et al., 2017 [RefID 4246]
The testicular toxicity of BPA (99% purity) was compared with that of BPS, BPAF, and tetrabromobisphenol A (TBBPA) on the C18-4 spermatogonial cell line. The authors developed and validated an automated multi-parametric high-content analysis to study nuclear morphology, DNA content, cell cycle progression, DNA synthesis, cytoskeleton integrity, and DDRs induced by these compounds. The only marker of DNA damage analysed was the formation of γH2AX foci. BPA showed a small increase at a single dose of 50 µg/mL after 48 and 72h exposure (doses tested: 0.1, 1, 5, 10,25, 50 µg/mL). The study presents several limitations in the reported methodology.

Mahemuti et al., 2018 [RefID 11171]
The aim of this study was to investigate the key molecular pathways involved in the developmental effects of BPA on human fetal lung and their potential implications in the link between pre-natal exposure to BPA and increased sensitivity to childhood respiratory diseases. Global gene expression profiles and pathway analysis was performed in cultured HFLF exposed to non-cytotoxic concentrations of BPA (0.01, 1 and 100 µM BPA for 24 h, 99% purity, Sigma-Aldrich). Molecular pathways and gene networks were affected by 100, but not 0.01 and 1 µM BPA. These changes were confirmed at both gene and protein levels. The pathways affected by BPA included the cell cycle control of chromosome replication and a decreased DDR. BPA increased DNA DSBs as shown by phosphorylation of H2AX and activated ATM signaling (increased phosphorylation of p53). This resulted in increased cell cycle arrest at G1 phase, senescence and autophagy, and decreased cell proliferation in HFLF. Finally, BPA increased cellular ROS level and activated Nrf2-regulated stress response and xenobiotic detoxification pathways. The authors suggest that pre-natal exposure to BPA may affect fetal lung development and maturation, thereby affecting susceptibility to childhood respiratory diseases.

Kim et al., 2018b [RefID 11137]
BPA (> 99% purity, Sigma-Aldrich) promoted cell proliferation in undifferentiated and differentiated human hepatocyte cell lines (HepG2 and NKNT-3, respectively) at submicromolar concentrations (0.3-5 µM for 24 h). The proliferative effects of BPA disappeared at concentrations higher than 5 µM (cell viability decreased at concentrations higher than 10 µM). Exposure to BPA in the submicromolar range induced DNA damage in both cell lines as shown by a dose-dependent increase in phosphorylation of histone H2AX (γH2AX), p53 activation and induction of cyclin B1. Increased levels of γH2AX were also observed in liver tissue of juvenile rats (PND 9) orally exposed to a relatively low dose of BPA (0.5 mg/kg for 90 days). At a higher BPA dose (250 mg/kg) no increase in hepatocyte proliferation or cyclin B1 was observed. BPA promoted ROS generation as measured by DCF-DA-enhanced fluorescence in HepG2 cells. Increased levels of ROS were suggested to play a role in BPA-induced proliferation and DNA damage as shown by the partial reversion of both processes upon pre-treatment with NAC.

Hercog et al., 2019 [RefID 287-G]
With the aim of comparing the toxicological profiles of possibly safer analogues of BPA, the authors investigated the cytotoxic/genotoxic effects of BPS, BPF and BPAF and their mixtures in human hepatocellular carcinoma HepG2 cells. Single exposure to BPA (99% analytical purity, Sigma-Aldrich) did not induce any significant changes in cell viability at the tested concentrations (2.5, 5, 10, 20 µg/mL for 24 or 72 h). Induction of a significant increase in DNA double strand breaks, as determined by

γH2AX assay, was observed only at the highest dose (20 µg/mL for 72 h). BPA (tested at the 10 µg/mL concentration) induced changes in the expression of some genes involved in the xenobiotic metabolism (*CYP1A1, UGT1A1*, but not *GST1*), response to oxidative stress (*GCLC* but not *GPX1, GSR, SOD1, CAT*), while no changes were observed in any of the genes involved in the DDR (*TP53, MDM2, CDKN1A, GADD45A, CHK1, ERCC4*). Similar results were obtained when cells were exposed to BPA as a single compound or in mixtures with its analogues at concentrations relevant for human exposure (10 ng/mL). The relevance of these changes is of uncertain biological significance.

Hercog et al., 2020 [RefID 288-G]

In a follow-up study by Hercog et al. (2020) the genotoxic effects induced by co-exposure of the cyanotoxin cylindrospermopsin (CYN)(0.5 µg/mL) and BPA (Sigma-Aldrich), BPS and BPF(10 µg/mL, 24 and 72 h exposure) were investigated on HepG2 cells using the same techniques and experimental conditions of Hercog et al. (2019). The results obtained with BPA confirm the previously published observations, but the relevance of these changes remains of uncertain biological significance.

Yin et al., 2020 [RefID 474-G]

The scope of the study was developing a novel *in vitro* three-dimensional testicular cell co-culture mouse model that enables the classification of reproductive toxic substances. BPA (99%, Sigma-Aldrich) as well as BPS, TBBPA, and BPAF were used as model compounds. A concentration-dependent increase in BPA toxicity was found in the range 2.5 - 400 µM following 24, 48 and 72 h exposures. The large variations in the number of gH2AX foci observed at 72 h make the relevance of these results questionable. No increase in gH2AX used as marker of DNA damage was found up to a dose of 100 mM (70% cell viability).

Nair et al., 2020 [RefID 367-G]

The effects of BPA (Sigma-Aldrich) as a single agent, or in combination with 4-tert-octylphenol (OP) and hexabromocyclododecane (HBCD), were studied in the HME1 mammary epithelial cells and in the MCF7 breast cancer cell line. Following a 2-month exposure to a low non-toxic BPA concentration (0.0043 nM), increased levels of DNA damage were evidenced by upregulation in both cell lines of phosphorylated DNA damage markers (γ-H2AX, pCHK1, pCHK2, p-P53). Disruption of the cell cycle was observed both after short exposures (24 h and 48 h, G2/M arrest) as well as after the 2-month exposure treatment (G1 and S phase increases). BPA increased cellular invasiveness through collagen. Methylation changes were investigated by Methylation Specific Multiplex-Ligation Dependent Probe Amplification (MS-MLPA) using a panel of 24 tumour suppressor genes (all hypomethylated) and identified hypermethylation of *TIMP3, CHFR, ESR1, IGSF4* in MCF7 cells and *CDH13* and *GSTP1* genes in HME1 cells. Finally, BPA induced phosphorylation of six protein kinases in HME1 cells (EGFR, CREB, STAT6, c-Jun, STAT3, HSP60) and increased levels of several other proteins involved in potential oncogenic pathways (HSP27, AMPKα1, FAK, p53, GSK-3α/β, and P70S6).

Yuan et al., 2021 [RefID 477-G]

This study investigated the combinatorial toxicity of BPA (≥ 99.8% purity), decabrominated diphenyl ether and acrylamide to HepG2 cells. Increased number of γH2AX foci were induced in HepG2 by a 24h exposure to a single BPA dose that induced 25% toxicity. The majority of the data (ROS measurements, Ca2+ flux, DNA damage, Caspase-3 and decreased mitochondrial membrane potential) refers to additive/synergistic effects induced by varying combinations of contaminants. The authors conclude that BPA induced an increase in γH2AX fluorescence and in the number of γH2AX foci/nucleus. However, this conclusion is not fully supported by the data presented.

Escarda Castro et al., 2021 [RefID 266-G]

The ability of BPA to induce genotoxic and epigenetic changes was investigated before and during cardiomyocyte differentiation in H9c2 rat myoblasts exposed to 10 and 30 µM BPA (92% and 73% of cell viability, respectively). Exposure to BPA (no information on purity or the supplier company) before differentiation repressed the expression of the *Hand2* and *Gata4* heart transcription factors and three genes belonging to the myosin heavy chain family *(Myh1, Myh3, and Myh8),* whereas exposure after the 5 days of differentiation reduced the expression of cardiac-specific *Tnnt2, Myom2, Sln,* and *Atp2a1* genes. BPA did not induce ROS and did not increase DNA 8-oxodG levels (as measured by

immunostaining) in either myoblasts or cardiomyocytes. After BPA exposure the percentage of DNA repair foci formed by co-localization of the γH2AX and 53BP1 proteins increased in a concentration-dependent manner in myoblasts (from 44% in the control group to 61% and 86% at 10 and 30 µM BPA, respectively), with no increase in MN. Repair foci also increased in cardiomyocytes (from 45% in the control group to 59% and 72% at 10 and 30 µM BPA, respectively). A small increase (up to 13%) in MN was also reported only in cardiomyocytes treated with 10 µM BPA. A decrease in the epigenetic markers H3K9ac and H3K27ac was also reported. The authors concluded from these *in vitro* data that BPA interferes with the process of cardiomyocyte differentiation. However, the reliability and significance of the data on BPA-induced DNA damage is questioned by several negative factors (high background levels of DNA repair foci, lack of information on methods for micronucleus assays and the small increase of MN over high background).

E.2.5. DNA oxidative damage

Barbonetti et al., 2016 [RefID 419]

With the aim of studying whether *in vitro* exposure to BPA affected human sperm integrity, the authors investigated the induction of pro- oxidative/apoptotic mitochondrial dysfunction. The decrease in the mitochondrial membrane potential of motile sperm observed following a 4 h exposure to 300 µM BPA (Sigma-Aldrich) was associated with increased mitochondrial generation of superoxide anion, caspase-9 and caspase-3 activation and motility decrement. Loss in sperm vitality associated with a complete sperm immobilization was observed following a 20h exposure to 300 µM BPA. This was accompanied by a significant increase in the formation of DNA 8-hydroxy-2′-deoxyguanosine. The significance of the increase levels of oxidative DNA damage in these extreme experimental conditions (no viability and no motility) is questionable.

Budiawan et al., 2018 [RefID 757-G]

The formation of the oxidized base 8-oxodG was analyzed by reacting dG with BPA with the addition of Fenton's reagent. DNA adduct 8-oxodG was analyzed by reverse phase HPLC with a UV/vis light detector at 254 nm. The results indicate that BPA acts as prooxidant because of the increase yield of 8-oxodG. The concentration of DNA 8-oxodG increases with increasing pH, temperature and length of incubation time. In the presence of BPA and Fenton's reagent the highest DNA 8-oxodG levels (42.258 ppb) are detected at pH 7.4, 60°C and 12 h incubation time (these are very low levels of DNA adduct formation). The relevance of this short report published in a conference proceedings is limited.

