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A statement on the developmental immunotoxicity of bisphenol A (BPA): answer to the question from the Dutch Ministry of Health, Welfare and Sport

Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF), Vittorio Silano, Claudia Bolognesi, Laurence Castle, Jean-Pierre Cravedi, Karl-Heinz Engel, Paul Fowler, Roland Franz, Konrad Grob, Rainer Gürtler, Sirpa Kärenlampi, Wim Mennes, Maria Rosaria Milana, André Penninks, Andrew Smith, Maria de Fátima Tavares Poças, Christina Tlustos, Detlef Wölfle, Holger Zorn, Corina-Aurelia Zugravu, Stacey Anderson, Dori Germolec, Raymond Pieters, Anna F Castoldi and Trine Husøy

Abstract

This statement addresses a request to EFSA from the Dutch Ministry of Health, Welfare and Sport to assess the impact of recent evidence underlying the conclusions of the 2016 RIVM report on the temporary tolerable daily intake (t-TDI) for BPA of 4 μg/kg bw per day set by EFSA in 2015. The CEF Panel has then evaluated the results of two studies published by Ménard et al. in 2014, suggesting food intolerance and impaired immune response to parasitic infection in rats exposed perinatally to BPA doses in the μg/kg bw per day range. The same appraisal criteria and weight-of-evidence analysis used for the 2015 EFSA opinion on BPA were applied to these studies. This new evidence adds to the indications of immunotoxicity of BPA in animals reported in previous reviews. For the only endpoint for which multiple BPA doses were tested (immunoglobulin G (IgG) levels), a benchmark dose analysis of the dose–response data was carried out. Due to the high interanimal variability within the treatment groups resulting in wide confidence intervals and the limited dose–response, the CEF Panel concluded that these data on anti-ovalbumin IgG antibodies are not suitable to derive a reference point for BPA on immunotoxicity. Furthermore, the limitations identified in both the Ménard et al. studies confound the interpretation of the results and prevent the assessment of the relevance to human health. The CEF Panel overall considers that the results from the two Ménard et al. studies are not sufficient to call for a revision of the EFSA t-TDI for BPA. EFSA will start a review of all the scientific evidence published after 2012 and relevant for BPA hazard assessment (including immunotoxicity) in 2017. The results of immunological studies, such as the two evaluated here, would form a useful contribution to this evaluation provided that the limitations identified herein were addressed.

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Keywords: bisphenol A, immune system, *in vivo*, perinatal

Requestor: Ministry of Health, Welfare and Sport, The Netherlands

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1. Introduction

1.1. Background and Terms of Reference as provided by the Dutch Ministry of Health, Welfare and Sport

The Background and Terms of reference for this statement is provided in a letter sent to EFSA from the Dutch Ministry of Health, Welfare and Sport on 19 April 2016 and stating the following:

'Please find enclosed the report "Bisphenol A, Part 2 Recommendations for Risk Management" drawn up by the Dutch National Institute for Public Health and the Environment (RIVM). In this report, the RIVM has made an assessment of environmental and health risks of bisphenol A (BPA) and possible risk management measures. BPA is in many different products and has an effect on the endocrine system.

In 2014 and 2015 European standards for safe exposure to BPA for workers and consumers are strengthened. Recent scientific research shows that BPA can damage the immune system of the fetus or young children at lower exposure levels than to which the current standards for BPA are based. This lower level of exposure is at approximately the same level as the daily exposure of consumers and workers to BPA. As a result of this exposure people have possibly more chance to develop food intolerances and they can be more susceptible to infectious diseases.

The RIVM concludes that these new findings constitute sufficient reason to consider further tightening of standards and suggests to take additional measures at the short term, to further reduce exposure to BPA.

I would kindly but urgently request you to carefully examine the results of the RIVM study and take appropriate actions. The content of this report is an important addition to the existing knowledge about BPA'.