E.2.6. Effects on meiotic cells

Karmakar et al., 2017 [RefID 3388]

The objective of this *in vitro* study was to investigate the proliferation, survivability and apoptotic rate of mouse testicular germ cells (CD-1 and C57-GFP) exposed to BPA (information on purity no reported) and to examine the differential expression of germ cell markers in these cultured cells. Cell viability and proliferation were not affected by low BPA concentrations (0.01, 0.1, 1, and 10 µM). Germ cell self-renewal and expression of differentiation related marker proteins were also found to be unchanged at these concentrations. In contrast, a significant reduction in survival was observed at 100 µM (increased apoptosis). When *in vitro* treated BPA germ cells were transplanted into recipient testes, fewer colonies were observed at high BPA concentrations (10 and 100 µM). A significant frequency of recombination failure during meiosis (production of "crossover-less" synaptonemal complex in pachytene spermatocytes) was observed in 10 µM BPA-exposed germ cell transplanted recipient. Experiment on continuous BPA-exposed and 100 µM BPA- recovered germ cells suggested that spermatogonial stem cells have a larger potential to survive in adverse environment. Finally, in a comparative proteomic analysis several differentially expressed cellular proteins were identified after BPA treatment of germ cell in culture.

E.2.7. Epigenetics

De Felice et al., 2015 [RefID 1462]

The aim of this study was to investigate whether a) BPA exposure was associated to deregulated expression of microRNAs and b) these epigenetic effects induced alterations in gene expression able to persist throughout a lifetime. Placenta samples from pregnant women living in a polluted area (40 patients subjected to therapeutic abortion for fetal malformation) and placenta samples from a control group of women living in a non-polluted area (40 pregnant women with a healthy pregnancy) were used for genome-wide miRNA expression profiling using microarray technology. This approach allow identification of miRNAs that were aberrantly expressed in placentas from malformed fetuses. Twelve miRNAs were upregulated and six miRNAs were downregulated in placentas samples from malformed fetuses. BPA was absent in the control group, while was detected only in patients subjected to therapeutically abortion. High levels of BPA were associated with a significantly increased miR-146a expression. The authors conclude that miR-146a, which correlates with BPA accumulation in the placenta, is a measure of fetal exposure related to fetal malformations. Finally, the use of bioinformatics tools allowed prediction of the target genes of miR-146a and exploring their functions including the downstream pathways. Several pathways involved in immune/ inflammatory, cancer and neural diseases were identified.

Karaman et al., 2019 [RefID 269-G]

The aim of this study was to investigate the epigenetic regulation induced by *in vitro* exposure to BPA to reveal a possible role on the progression of prostate cancer. Changes in gene expression of chromatin modifying enzymes, promoter methylation of tumour suppressor genes and histone modifications were studied in the PC-3 human prostate carcinoma cell line. A significant decrease in global levels of 5-methylcytosine and an increase in 5-hydroxymethylcytosine were observed following exposure to 10 µM of BPA (99% purity, Sigma-Aldrich) for 96 h (in these experimental conditions no changes in cell proliferation were observed). A significant increase in DNA methylation of the *p16* promoter region, with a concomitant decrease in *p16* gene expression, was observed (1 and 10 µM), while no changes were found for other genes involved in cell cycle control (*Cyclin D2* and *Rassf1)*. Significant changes were observed in global histone modifications (H3K9ac, H3K9me3, H3K27me3, and H4K20me3) after 48 and 96 h exposures to BPA (0.1, 1 and 10 µM). Analysis of the promoter methylation status of 94 tumour suppressor genes identified significant changes in *BCR, GSTP1, LOX, NEUROG1, PDLIM4, PTGS2, PYCARD, TIMP3, TSC2 and ZMYDN10* (including the *MGMT* DNA repair protein). The authors conclude that epigenetic signatures such as DNA methylation and histone modifications could be proposed as molecular biomarkers of BPA-induced prostate cancer progression.

E.3. *In vivo* studies

E.3.1. Chromosomal damage

Masuda et al., 2005

The study investigated the systemic genotoxicity elicited in mice from BPA treated with nitrite under acidic conditions to simulate the stomach environment. BPA, purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan), was dissolved in DMSO and co-incubated for 1 h at 37°C in buffer (1:100 v:v) containing 100 mM sodium nitrite at pH 3.0-5.0. The reaction mixture was extracted with ethyl acetate, evaporated to dryness, and the residue dissolved in DMSO for the mutagenicity tests: Ames test (reported under *in vitro studies*) and micronucleus test in mouse peripheral blood reticulocytes. For the micronucleus test, samples of nitrite-treated BPA dissolved in DMSO were administered by gastric intubation to male ICR mice (five per experimental group) at a dose of 228 mg/kg body weight (bw); untreated BPA dissolved in DMSO (228 mg/kg bw) served as control. Five microliters of peripheral blood were collected from a tail blood vessel at 24, 48 and 72 h after sample administration. Blood samples were stained on acridine orange and at least 1000 RNA-containing erythrocytes were observed by fluorescence microscopy for micronucleus evaluation. Results obtained indicated that BPA under the condition of the study did not induce a detectable increase of micronucleated reticulocytes in treated mice. Conversely, nitrite-treated BPA significantly increased the frequency of MNRETs compared with vehicle controls and untreated BPA at 48 and 72 h after oral administration.

Naik and Vijayalaxmi, 2009

This study evaluated potential genotoxic effects of BPA by induction of chromosomal aberrations and MN in bone marrow cells of Swiss albino mice. To assess for potential interference of BPA with mitotic spindle apparatus, induction of c-mitoses was also performed. BPA (Loba Chemie, Mumbai, India) 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 five consecutive days. In single treatment schedule, sampling of bone marrow was performed at 6, 24, 48 and 72 h from beginning of treatment for both micronucleus and chromosome aberration assays. In cumulative treatment schedule, bone marrow was sampled in both assays 24 h 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 h. Results showed that no significant increases of chromosomal aberrations or MN were induced at any dose-level and sampling time used. Conversely, significant increases in the frequencies of gaps were observed in all dose-levels assayed at the 48 and 72 h sampling time and at the two higher dose-levels (50 and 100 mg/kg bw) at the 24 h sampling time. The significant increases of achromatic lesions (gaps) are not considered relevant for clastogenicity. In addition, BPA also induced c-mitotic effects through increases of mitotic indices and decrease in anaphase for both higher dose-levels at 24, 48 and 72 h sampling times.

De Flora et al., 2011

The study evaluated the MN frequency in bone marrow cells and DNA damage by the alkaline comet assay in peripheral blood lymphocytes, in male Sprague-Dawley rats treated with BPA (Sigma-Aldrich) via drinking water for a calculated daily intake of 200 mg/kg bw for 10 consecutive days and sacrificed at the end of treatment. Eight rats were used for each experimental group (control and BPA treated animals). No increase of MN frequency was reported in the bone marrow. No increase of DNA damage, expressed as tail moment, was observed in peripheral lymphocytes.

Tiwari et al., 2012

This study was aimed to assess potential genotoxic effects of BPA (Sigma-Aldrich) in rats (five males and five females per group) 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 MN 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. Results obtained for genotoxicity endpoints show marked dose-related increases of both MN 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 that were statistically significant at dose-levels as low as 10 µg/kg bw per day. Significant increase in plasma concentration of 8-OHdG was detected only at 50 mg/kg bw. A dose-related increase of malonaldehyde and decrease of glutathione were observed in liver.

Gajowik et al., 2013 [RefID 2120]

The study investigated the effects of 2-week exposure to BPA, either alone or in combination with X-rays, on the induction of genotoxic effects in different tissues of female mice. Outbreed female mice were treated with BPA (no information on purity) at 5, 10, or 20 mg/kg in drinking water for 2 weeks. The statistically significant increase in DNA damage, evaluated by comet assay was observed in lung only at 5 and 10 mg/kg. Negative results were reported in all the other organs analyzed in the same animals (spleen, kidneys, liver and bone marrow). Increase of MN frequency was observed in peripheral blood reticulocytes, but not in the bone marrow after 2 weeks of exposure. No increase was detected after 1 week of treatment. No clear results were reported for the combined exposure BPA and X-rays for both the biomarkers.

Srivastava and Gupta, 2016 [RefID 9611]

The genotoxic effects of repeated oral exposure to BPA in adult male Wistar rats was evaluated. Groups of 10 animals were administered with BPA (Sigma-Aldrich) dissolved in olive oil at three dose levels (5 µg, 50 µg and 100 µg/100 g bw), with and without vitamin E (4 mg/100 g bw), once a day for 90 days and sacrificed on day 91. The positive control group was injected with mitomycin C (3 µg/g bw) 48 h before sacrifice. Bone marrow was collected and micronucleus frequency determined in polychromatic and normochromatic erythrocytes in bone marrow smears stained with May-Gruenwald and Giemsa. A slight increase of MN in polychromatic (three-fold) and normochromatic (two-fold) erythrocytes was observed comparing the overall incidence of MN in vehicle controls and high dose BPA in PCE (4/2362 vs 12/2456) and NCE (5/9635 vs 11/9712). No statistical analysis was performed. The effect was not observed when animals were co-administered with BPA + vitamin E. No deviation of PCE/NCE ratio was observed.

Fawzy et al., 2018 [RefID 270-G]

The study was conducted to evaluate the protective action of pumpkin seed oil (PSO) against adverse effects induced by BPA. BPA (Sigma-Aldrich) was administered orally to male Swiss albino mice at 50 mg/kg bw once a day for 28 days. PSO was administered at 1 mL/kg bw either before, with or after treatment of BPA, for 28 days. Seven groups of animals (n = 10) were treated: group 1 (control); group 2 (vehicle); group 3 (PSO); group 4 (BPA); group 5 (PSO before BPA); group 6 (PSO with BPA) and group 7 (PSO after BPA). DNA damage was evaluated by comet assay in liver and testes. Fifty randomly selected nuclei per experimental group were analysed. MN frequencies were evaluated in bone marrow. Two thousand polychromatic erythrocytes (PCE) were scored per animal. A significant ($p<0.05$) increase of tail DNA % in liver and testes of BPA-treated group with respect to controls (19.93 ± 0.68 vs 13.15 ± 0.22 and 23.56 ± 0.45 vs 15.00 ± 0.50) was observed. A significant increase of MNPCEs (66.40 ± 9.94 vs 10.40 ± 2.96) and a decrease in the ratio of PCE/NCE were also detected. The histopathological examination revealed hepatocyte vacuolar degeneration with many necrotic cells. A defective spermatogenesis was also observed characterized by severe necrosis and loss of the spermatogonial layers with multiple spermatid giant cells formation in most of the seminiferous tubules and a congestion of the interstitial blood vessels. The treatment with PSO reduced the genotoxic effects induced by BPA. PSO before BPA treatment was the best regimen in the alleviation of the adverse effects.

Panpatil et al., 2020 [RefID 379-G]

The study evaluated the protective action of turmeric acid on the genotoxic effects of BPA in Wistar rats. Six groups of six animals were administered with BPA (Sigma-Aldrich) at 0, 50 and 100 µg/kg by oral gavage for a period of 4 weeks: three groups were fed with a normal diet, the others with a diet containing 3% turmeric. At the end of the experiment the animals were sacrificed. Urine was collected 24 h before the sacrifice. 8-OHdG was measured in urine using an ELISA kit. DNA damage by comet assay was evaluated in blood, liver and kidney: 50 cells per slide were counted twice. Micronucleus assay was applied in bone marrow: 2000 PCE were evaluated. A weak but statistically significant and dose related increase of tail length was observed in liver. In kidney an increase of DNA damage was observed only at the dose of 50 µg/kg. A dose related increase of 8-OHdG in urine and of the concentration of MDA in blood serum was observed. A dose related increase of MNPCE was reported associated with a low decrease of the PCE/NCE ratio. A significant decrease of the genotoxic effects was observed in animal fed with diet with turmeric.

E.3.2. DNA damage on somatic cells

Ulutaş et al., 2011

The authors aimed to assess potential genotoxicity of BPA (purity > 99%) in peripheral blood nucleated cells of rats by comet assay. Groups of six 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) used.

Dobrzyńska and Radzikowska, 2013

This study investigated the effects of BPA (no information on purity) 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 2 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. For comet assay animals were sacrificed 24 h after the last treatment and DNA tail moment was used to assess the levels of DNA breakage induced in cells isolated from liver, spleen, bone marrow, lungs, and kidneys. 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-levels assayed. No DNA breakage was detected in liver cells.

Zhou YX et al., 2017 [RefID 9083]

The study investigated the neurotoxicity of low-dose exposure to BPA in a mouse model, examining brain cell damage and the effects of learning and memory ability after 8 weeks exposure to BPA at 0.5, 50 and 5000 µg/kg bw (daily dose, by gavage). The comet assay was used to detect brain cell damage. At the end of treatment 11 mice per group were sacrificed and brain processed for comet assay. Forty cells from each brain were analyzed. Based on tail DNA percentage, the damage level was divided into five grades, from 0 (undamaged) to 4 (maximum damage). The results obtained indicated that with increasing exposure concentrations the fraction of damaged cells (all types) increased significantly from 23.0% in the control group to 47.3%, 66.6% and 72.5% in the low-, medium and high exposed groups, respectively. Also, the severity of DNA damage, expressed as arbitrary units (AUs), increased with AUs of 0.28 in the control to AUs of 0.59, 0.96 and 1.28 in the low-, medium and high-exposed groups, respectively.

Abdel-Rahman et al., 2018 [RefID 199-G]

The study evaluated the protective action of lycopene (LYC), an antioxidant agent, on the toxic effects of BPA (Sigma-Aldrich). Four groups of seven Wistar rats were treated daily for 30 days via gavage: the first group (controls) received corn oil, the second group was given lycopene at a dose of 10 mg/kg bw, the third group was given BPA at 10 mg/kg bw, the fourth group was administrated both BPA and LYC at the 10 mg/kg. Rats were sacrificed immediately after the last administration. Liver was frozen at -80 °C. Single-cell suspensions for comet assay were prepared from frozen livers. No positive controls were used. The comet method applied was not reported. A significant ($p < 0.05$) increase of tail DNA % in liver of BPA-treated group with respect to controls (25.05 vs 6.68) was observed. Higher activities ($p < 0.05$) of liver enzymes (serum ALT, alkaline phosphatase (ALP) and GGT and lower levels of total protein and albumin than control rats were detected in serum. Antioxidant enzymes (GPx, SOD and CYPR450 activities) significantly ($p < 0.05$) decreased while MDA level significantly increased in liver of BPA treated animals. Caspase-3 protein in liver of BPA-treated rats is overexpressed. Histopathological analyses showed deleterious hepatic changes ranging from hepatocytes' vacuolization and eccentric nuclei to focal necrosis and fibrosis. LYC administration reduced the cytotoxic effects of BPA on hepatic tissue, through improving the liver function biomarkers and oxidant-antioxidant state as well as DNA damage around the control values.

Kazmi et al., 2018 [RefID 315-G]

The study evaluated the protective role of *Quercus dilatate* extracts against BPA (no information on purity) induced hepatotoxicity. Ten groups of SD rats (7 animals/group) were considered, including untreated control group and a group receiving the vehicle. The distilled water-acetone (QDDAE) and methanol-ethyl acetate (QDMEtE) extracts were administered in high (300 mg/kg bw) or low (150 mg/kg bw) doses to SD rats, intraperitoneally injected with BPA (25 mg/kg bw). A group of rats was treated only with BPA. Rats were sacrificed after 4 weeks of treatment and blood and liver were collected. The comet method applied was not sufficiently detailed. An increase of DNA strand breaks in hepatocytes was reported for animals treated with BPA alone. However, the results reported using the different parameters (tail length, % of DNA in tail, tail moment) are not consistent. The % of DNA in tail is 28.35 ± 1.2 in BPA treated animals vs 0.01 ± 0.005 in controls. The value of % of DNA in tail in controls is extremely low with respect to the data reported in the scientific literature. Significant reduction in haemoglobin level, red blood cells and platelet count, whereas elevated levels of white blood cells and erythrocyte sedimentation rate (ESR) were observed in the BPA treated group.

Administration of BPA significantly ($p < 0.05$) decreased the endogenous antioxidant enzyme (CAT,GPx, superoxide dismutase (SOD) and GSH) levels compared with control group. In addition, in the BPA treated group, H_2O_2, nitrite and TBARS levels in the hepatic tissue were found to be higher when compared with controls. Histopathological examination of BPA treated animals revealed intense hepatic cytoplasm inflammation, centrilobular necrosis, cellular hypertrophy, fatty degeneration, vacuolization, steatosis and distortion of portal vein. A dose dependent hepatoprotective activity was exhibited by both the extracts of *Quercus dilatate* in different extent for the parameters analysed.

Elhamalawy et al., 2018 [RefID 510-G]

The study was aimed to evaluate the protective effects of sesame oil (SO) against BPA-induced hepatotoxicity in mice. Groups of male Swiss albino mice (n = 10) were given BPA (from Sigma-Aldrich, dissolved in ethanol and diluted in corn oil) by gavage at 50 mg/kg bw, with/without SO (1 ml/kg bw), once a day for 28 successive days. SO was administered through three regimens (before, with or after treatment with BPA). Liver DNA damage was evaluated by alkaline comet assay in 50 nuclei per experimental group. Mean tail length, tail moment and % tail DNA were significantly increased ($p < 0.05$) in liver of BPA treated mice. The effect was partially alleviated by SO administration at all the three regimens.

Sharma et al., 2018 [RefID 662-G]

The *in vivo* genotoxic potential of BPA in mouse organs was investigated using the alkaline comet assay. Male CD-1 mice (5 per group) were administered by gavage with BPA (Sigma-Aldrich) suspensions in corn oil prepared by ultrasonication at three dose levels (125, 250 and 500 mg/kg bw), twice 24 h apart. Ethyl methane sulphonate, given once by gavage at 300 mg/kg bw, served as positive control. Animals were sacrificed 3 h after the last treatment and DNA damage investigated by a commercial kit for comet assay in liver, kidney, testes, urinary bladder, colon and lungs cells. For each mouse, 200 cells were analysed (100 per gel) using an automatic comet assay scoring imaging system. Median values for each tissue from each animal were used, and the mean of the median values was evaluated in a statistical analysis. The results of comet assay did not show BPA related effects in any tissue, except for the testes, in which an increased level of DNA strand breaks ($p < 0.01$ compared with control group) was observed at the lowest dose; however, no dose response relationship was observed as the effects at the medium and highest doses were at the same level as the control group. A modified alkaline comet assay was conducted on human sperm cells treated with BPA 0, 1, 1.5, 2 and 3 µmol/L for 1h. BPA 3 µmol/L reduced cell viability to 60%, therefore it was the highest concentration tested. Ethyl methanesulfonate (EMS) was used as positive control. In total, 600 cells were scored for each concentration. No increase in % tail DNA was observed compared with the negative control.

Amin, 2019 [RefID 216-G]

The aim of the study was to evaluate the cardiotoxicity of BPA and to assess copeptin as a cardiotoxic diagnostic and prognostic biomarker. Three groups of Wistar rats were treated subcutaneously (SC) once daily (6 days/week) for 4 weeks: group I, naive group received regular diet and water; group II, vehicle group administered corn oil; and group III received BPA (Sigma-Aldrich) daily (30 mg/kg bw per day SC). Rats were sacrificed immediately after the last administration: blood samples were collected for estimating serum copeptin levels and the hearts were subjected to histological, immunohistochemical, and electron microscopic examination. Hearth cells were isolated and processed for comet assay. A statistically significant increase of % of DNA in tail was detected in BPA treated animals with respect to controls (6.88 vs 1.67). BPA induced a significant increase in mean values of serum copeptin level and histopathological changes with dilated congested blood vessels and extensive collagen fiber deposition in the myocardium. Light microscopic examination revealed focal disruption of cardiomyocytes with some nuclear changes, such as karyolysis and pyknosis and sarcoplasmic vacuolization. The mitochondria appeared swollen and deranged with different sizes and shapes.