1.2. Interpretation of the Terms of Reference

This statement only addresses the urgent request of the Dutch Ministry of Health, Welfare and Sport 'to carefully examine the results of the RIVM study' (RIVM, 2016). Accordingly, the current evaluation focuses only on the two studies by Ménard et al. (2014a,b) on BPA immunotoxicity underlying the conclusions of the RIVM report, leading RIVM to suggest a reconsideration of the EFSA t-TDI.

2. Data and methodologies

2.1. Data

- Ménard et al. (2014a) paper and original data
- Ménard et al. (2014b) paper

2.2. Methodologies

The methodology used including the criteria and principles set for reviewing the experimental studies and the weight of evidence (WoE) approach applied to hazard identification, is the same as that used in the EFSA opinion on BPA of 2015 (EFSA CEF Panel, 2015). This is described in detail in Appendix A.

3. Assessment

3.1. Review of the two studies by Ménard et al. (2014a,b)

3.1.1. Ménard et al. (2014a) Food intolerance at adulthood after perinatal exposure to the endocrine disruptor bisphenol A. The FASEB Journal, 28, 4893–4900

Ménard et al. (2014a) studied multiple immune parameters to address the effects of exposure to BPA on the response to dietary antigens. In the first study, pregnant and lactating Wistar rats were treated orally (most likely gavage but not further specified by the authors) with BPA (0.5, 5 or 50 μg/kg body weight (bw) per day) or vehicle (4% ethanol in corn oil) for approximately 30 days from gestation day 15 until pup weaning at postnatal day (PND) 21.

To evaluate the immune response to the food antigen ovalbumin (OVA), adult female offspring from these dams were fed 20 mg OVA (tolerised) or bicarbonate buffer vehicle control (immunised) via oral gavage on PND 45 (~ 6.5 weeks). All rats were given a subcutaneous (s.c.) injection of 100 μg OVA on PND 52 (plus Complete Freund Adjuvant) and PND 66, and euthanised on PND 73 (10.5 weeks). Following this treatment, serum OVA-specific antibody levels were examined along with splenic *ex vivo* proliferation and cytokine production following OVA stimulation.

BPA treatment at all doses significantly increased anti-OVA immunoglobulin G (IgG) antibodies both in OVA-immunised and OVA-tolerised animals as compared with animals without BPA exposure. Irrespective of the BPA treatment, the tolerised rats had lower levels of OVA-specific IgG than their immunised counterparts. The authors claimed a non-monotonic BPA dose–response relationship with the highest antibody titres being observed for the 5 μg group (for both tolerised and immunised animals). The isotype of IgG was not indicated and the authors stated that no increases in immunoglobulin E (IgE) were observed.

Additional endpoints were evaluated in adult females following perinatal exposure to 5 μg BPA/kg bw per day only. *Ex vivo* assays were conducted to assess multiple measures of cell responsiveness and activation responses in splenic lymphocytes obtained from control and OVA-immunised/tolerised animals exposed to BPA and following restimulation with OVA *in vitro*.

For the OVA-specific splenocyte proliferation, a significant increase in cell proliferation was observed for the BPA/tolerised rats as compared to the non-BPA-exposed counterpart. There were no changes in proliferation between the BPA-treated and -untreated rats for the immunised group. A significant increase in interferon gamma (IFNγ) production was observed for the BPA/immunised rats when compared to the non-exposed immunised rats. This change was not observed for the tolerised rats. No changes in interleukin (IL)-10 were observed for any group.

Immune phenotyping was conducted on splenocytes from PND 45 rats (these spleens were from rats in a separate study that were not exposed to OVA). A significant increase in activated splenocytes was observed along with no change in regulatory T-cells following exposure to BPA when compared to vehicle controls. This analysis was not conducted on OVA-tolerised/immunised rats.