Majid et al., 2019 [RefID 354-G]

The study evaluated the protective role of sweet potato (*Ipomoea batatas* L. Lam.) against BPA-induced testicular toxicity. Sixteen groups of seven Male SD rats were established, including controls, animals treated with the vehicle, with ethyl acetate and methanol extracts from tuber and aerial part of *Ipomoea batatas,* with BPA (Merck KGaA) and with BPA and different extracts of *Ipomea batatas*. The BPA group

received 50 mg/kg bw dissolved in 10% DMSO, injected intraperitoneal on alternate days for 21 days. The rats were sacrificed 24 h after the last treatment. Comet assay was applied to evaluate the DNA damage. An average 50–100 cells were analysed in each sample for comet parameters (head length, comet length, tail moment, tail length, and amount of DNA in head) of gonadal cell's nuclei. A statistically significant increase of % DNA in tail (3 folds with respect to the control value) was reported in the group of rats treated with BPA. Endogenous antioxidant enzymes were measured in supernatant from the testicular homogenates: BPA decreased the levels of peroxidases (POD), CAT, SOD. BPA induced also gonadotoxicity measured as size and weight of testes and epididymis, concentration and quality of sperms. The treatment with extracts of *Ipomea batatas* significantly reduced the gonadotoxicity induced by BPA, the DNA damage and restored the levels of antioxidant enzymes.

Mohammed et al., 2020 [RefID 363-G]

The study evaluated the protective role of ginger extract (GE) against BPA-induced toxic effects on thyroid. Four groups of 20 male albino rats were treated orally with BPA (Sigma–Aldrich), GE or both once a day for 35 days as follow: Control group: 0.1 ml/rat of corn oil; BPA group: 200 mg/kg bw per day (1/20 of the oral LD50); GE group: ginger extract 250 mg/kg bw; BPA + GE group: ginger extract followed by BPA after 1 h with the same doses as the other groups. The animals were sacrificed 24 h after the last administration. DNA damage was evaluated by comet assay. A statistically significant increase of DNA damage expressed as tail % DNA, tail length and tail moment were shown in thyroid follicular cells of animals treated with BPA. A concurrent increase of MDA and a decrease of GSH, and SOD were also observed. Adverse effects on the thyroid gland were reported with a significant decrease in serum levels of T_3 and T_4 accompanied by a significantly increase in serum TSH level. A decrease of Nrf-2 mRNA relative expression and protein concentration and of HO-1 mRNA expression in the BPA-induced thyroid injured rats were also described. The histopathological analysis revealed an alteration of the thyroid gland follicles most of which containing scanty colloid secretion and some others atrophied. The treatment with GE significantly reduced the genotoxic damage and the alteration of thyroid hormones regulating genes.

Zhang et al., 2020 [RefID 485-G]

The study investigated the long-term neurotoxicity of maternal exposure to BPA in mouse offspring. Pregnant mice (F0) were orally dosed with BPA (from Sigma-Aldrich) dissolved in tea oil at 0.5, 50, 5000 µg/kg bw per day until weaning. Then, the first generation (F1) of mice were used to generate the F2. DNA damage in brain cells was evaluated by comet assay in eight male and eight female mice from both F1 and F2. DNA damage, expressed as AU, was slightly (less than 2-fold) increased in the F1 male mice at the lowest dose and in females at the intermediate dose. No effect of BPA exposure was observed in the F2 mice.

E.3.3. DNA damage on germ cells

Ullah et al., 2019 [RefID 443-G]

The study evaluated the effect of subchronic exposure to BPA (Santa Cruz Biotechnology) and the analogues bisphenol B, F and S, on DNA integrity of rat spermatozoa. Sprague-Dawley rats (7 per group) received daily administrations of bisphenols by gavage in 0.1% ethanol solution at 5, 25 and 50 mg/kg bw per day. Animals were sacrificed on day 29th and DNA damage in spermatozoa from the cauda epididymis evaluated using a modified neutral comet assay scoring 100 cells per animal. Both tail moment and % tail DNA were significantly ($p < 0.05$) increased in the BPA 50 mg/kg bw per day group compared with vehicle controls, while no significant difference with controls was observed in the BPA 5 and 25 mg/kg bw per day groups. Comet assay was also performed on rats sperm cells treated *in vitro* with BPA 0, 1, 10, and 100 µg/L for 2h. A statistically significant increase in % tail DNA was observed at the highest concentration tested. Under the same treatment conditions statistically significant increases in SOD, TBARS and total ROS were observed at BPA 100 µg/L.

Zhang S et al., 2019 [RefID 486-G]

The study investigated the effects of maternal exposure to BPA on testicular development in offspring males. Pregnant Kunming mice were randomly divided into seven groups with 20 mice in each group. One group served as control, the others received BPA (Sigma, USA) in drinking water at 0.05, 0.5, 5,

10, 20, and 50 mg/kg bw per day, for 40 days from gestation day 0 to lactation day 21. F1 male mice were sacrificed at weaning (PND21). Testicular DNA damage was evaluated by comet assay and expressed as OTM. The results obtained showed that the testicular germ cell damages of group D, group E, group F and group G (5, 10, 20 and 50 mg/kg bw per day, respectively) were significantly higher than that of the control group ($p < 0.05$). No experimental data are shown.

Pan et al., 2020 [RefID 377-G]

The study evaluated the effect of BPA on DNA integrity and protamination of mouse sperm cell. Newborn male mice were subcutaneously injected with BPA (from Sigma-Aldrich, 0.1 and 5 mg/kg bw, n = 15) or corn oil (control group, n = 20) daily from PND 1 until 35. At PND 70, epididymis caudal spermatozoa and testes were collected. A flow cytometry sperm chromatin structure assay (SCSA) was performed to evaluate DNA fragmentation of sperm cells. DNA fragmentation was expressed as DNA Fragmentation Index (DFI), calculated as the ratio between sperm cells with strands DNA. Apoptosis of spermatogenic cells (spermatogonia, spermatocytes, round spermatids, and elongated spermatids) was determined by the TUNEL method. Exposure to BPA was associated with a significant ($p < 0.01$) and dose-related increase of the DFI in sperm cells and apoptosis in spermatogenic cells ($p < 0.05$).

Sahu et al., 2020 [RefID 405-G]

The study investigated BPA toxicity in sperm cells of juvenile Sprague-Dawley rats. BPA (Sigma-Aldrich) was administered by gavage daily for 5 days per week for 8 consecutive weeks to 4 rat groups (7 animals/group, of which only 4 used for genotoxicity assessment): i) Control (normal feed and water); ii) BPA (100 mg/kg bw per day); iii) control fed with a zinc deficient diet (ZDD); IV) BPA + ZDD. Sperm DNA damage was evaluated by the comet and Halo assays, apoptosis in testes cells was quantified by TUNEL assay, and testicular levels of 8-OHdG were determined by immunohistochemistry. All comet assay parameters (tail length, OTM and % DNA in the tail) and the nuclear diffusion factor in the halo assay, were slightly but not significantly increased in testes cells of BPA treated rats compared with controls. When associated with a ZDD, BPA exposure resulted in a significant ($p < 0.05$) effect in both assays. TUNEL-positive cells and percent of 8-OHdG positive areas in testicular tissue were also slightly but non significantly increased in BPA treated rats fed with standard diet, while the effect was statistically significant in rats receiving a ZDD.

Zahra et al., 2020 [RefID 481-G]

The study aimed to evaluate the protective potential of methanol extract of the Apocynaceae plant *Vincetoxicum arnottianum* (VAM) on BPA induced testicular toxicity in male SD rat. BPA (source and purity not specified) diluted in 10% DMSO was injected intraperitoneally at 25 mg/kg on alternate days for 30 days, with or without the oral co-administration of VAM extracts (150 and 300 mg/kg bw) to groups of seven rats. Sperm DNA damage was evaluated by comet and DNA ladder assays. Subchronic administration of BPA was associated with a significant ($p < 0.01$) increase of all comet parameters compared with vehicle controls. The effect was attenuated by co-administration of VAM extract in a dose-related manner. Electrophoresis on agarose gel showed extensive DNA fragmentation in testes of BPA treated rats.

Tiwari and Vanage, 2013

This study investigated the induction by BPA of dominant lethal mutations in the different stages of spermatogenesis in the rat. Furthermore, the induction of DNA damage by BPA in epididymal sperm was investigated. Holtzman male rats (7 per group) were treated by oral gavage with BPA (Sigma Chemical Co.) dissolved in ethyl alcohol and diluted in sesame oil, at dose-levels of 10 µg/kg bw and 5 mg/kg bw once a day for 6 consecutive days. Negative controls were treated with vehicle. Each treated male was mated with two females per week over a period of eight weeks. The mated females were then sacrificed on the day 15^{th} of their gestation and uterine content examined. DNA damage in epididymal sperm was evaluated by alkaline comet assay in sperm samples from treated males (4 animals per group) sacrificed after completion of the mating phase. In the dominant lethal study, a significant decrease in total implants/female and live implants/female, with a concurrent significant increase in the number of resorbed embryos per female, was observed during the fourth week and sixth week in females mated with males treated with 5 mg BPA/kg bw, suggesting the induction of

post-implantation loss due to dominant lethal mutations in mid-spermatids and spermatocytes. No significant change was observed in the pre-implantation and post-implantation losses in pregnant female mated with males exposed to 10 µg/kg bw of BPA. In the comet assay with epididymal sperm, a significant increase in comet parameters (tail length, tail moment and % tail DNA) was observed in rats treated with 5 mg/kg bw compared with control.

E.3.4. Effects on meiosis

Pacchierotti et al., 2008

The study evaluated the potential aneugenic effects of BPA on mouse male and female germ cells and bone marrow cells following acute, subacute or subchronic oral exposure. For experiments with acute and subacute exposure, female C57BL/6 mice were treated by gavage with BPA (from Sigma-Aldrich) dissolved in corn oil once with 0.2 and 20 mg/kg bw, or with seven daily administrations of 0.04 mg/kg bw. In subchronic experiments, mice received BPA in drinking water at 0.5 mg/L for 7 weeks. The dose levels tested for subacute effects in bone marrow and male germ cells were 0.002, 0.02 and 0.2 mg/kg bw for 6 days. For the assessment of aneugenicity in female germ cells, M II oocytes were harvested 17 h after induced superovulation, and cytogenetically analyzed after C-banding. The percentages of metaphase I-arrested oocytes, polyploid oocytes and oocytes that had undergone Premature Centromere Separation (PCS) or Premature Anaphase II (PA) were calculated. To evaluate the aneugenic effects of BPA upon the second meiotic division, zygote metaphases were prepared from superovulated females mated with untreated C57Bl/6 males. Zygote metaphases were prepared, C-banded and cytogenetically analyzed for the occurrence of polyploidy and hyperploidy. Experiments on male germ cells were performed with 102/ElxC3H/El)F1 males. Epididymal sperms were collected and hybridized with fluocrochrome-labelled DNA probes for chromosomes 8, X and Y and 10,000 sperm per animal were analyzed to evaluate the incidence of hyperhaploid (X88, Y88, XY8) and diploid (XY88, XX88, YY88) sperm cells. Micronucleus test was performed with four groups of five (102/ElxC3H/El)F1 male mice treated with 0, 0.002, 0.02 or 0.2 mg/kg BPA by gavage on 2 consecutive days and sacrificed 24 h after the second administration. In total, 2000 PCE from two slides were scored per animal for the presence of MN. No significant induction of hyperploidy or polyploidy was observed in oocytes and zygotes at any treatment condition. The only detectable effect was a significant increase of M II oocytes with prematurely separated chromatids after chronic exposure; this effect, however, had no consequence upon the fidelity of chromosome segregation, as demonstrated by the normal chromosome constitution of zygotes under the same exposure condition. Similarly, with male mice no induction of hyperploidy and polyploidy was shown in epididymal sperm after six daily oral BPA doses, and no induction of MN in PCE.

Liu C et al., 2014 [RefID 4378]

The study authors exposed 9-week-old male Wistar rats to BPA (Sigma-Aldrich) by gavage at 20 µg/kg bw per day for 60 consecutive days to coincide with one cycle of spermatogenesis. At the end of treatment testes were removed for the analysis of the staging of the seminiferous epithelium and microscopic analysis of meiotic chromosomal spreads. The formation of DNA double strand breaks in meiotic cells was assessed by immunofluorescence staining with anti-γH2AX antibodies. Immunostaining for synaptonemal complex protein 3 (SCP3), ATM kinase and pCHK1 was also performed on the spread nuclei. The assessment of the relative frequency of the 14 stages of the cycle of the seminiferous epithelium showed significant increases in stage VII and decreases in stages VIII and XIV, indicating an impairment of spermatogenesis following BPA exposure. Meiotic recombination, initiated by programmed double-strand breaks (DSBs) and marked by γH2AX expression, was also impaired in BPA-exposed rats. BPA exposure resulted in meiotic DSBs accumulation in pachytene spermatocytes. These cells exhibited chromosomal abnormalities, including asynapsis, end-to-end associations and interrupted regions of SCP3 staining, and increased activation of ATM. The authors concluded that BPA exposure at an environmentally relevant dose (20 µg/kg bw per day) induced meiotic abnormalities in adult male rats, with inhibition of spermiation and disruption of spermatogenesis. BPA exposure resulted in the delayed initiation of meiosis in the early meiotic stage and chromosomal abnormalities and meiotic DSBs accumulation in the late meiotic stage, which subsequently activated the ATM DNA damage checkpoint kinase.

Vrooman et al., 2015 [RefID 7521]

The aim of the study was to test the effect of estrogenic chemicals on meiotic chromosome dynamics during neonatal development when the first cells initiate meiosis. Outbred CD-1 and inbred C57BL/6J (B6) newborn male mice were given single oral doses of 20 or 500 ng/g per day BPA (supplied by NIEHS), ethinyl estradiol (EE) as positive control 0.25 ng/g per day or vehicle from 1–12 days post-partum (dpp). In total, six to 12 males (one to three males per litter from at least three litters) for each exposure group at 20 dpp at 12 weeks or 1 year of age (CD-1 only) were analyzed to assess the effects on synapsis and recombination. Immunostaining surface spread preparations of meiotic cells with antibodies for SYCP3 (a component of the synaptonemal complex) and MLH1 (DNA mismatch repair protein that localizes to the large majority of sites of meiotic exchange) was applied to evaluate the synapsis and recombination foci. Perturbations in synapsis were not observed in either strain, but MLH1 foci were significantly reduced in BPA and EE exposed CD-1 males. Mean MLH1 counts for juvenile CD-1 males were 22.27 ± 0.12, 21.50 ± 0.10, 21.87 ± 0.10, and 20.85 ± 0.10 for placebo, 20 ng BPA, 500 ng BPA, and 0.25 ng EE-exposed, respectively ($p < 0.0001$). No differences were detected in B6 males with any exposure. Similar reductions were observed in CD-1 males at 12 weeks and at 1 year. Insensitivity of B6 males to estrogen was considered as a reflection of genetic differences. The study shows that a brief exposure of newborn male mice to exogenous estrogen reduces the meiotic recombination increasing the incidence of meiotic errors and affecting permanently the spermatogenesis in the adult. The effect induced by BPA is similar to the positive control, no dose-response was observed for the BPA doses tested.

Zhang MQ et al., 2017 [RefID 8813]

The study investigated the protective role of melatonin against the adverse effects induced by BPA on the female reproductive system in mice. Three groups of female ICR mice were used: a control group, a BPA group treated daily with oral doses of BPA (supplier company and purity not reported) at 100 µg/kg bw and a group treated with BPA+melatonin (administered daily at 15 or 30 mg/kg bw) for 7 days preceding oocyte collection and analysis. The number of animals in each group was not reported. Oocytes were immunostained with anti-α-tubulin-FITC antibody to observe the spindle morphologies and counterstained with Hoechst to visualize the chromosome alignment. The treatment with BPA was shown to disrupt normal spindle assembly (70% of oocytes had disrupted spindles) chromosome alignment (80% of oocytes exhibited misaligned chromosomes). Metaphase I oocytes were briefly chilled to induce the depolymerization of microtubules that are not attached to kinetochores and then immunostained with CREST to detect kinetochores, with anti-α-tubulin-FITC antibody to visualize the microtubules and counterstained with Hoechst to observe the chromosomes. An increased frequency of kinetochores without attachment by microtubules was observed in BPA-exposed oocytes (76.1 ± 6.1% n = 99 vs 19.7 ± 1.8%, n = 92). A statistically significant higher frequency of aneuploid oocytes in M II was also observed in BPA-exposed oocytes (42.6 ± 1.8%, n = 94 vs 3.7 ± 3.7%, n = 82). In BPA-exposed oocytes, the fluorescence intensity of ROS was increased compared with controls (153.8 ± 7.7, n = 138 vs 70.9 ± 3.1, n = 96). An increase of early apoptosis was shown by the annexin-V signal. In addition, BPA-exposed eggs had significantly reduced fertilization rates (41.1 ± 1.5%, n = 65 vs 87.2 ± 0.9%, n =72). The melatonin administration significantly improved the oocyte quality via reduction of ROS levels and inhibition of apoptosis.

Horan et al., 2018 [RefID 291-G] [23]

In a comparative study on the meiotic effects in mice of BPA and its analogues, oral doses of 20 ng/g BPA (purity > 99%)[24] or placebo (vehicle only) control were administered on 14 and 15 days post coitum (dpc) to coincide with the time of meiotic entry in the fetal ovary. Variation in meiotic recombination was measured by the number of MLH1 foci in pachytene stage meiocytes. By comparison with unexposed female fetuses, BPA induced a significant increase in mean MLH1 counts ($p < 0.01$), indicating increased levels of meiotic recombination in developing oocytes as a result of maternal BPA

[23] Information on BPA purity provided by the study authors on 4 November 2021, upon EFSA request

exposure. According to the study authors, the subtle changes in meiotic recombination induced are compatible with continued oocyte survival, but increase the frequency of aneuploid eggs and embryos produced by the adult female. In contrast, in male neonatal exposure to BPA causes a permanent reduction in recombination levels spermatocytes, with an increase in the frequency of spermatocytes with at least one synaptonemal complex lacking an MLH1 focus. Low recombination rates were also considered deleterious because spermatocytes with homologues that failed to undergo recombination faced death due to the actions of the spindle assembly checkpoint mechanism that causes arrest of cells with unpartnered chromosomes at metaphase. Further data are presented in this work on the effects on male meiotic recombination in the progeny of 129S1/SvlimJ males (3 animals) inadvertently exposed to BPA released by PC cages, showing that the reduction of meiotic recombination rates persists for several generation (up to F3).

E.4. Other studies

E.4.1. DNA adducts

Izzotti et al., 2009

The study evaluated the formation of DNA adducts in female CD-1 mice receiving BPA (from Sigma Chemical Co.) in their drinking water (200 mg/kg bw) for 8 consecutive days by the ^{32}P postlabelling method. Results obtained indicated that administration of BPA, under the experimental conditions of the study, 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). The level of DNA adducts in the liver of BPA-treated mice, as evaluated in three replicate analyses, was 5.88 ± 0.29/10^8 nucleotides (means ± SE of the data obtained in five mice) vs. 1.71 ± 0.14 in controls ($p < 0.001$). The level of DNA adducts in the pooled mammary cells of BPA-treated mice was 4.97 ± 0.61/10^8 nucleotides (means ± SE of three replicate analyses) vs. 1.05 ± 0.13 in controls ($p < 0.001$). DNA adducts were not chemically characterized, but the authors noted that DNA adducts formed in liver and mammary cells had the same chromatographic mobility as those formed *in vitro* by the reaction of BPA with calf thymus DNA in the presence of S9 mix .