A long-term OVA oral challenge was also conducted to explore physiological consequences of developmental BPA exposure on food intolerance. Adult female offspring from dams treated with 5 μg BPA/kg bw per day (from gestational day 15 to PND 21 as described above) were fed 20 mg OVA (tolerised) or bicarbonate buffer vehicle control (immunised control) *via* oral gavage on PND 45, immunised on PND 52 (s.c. injection of 100 μg OVA + Complete Freund Adjuvant), and received an oral challenge *via* gavage with 50 mg OVA on PND 59, 61, 63, 65 and 67. The animals were euthanised on PND 67 (9.5 weeks). Colon samples were evaluated for cytokine production and myeloperoxidase (MPO) activity. Antibody levels were not evaluated to confirm tolerance.

OVA-tolerised rats perinatally exposed to BPA had increased MPO activity and elevated concentrations of the cytokines IL-10 and IFNγ in colonic tissues as compared to the unexposed counterpart. No changes were identified in the immunised rats. Levels of transforming growth factor beta (TGFβ) were significantly decreased in both OVA-tolerised and OVA-immunised rats.

It was indicated that 7–26 female offspring/group were included for the studies described above.

In summary, the authors conclude that low-dose exposure to BPA induced the failure of oral tolerance in adult life and colonic inflammation following oral challenge.

The strengths and weaknesses identified by the EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF Panel) in this study are listed in Table 1.

Comments from the CEF Panel

The authors measured the critical cell populations, cytokines, immunoglobulins and MPO that provide a mechanistic framework underlying the immune-specific response to food allergens and the inflammatory response in the intestine using relevant models. There was some internal consistency in this study supporting the biological plausibility of the findings.

The enhancement of OVA-specific IgG in the plasma of BPA-treated offspring may suggest a dysregulation of antigen-specific tolerance in the gastrointestinal (GI) tract. However, looking at the individual animal data, a high level of variability in the data set was observed. The study would benefit from additional controls including non-tolerised/non-immunised BPA controls and tolerised/non-immunised controls. In the measurement of OVA-specific IgG where three doses were used, the authors claim a non-monotonic dose–response curve, but no statistical support for this conclusion was provided. No power calculation was presented and the number of animals per experimental group varied substantially (n = 11–26 female offspring/group). The authors do not report on the allocation of

the pups to the experimental groups and whether litter effects were controlled for. Neither was this evident from the raw data. Also, the number of dams was not reported, and given the number of pups used per group that of dams may have been as low as two or three per group.

Only spleen cells were evaluated for *in vitro* OVA restimulation and proliferation (information from mesenteric lymph node (MLN) would be beneficial). Cell proliferation was evaluated following 6 days of OVA stimulation. However, cell viability was not reported.

The above findings may be supported by the observed increase in OVA-specific cell proliferation in the BPA-exposed OVA-tolerised rats and the corresponding increase in the number of activated T-lymphocytes in the spleens from these animals. However, a lack of the expected response in immunised animals raises some doubts about the reliability of the increased cell proliferation reported for the BPA-exposed groups. The authors evaluated specific endpoints in the GI tract including cytokine and MPO production that suggest alterations in inflammatory responses in that organ, but no confirmatory pathology was provided.

It is a significant limitation that the authors conducted the majority of their studies with only one dose of BPA (5 μg/kg bw per day). The study would have been considerably strengthened if all of the endpoints had been tested at multiple doses. BPA measurements in biological samples were not performed. Overall, the paper lacks details in the study design and reporting. Assessment of only one gender and use of an outbred strain (not stated by the authors but normally the Wistar strain is outbred unless differently specified) limits interpretation of the findings. Some of the data (cytokines and MPO) were inconsistent within the study. The lack of standard toxicological parameters (body and organ weights and histology of the spleen, thymus and intestine) is an additional limitation of the study.

Methods and statistics conducted for the flow cytometry study were unclear, i.e. the number of gated events and cells were not reported and it is unclear if the percentages reported reflect the total number of cells or CD4+ cells). Immune phenotyping was not evaluated on OVA-tolerised/immunised animals and this was only conducted on spleens (information from MLN would be beneficial).