E.4.2. γH2AX

Chianese et al., 2018 [RefID 249-G]

The study investigated the effects of chronic exposure to the low dose of BPA, from foetal period to sexual maturation, on post-natal testis development. Wistar rats were used. BPA (Sigma Aldrich) at 0.1mg/L BPA was orally administered to dams or weaned offspring *via* drinking water. The daily dose of 10 µg/kg bw was calculated based on daily drinking consumption (the BPA dose used was lower than or within the reference limit for humans, currently considered "safe" by ESFA and by EPA). To analyze the possible effects of BPA on the first round of spermatogenesis, the male newborns were sacrificed at 17 PND (late infantile), 45 PND (pubertal), or 60 PND (young adult), randomly choosing a total of five animals/treatment/time point from different litters. Serum levels of BPA did not differ between exposed and control groups. The cytoarchitecture of the seminiferous epithelium was impaired in BPA-exposed animals due to low expression and scattered localization of connexin 43 (Cx43) and zonula occludens 1 (ZO-1), well-known markers of the BTB. DNA breaks were detected by immunofluorescence for γH2AX in spermatocytes and in round spermatids. A statistically significant increase of the expression rate of the crossover-associated protein *Mlh1*, a marker of DNA mismatch repair system and of *Rad51*, which encodes DNA repair protein (detected by quantitative real-time RT-PCR) was observed in all BPA-exposed rats. Massive and diffuse ROS production was observed in the testis of BPA-exposed animals. CAT and SOD significantly decreased at both 45 and 60 PND in BPA-exposed animals, compared with controls. Oxidative stress at DNA level induced by BPA was determined by 8-OHdG immunostaining. Immunofluorescence for 8-OHdG revealed diffused DNA damage in the germinal epithelium of treated animals. TUNEL signal was detected in the germinal compartment at basal levels and in Sertoli cells in BPA-exposed animals at 45 and 60 PND. A significantly higher fraction of apoptotic cells/tubule was detected in BPA-exposed animals. A different pattern of *sirt1* mRNA expression was observed in BPA-treated rats with respect to the controls during the first round of spermatogenesis at

17, 45 and 60 PND with highest expression levels observed at 45 PND and lowest expression rate observed at 60 PND. SIRT1 has a recognized role in spermatogenesis in the regulation of the hypothalamus-pituitary-gonad (HPG) axis. *SIRT1* impairment may affect the progression of spermatogenesis towards late meiotic and post-meiotic stages, and the maturation of spermatozoa.

Yang et al., 2021 [RefID 471-G]

The aim of this study was to investigate the mechanisms underlying BPA-aggravated atherosclerosis. Four-week-old male Ldlr$^{-/-}$ C57BL/6 mice were administered 250 mg/L BPA (Sigma-Aldrich) via drinking water for 30 weeks with or without a western diet and/or resveratrol (RESV) for 12 weeks. The results indicate that chronic BPA exposure significantly aggravated atherosclerosis, enhanced the production of inflammatory cytokines and promoted macrophage infiltration into plaque areas. Peritoneal macrophages isolated from BPA-exposed mice exhibited a more pro-inflammatory phenotype in response to cholesterol crystal treatment than those from control mice. In comparison with the control group no change in the levels of DNA breaks (as measured by tail moment or tail DNA % in comet assays) was found in macrophages isolated from BPA-treated mice (data are not reported). The authors conclude that the DNA repair capacity of BPA-exposed macrophages, as measured by the kinetics of reduction in the number of DNA breaks following methyl methanesulfonate treatment, are reduced in comparison to the control group. However, the significance of a delay in DNA strand joining is uncertain and no quantitative evaluation of the data is presented. The overall significance of these contradictory results is uncertain.

E.4.3. DNA oxidative damage

Esplugas et al., 2018 [RefID 267-G]

The aim of the study is to investigate the renal and hepatic effects induced by a co-exposure to low doses of ionizing radiation and BPA. Sixty male mice (C57BL/6J) were randomly assigned to six experimental groups (n = 10) at PND 10 and received a single subcutaneous dose of 0.9% saline solution, BPA (Sigma–Aldrich) (25 µg/kg bw), cesium at two different doses or combined BPA and cesium. Urine (24 h) and blood were collected after 2 months. No increase of 8-OHdG in liver and kidney was observed in BPA treated mice. Following exposure to BPA the mRNA expression of CYP1A2 in liver decreased in respect to that in the control group. Limitations: only a single subcutaneous dose was applied; the analyses were carried out after 2 months. The study has a low relevance for the genotoxicity evaluation.

Franklyn et al., 2020 [RefID 521-G]

The study investigated the oxidative damage induced *in vivo* in rats by the subchronic administration of BPA, alone or in combination with chromium (VI), through the determination of 8-OHdG adducts in urine. Groups of five young adult rats received water (10 ml/kg bw per day) and served as controls, or BPA (from Sigma-Aldrich) (dissolved in DMSO and water at 2 mg/kg bw per day) or BPA (4 mg/kg bw per day) together with potassium dichromate (1.28 µg/kg bw per day) by gavage for 28 consecutive days. Urine was collected every week and the formation of 8-OHdG analyzed using LC-MS/MS with reverse-phase chromatography. The formation of 8-OHdG was detected in urine samples from BPA treated rats from week 1 onwards, with a slight increasing trend. The lowest and highest 8-OHdG concentrations were equal to 27.597 ng/mL and 31.683 ng/mL, respectively. 8-OHdG level in urine of control animals (only shown in a graph) was apparently zero. 8-OHdG levels in urine of rats treated with BPA and CrVI were higher than in the BPA group only at week 4. The authors concluded that CrVI had a synergistic effect on the formation of 8-OHdG by BPA, but the lack of a groups treated with CR VI alone, and the different BPA levels administered without (2 mg/kg bw per day) and with Cr VI (4 mg/kg bw per day) prevent a firm conclusion on this aspect. This study did not follow a validated test procedure for which an OECD TG is available. No positive control was used. The study has a low relevance for the genotoxicity assessment due to the experimental limitations.

E.5. Epidemiological studies

Lv YS et al., 2017 [RefID 4702]

The study investigated the contribution of BPA exposure via dermal contact route to human body burden and the relationship between BPA exposure level and oxidative DNA damage. Six male students living at the university were recruited and required to simulate the cashiers' work in handling the thermal receipts for 8 h/day for a period of 5 days. The volunteers were provided the same foods and drinks during the experimental periods to control the variations of BPA exposure levels from dietary and dust ingestion. Urine were collected for 5 days before the simulation, during the experimental period, and for 2 days after the simulation. BPA and 8-hydroxy-2′-deoxyguanosine (8-OHdG) concentrations were determined by high performance liquid chromatography/tandem spectrometer (LC/MS/MS). BPA concentrations was shown to increase four times during the simulation period compared with those before the experimental period. A similar trend was observed also for 8-OHdG. No significant decrease of both the parameters was reported in the post-experimental period. It was estimated that the contribution ratio of BPA via dermal contact to urinary BPA was 70.9%. BPA levels for individuals showed a similar trend with a large interindividual variability. A weak correlation, although statistically significant, was observed between urinary BPA and urinary levels of 8-OHdG (R^2= 0.237, $p < 0.001$). The study presents several limitations such as the low number of the subjects, the short post-experimental period, and does not allow any clear conclusion to be drawn.

Huang YF et al., 2017b [RefID 2926]

In this study the association between pre-natal exposure to nonylphenol (NP) and BPA and inflammation biomarkers was investigated in 241 mother-fetus pairs. Third-trimester urinary NP and BPA levels, and urinary biomarkers of oxidative/nitrative stress, were simultaneously measured in 233 urine samples. The biomarkers included products of oxidatively and nitratively damaged DNA (8-hydroxy-2′-deoxyguanosine (8-OHdG) and 8-nitroguanine (8-NO_2Gua)), as well as products of lipid peroxidation (8-isoprostaglandin F2α (8-isoPF2α) and 4-hydroxy-2-nonenal-mercapturic acid (HNE-MA)), analyzed by HPLC electrospray ionization (ESI)-MS/MS. Inflammatory biomarkers (IL-6, TNF-α and C reactive protein) were analysed in maternal and umbilical cord plasma samples by immunoassays, and the antioxidant GPx by a commercial kit. Maternal urinary NP and BPA levels were detectable in 99.2% and 82.2%, respectively. The geometric mean of the NP and BPA levels were 3.99 and 2.24 μg/g creatinine, respectively. After controlling for covariates, NP was significantly associated with increases in 8-NO_2Gua and 8-OHdG and decreases in TNF-α in the pregnant women. BPA was significantly associated with increased maternal 8-isoPF2α levels and decreased maternal and cord blood GPx levels. This study did not follow an internationally agree guideline, not available for the effects evaluates, but it was adequately performed and reported.

Radwan et al., 2018 [RefID 390-G]

The study examined the associations between urinary BPA concentration and male fertility in 315 men under 45 years of age with normal sperm concentration, attending a male reproductive health clinic for diagnostic purposes. Participants provided urine and semen samples on the same day of their clinic visit. BPA urinary concentrations were measured using gas chromatography coupled with tandem mass spectrometry. Semen parameters (sperm concentration, motility and morphology) were analyzed by standard procedures. Sperm DNA damage was assessed by a flow cytometry based sperm chromatin structure assay(SCSA). Sperm aneuploidy was evaluated by multicolour FISH analysis using DNA probes specific for chromosomes 13, 18, 21, X, Y; based on the information on six types of chromosome disomies, total disomy rates were calculated. A multiple linear regression analysis identified a positive association between the urinary concentrations of BPA in the 25th to –50th percentile and total sperm sex chromosome disomy ($p = 0.004$), but not with the higher quartiles (50th to -75th and > 75th). When modelled as continuous variable, urinary BPA concentration was associated with increased sperm sex chromosome disomy ($p = 0.01$). Such statistically significant relation was not noticed in case of sperm total chromosome disomy. Urinary concentration of BPA was also associated with increased percentage of immature sperms ($p = 0.018$) and decrease sperm motility ($p = 0.03$). No statistically significant association was observed between urinary BPA and sperm DFI. This study did not follow an internationally agreed guideline, not available for the effects evaluates, but it is adequately planned and reported. As acknowledged by the study authors the findings reported need to be confirmed in future studies. The lack of association between sperm disomy and BPA concentration in the higher quartiles is noticed.

Omran et al., 2018 [RefID 373-G]

The aim of the study is to evaluate a possible role of BPA exposure to the infertility in men. This case-control study involved 50 infertile patients and 50 matched controls. Sperm concentration, morphology, and motility were evaluated to estimate the semen quality. Urinary BPA levels were measured by high-performance liquid chromatography. Sperm DNA damage was determined by alkaline comet assay. Oxidative stress (total antioxidant activity and MDA levels) was also determined. BPA concentrations were similar in urine samples from all infertile patients and fertile controls, with median values of 24.2 µg/L and 20.9 µg/L, respectively. BPA levels were negatively associated with semen quality and antioxidant levels. The parameters of comet assay were statistically significant higher in infertile patients with respect to the controls (mean tail DNA % was approximately three-folds). The correlations between BPA levels and the comet parameter were analysed: weak but statistically significant correlations were reported for tail DNA % in the control group and in the total cases (Spearman's correlation coefficient: 0.500 (p value < 0.001) and 0.199 (p value < 0.05), respectively). The study presents some limitations: number of subjects recruited, the lack of the analysis of possible confounding factors.

Rocha et al., 2018 [RefID 402-G]

In this study, concentrations of 40 EDCs, including BPA, were determined in urine samples collected from 300 Brazilian children of ages 6–14 years. Oxidative DNA damage was evaluated from the urinary concentrations of 8-hydroxy-2′-deoxyguanosine (8-OHdG). BPA was the major compound found in 98% of the samples analyzed at concentrations ranging from < LOD to 35.9 ng/mL with a geometric mean of 1.74 ng/mL. Statistically significant correlation based on conventional univariate statistics was found between log-transformed urinary concentrations of 8OHDG and BPA. The study has low relevance for risk assessment due to the limitations in identifying the association in a scenario with multiple exposures.

Kiwitt-Cárdenas et al., 2021 [RefID 321-G]

In this cross-sectional study the association between urinary BPA concentrations and human sperm DNA fragmentation was investigated. The study was conducted with 158 healthy university students (18–23 years) that provided urine and semen samples on a single day. Urinary BPA concentrations were measured by dispersive liquid–liquid microextraction and ultrahigh performance liquid chromatography with tandem mass spectrometry detection. Sperm DNA fragmentation was analyzed by a Sperm Chromatin Dispersion (SCD) test, which measures halos of DNA loop dispersion after sequential incubation in acid and lysis solution under fluorescence microscopy, using a commercial kit. A SDF (sperm DNA fragmentation) index was calculated as the percentage of sperm with fragmented DNA divided by the total sperm analyzed (300 for each sample). No association was found between urinary BPA concentrations and SDF index in the total study population. A significant positive association with urinary BPA levels was only observed in the subgroup of men with SDF index > 30% (59 subjects). This study used a non-standards method to evaluate sperm DNA fragmentation that is not validated for regulatory purpose.

E.5.1. Polymorphisms in DNA repair genes

Kim and Hong, 2020 [RefID 564-G]

This study investigated the possible role of nine polymorphisms in three DNA repair genes (poly(ADP-ribose) polymerase family member 4 (*PARP4*); X-ray repair cross complementing 3 (*XRCC3*); RAD51 recombinase (*RAD51*)), on the relationship between BPA exposure and liver abnormalities in an elderly population. In total, 502 older adults without liver disease or renal failure aged 60 or over were included in the study. Urinary BPA, malonaldehyde (MDA) and cotinine levels (to monitor tobacco exposure) were measured. The serum levels of ALT, AST, and γ-GTP enzymes were used to detect liver abnormalities. LDL cholesterol levels were also measured. A significant association between BPA levels and liver abnormality was found only in elders with the PARP4 G-C-G haplotype, XRCC3 G-A-G haplotype, or RAD51 T-A A haplotype (OR = 2.16 for PARP4; OR = 1.57 for XRCC3; OR = 1.43 for RAD51). Particularly, PARP4 and XRCC3 showed significant interactions with BPA exposure in relation to liver abnormality (p < 0.05 for both genes). These results suggest that *PARP4*, *XRCC3*, and *RAD51*

gene polymorphisms may have modification effects on the relationship between BPA exposure and liver abnormality.

Appendix F – Benchmark dose analysis from which the reference point was selected (Luo et al., 2016 [RefID 4679])

F.1. Data Description

Th17 cell frequency in the spleen in offspring mice (%) was analysed. In the paper, the data are represented in a graph and the actual numbers were provided to EFSA by T. Shen. Based on the provided information, EFSA calculated the standard deviation (SD). The group, specified by sex and day of measurement, was used as a covariate. The 10/11 animals within each group are coming from different litters, supporting the independence assumption for which no litter effect was considered in the analysis. The data were treated as continuous data since the number of cells in the denominator were not available to consider the data as a quantal response. Both controls (blanc and vehicle) were not significantly different (t-test, p=0.89, 0.94, 0.74, 0.82 for male PND21, female PND21, male PND42, female PND42, respectively) and used in the analysis.

Table F1: Data used for analysis.

Dose (µg/kg bw per day)	Mean	SD	n	group
0.000	1.336	0.360	10	female PND21
0.000	1.348	0.310	10	female PND21
0.475	1.625	0.343	10	female PND21
4.750	2.200	0.663	10	female PND21
47.500	3.336	0.637	11	female PND21
0.000	1.222	0.233	10	female PND42
0.000	1.252	0.326	10	female PND42
0.475	1.541	0.325	10	female PND42
4.750	1.819	0.556	10	female PND42
47.500	2.477	0.477	11	female PND42
0.000	1.347	0.369	10	male PND21
0.000	1.327	0.282	10	male PND21
0.475	1.554	0.242	10	male PND21
4.750	1.774	0.501	10	male PND21
47.500	2.405	0.532	11	male PND21
0.000	1.194	0.235	10	male PND42
0.000	1.234	0.287	10	male PND42
0.475	1.426	0.278	10	male PND42
4.750	1.646	0.477	10	male PND42
47.500	2.226	0.526	11	male PND42

F.2. Selection of the BMR

An adverse outcome pathway for BPA leading to allergic responses that can be modelled to establish a BMD is currently not available. What can be stated is that T helper cells are key players in the immune-inflammatory chain of molecular events leading to amplification or suppression of specific immune elements, orienting the immune response towards effective resolution or chronic disease, and, according to an equilibrium in which these same cells and through the production of specific cytokines, restrict each other's own activity. Functionally, T helper 17 cells play a role in host defence against extracellular pathogens by mediating the recruitment of inflammatory cells to infected tissues. Aberrant regulation of Th17 cells plays a significant role in the pathogenesis of multiple inflammatory and autoimmune disorders. The most notable role of IL-17 produced by Th17 cells is its involvement in inducing and mediating proinflammatory responses, associated with allergic responses. IL-17 induces the production of many other cytokines (such as IL-6, G-CSF, GM-CSF, IL-1β, TGF-β, TNF-α), chemokines (including IL-8, GRO-α, and MCP-1), and prostaglandins (e.g., PGE2) from many cell types (fibroblasts, endothelial cells, epithelial cells, keratinocytes, and macrophages). As such, numerous

studies have shown that Th17 cells and their cytokines are also associated with the development of asthma (Doe et al., 2010). IL-17A is considered an important cytokine to induce the inflammatory response asthma. In the pathogenesis of asthma, Th17/IL-17A can induce the accumulation of inflammatory cells in the airway and participate in the process of asthma. In addition, the activation of Th17 cells and the secretion of IL-17 can increase the immune response of Th2 cells, thereby aggravating the severity of allergic asthma.

BPA exposure led to a dose-related increment of Th17 cells in mice. This effect was consistent with effects on cellular immunity based on Th17 cells and associated cytokines (IL-17, IL-21 and IL-23), as well as with effects of BPA in the cluster of allergic lung inflammation.

When using the benchmark approach, for dose-response analysis, a BMR needs to be selected. The EFSA guidance (EFSA Scientific Committee, 2017a) recommends a BMR of 5% as default. However, deviating from this default is possible, based on toxicological or statistical considerations. For Th17 cells, there is currently insufficient information available on the normal variability of this measure, either in the mouse strain used in the study, or other strains. In humans, a study published in 2016 reported a retrospective analysis on lymphocyte subpopulations, analysed over few years in an outpatient laboratory in Northeast Italy (Sorrenti et al., 2016) to provide reference ranges. In Caucasian patients (mean age 42 ± 8.5 years), mean values ± SD of Th17 in peripheral blood are 221.6 ± 90.2 cells/µL (10.5 ± 4.4%). Registered cases of lymphocyte associated diseases (immunodeficiencies and lymphoproliferative disorders) were excluded from the study, as well as samples with values of total erythrocytes, total leukocytes, total lymphocytes, and major lymphocyte populations (T cells, Th, Tc and B lymphocytes) outside the normal range according to guidelines.

Furthermore, the CEP Panel notes that the increment of Th17 cells is an intermediate endpoint, and some reserve capacity will exist. While considering that in the human population, for individuals a 20% increase may not necessarily imply an adverse condition for that person, given the pivotal role of Th17 cells in lung allergy, the CEP Panel considered that if the population at large showed a 20% increment in Th17 cells, individuals that are in the higher segment of the normal range, will be put out of the normal range, and as a consequence numbers of lung allergy cases would be expected to go up.

In conclusion, while the effect of BPA exposure on Th17 cells is clear, considering the standard deviation in the outcomes of the animal study (see the Table in Section 2.1.1 of Annex I), the CEP Panel considered 20% would be in line with the variability noted in the animal study and the wider normality range in humans, and considered it as adverse and took it as the BMR.

F.3. Results

Table F2: Fitted Models.

model	converged	loglik	npar	AIC
full model	yes	18.60	21	4.80
full-v	yes	18.65	24	10.70
null model	yes	−80.77	2	165.54
null model-a	yes	−76.25	5	162.50
Expon. M3-	yes	1.97	4	4.06
Expon. M3-a	yes	12.43	7	−10.86
Expon. M3-ab	yes	18.12	10	−16.24
Expon. M5-a	yes	12.43	8	−8.86
Expon. M5-ab	yes	18.12	11	−14.24
Hill m3-a	yes	12.43	7	−10.86
Hill m3-ab	yes	18.12	10	−16.24
Hill m5-a	yes	12.42	8	−8.84
Hill m5-ab	yes	18.12	11	−14.24
Inv.Expon. m3-a	yes	12.28	7	−10.56
Inv.Expon. m3-ab	yes	17.88	10	−15.76
Inv.Expon. m5-a	yes	12.21	8	−8.42
Inv.Expon. m5-ab	yes	17.74	11	−13.48
LN m3-a	yes	12.37	7	−10.74

LN m3-ab	yes	18.03	10	−16.06
LN m5-a	yes	12.33	8	−8.66
LN m5-ab	yes	17.97	11	−13.94

Table F3: Estimated Model Parameters.

EXP
estimate for var- : 0.04902
estimate for a-female PND21 : 1.291
estimate for a-female PND42 : 1.22
estimate for a-male PND21 : 1.299
estimate for a-male PND42 : 1.187
estimate for CED-female PND21 : 0.1863
estimate for CED-female PND42 : 0.4994
estimate for CED-male PND21 : 0.9104
estimate for CED-male PND42 : 0.8481
estimate for d- : 0.2959
HILL
estimate for var- : 0.04902
estimate for a-female PND21 : 1.291
estimate for a-female PND42 : 1.22
estimate for a-male PND21 : 1.299
estimate for a-male PND42 : 1.187
estimate for CED-female PND21 : 0.1867
estimate for CED-female PND42 : 0.5023
estimate for CED-male PND21 : 0.917
estimate for CED-male PND42 : 0.8541
estimate for d- : 0.2977
INVEXP
estimate for var- : 0.04913
estimate for a-female PND21 : 1.287
estimate for a-female PND42 : 1.222
estimate for a-male PND21 : 1.306
estimate for a-male PND42 : 1.193
estimate for CED-female PND21 : 0.2047
estimate for CED-female PND42 : 0.6054
estimate for CED-male PND21 : 1.177
estimate for CED-male PND42 : 1.089
estimate for d- : 0.0651
LOGN
estimate for var- : 0.04907
estimate for a-female PND21 : 1.289
estimate for a-female PND42 : 1.221
estimate for a-male PND21 : 1.303
estimate for a-male PND42 : 1.19
estimate for CED-female PND21 : 0.1977
estimate for CED-female PND42 : 0.5625
estimate for CED-male PND21 : 1.063
estimate for CED-male PND42 : 0.9869
estimate for d- : 0.1117

Table F4: Weights for Model Averaging.

EXP	HILL	INVEXP	LOGN
0.27	0.27	0.21	0.25

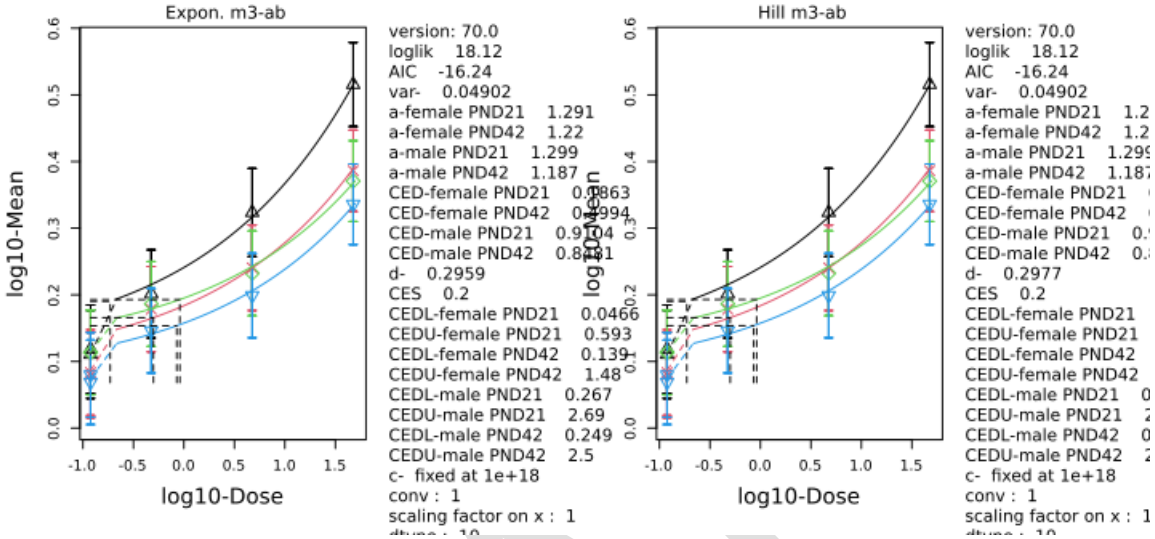
Table F5: Final BMD Values.

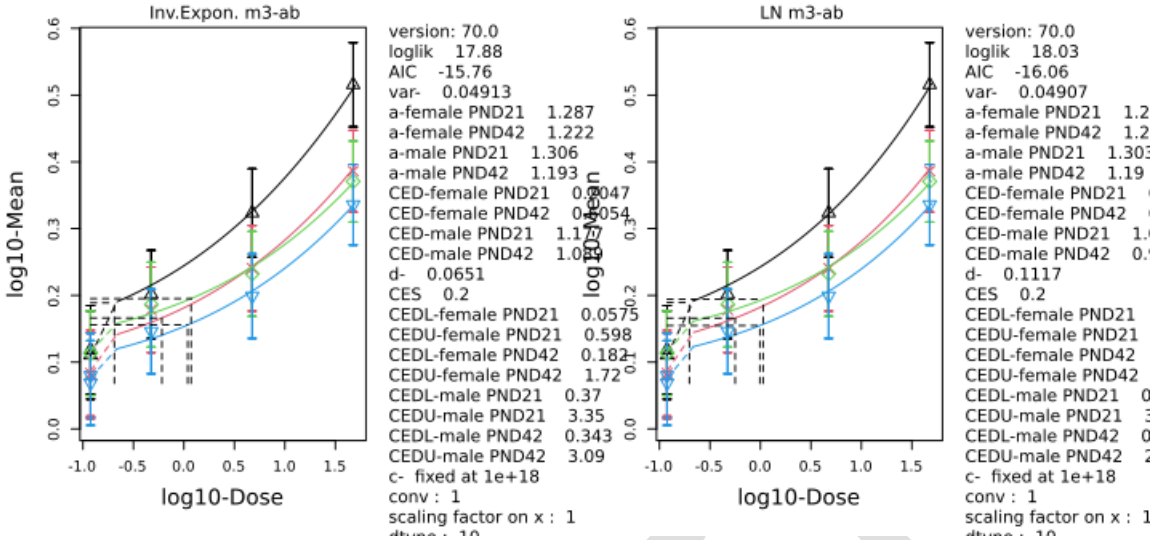
Subgroup	BMDL (µg/kg bw per day)	BMDU (µg/kg bw per day)

female PND21	0.06	0.74
female PND42	0.17	1.79
male PND21	0.30	3.39
male PND42	0.35	3.38

Confidence intervals for the BMD are based on 1000 bootstrap data sets.

Visualisation

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log10-Dose
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AIC -15.76
var- 0.04913
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a-female PND42 1.222
a-male PND21 1.306
a-male PND42 1.193
CED-female PND21 0.8047
CED-female PND42 0.8054
CED-male PND21 1.1
CED-male PND42 1.08
d- 0.0651
CES 0.2
CEDL-female PND21 0.0575
CEDU-female PND21 0.598
CEDL-female PND42 0.182
CEDU-female PND42 1.72
CEDL-male PND21 0.37
CEDU-male PND21 3.35
CEDL-male PND42 0.343
CEDU-male PND42 3.09
c- fixed at 1e+18
conv : 1
scaling factor on x : 1
LN m3-ab
log10-Mean
log10-Dose
version: 70.0
loglik 18.03
AIC -16.06
var- 0.04907
a-female PND21 1.2
a-female PND42 1.2
a-male PND21 1.303
a-male PND42 1.19
CED-female PND21
CED-female PND42
CED-male PND21 1.
CED-male PND42 0.
d- 0.1117
CES 0.2
CEDL-female PND21
CEDU-female PND21
CEDL-female PND42
CEDU-female PND42
CEDL-male PND21 0
CEDU-male PND21 3
CEDL-male PND42 0
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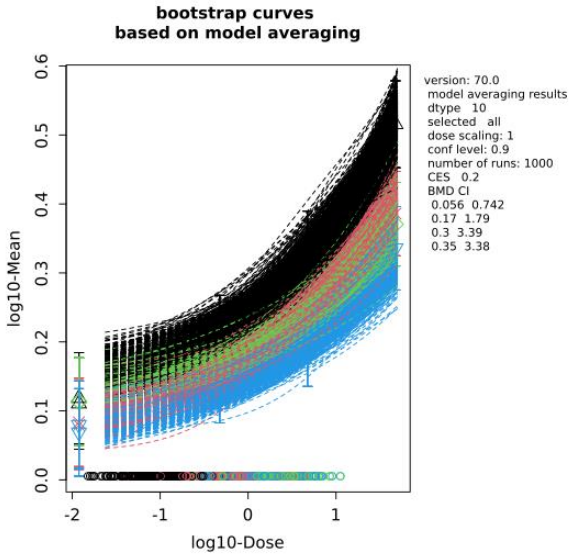
c- fixed at 1e+18
conv : 1
scaling factor on x : 1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log10-Dose
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selected all
dose scaling: 1
conf level: 0.9
number of runs: 1000
CES 0.2
BMD CI
0.056 0.742
0.17 1.79
0.3 3.39
0.35 3.38

Annexes

Annex A – Revised protocol on BPA hazard assessment

Annex B – Appraisal of internal validity of epidemiological studies

Annex C – Data extraction of epidemiological studies

Annex D – Weight of evidence from epidemiological studies

Annex E – Appraisal of internal and external validity of animal studies

Annex F – List of animal studies with endpoints appraised and relevant

Annex G – Data extraction of studies reporting relevant endpoints in animal studies

Annex H – Weight of evidence from animal studies

Annex I – Benchmark dose analysis

Annex J – Uncertainty analysis - blank excel templates including definitions

Annex K – Uncertainty analysis - additional results and calculation methods

Annex L – Weight of evidence on Genotoxicity

Annex M – Uncertainty analysis Genotoxicity - blank excel templates