Although the Panel noted significant limitations in this study, the reported alterations in endpoints associated with food allergy and intolerance suggest there may be some potential immunotoxic effects in rats associated with perinatal exposure to 5 μg BPA/kg bw per day.

3.1.2. Ménard et al. (2014b). Perinatal exposure to a low dose of bisphenol A impaired systemic cellular immune response and predisposes young rats to intestinal parasitic infection. PLOS One, 9, e112752

Pregnant and lactating Wistar rats were treated orally (most likely gavage but not further specified by the authors) with BPA (5 μg/kg bw per day) or vehicle (4% ethanol in corn oil) for approximately 30 days from gestation day 15 until weaning on PND 21.

To evaluate the immune response to dietary antigens in juvenile animals, female offspring from these dams were fed by gavage 20 mg OVA (tolerised) or control bicarbonate buffer (immunised) on PND 25 (~ 3.5 weeks). All rats were challenged on PND 32 (100 μg OVA s.c. injection + Complete Freund Adjuvant) and PND 46 (100 μg OVA s.c.), and euthanised on PND 53 (7.5 weeks).

After this treatment, serum OVA-specific antibody levels were examined along with splenic and MLN cytokine production following OVA stimulation.

Irrespective of BPA treatment, OVA-specific IgG titres were lower in tolerised than in immunised rats. Perinatal exposure to 5 μg BPA/kg bw per day did not affect the anti-OVA IgG antibodies either in immunised or tolerised animals. The isotype of IgG was not indicated and the authors stated that no increases in IgE were observed.

Ex vivo assays were conducted to assess multiple measures of cell responsiveness and activation responses in spleen and MLN cells obtained from control and OVA-immunised/tolerised animals exposed to BPA and following *in vitro* OVA restimulation. It was indicated that for these studies 12–17 female offspring were included per group.

Splenocytes from the BPA/immunised and BPA/tolerised rats produced reduced amounts of IFNγ as compared to cells obtained from the non-BPA-exposed counterparts. A similar BPA-induced decrease in IFNγ production was observed only in MLN cells obtained from OVA-immunised animals, and not in cells from OVA-tolerised animals. No changes in IL-10 were observed in the spleen or mesenteric lymph node.

Immune phenotyping was conducted on splenocytes from PND 25 rats (these spleens were from rats that were not exposed to OVA). A significant decrease in regulatory T-cells, T-helper cells (which

also were composed of T-regs) and dendritic cells was observed following exposure to BPA in the spleen and MLN compared to vehicle controls. This analysis was not conducted on OVA-tolerised/ immunised rats. It was indicated that 12 female offspring were included per group for the studies described above.

Host resistance to the helminthic parasite *Nippostrongylus brasiliensis* following developmental exposure (from gestational day 15 to PND 21 as described above) to 5 μg BPA/kg bw per day was also evaluated. At PND 25, female offspring were infected with 1,000 infective stage larvae *N. brasiliensis* subcutaneously and euthanised 1 week later (PND 32). Colon samples were evaluated for cytokine production, MPO activity and living larvae.

While there was an increase in IgE following infection, there was no difference in response to BPA exposure.

Rats perinatally exposed to BPA had elevated levels of living larvae in their faecal material as compared to controls. This was accompanied by a significant decrease in MPO activity and elevated levels of cytokines (IL-4 and IL-13 (TH2), IL-10 (anti-inflammatory) and growth-regulated oncogene/ keratinocyte chemoattractant (GRO/KC), and IFNγ (pro-inflammatory)) in the small intestine as compared to the non-BPA-exposed infected animals.

It was indicated that 7–8 female offspring were included per group for the studies described above.

In summary, the authors conclude that in juvenile rats, low-dose perinatal exposure to BPA resulted in normal responses to food antigen but failed to induce a proper cellular immune response following systemic immunisation and suggest an immunosuppressive effect. In addition, they report a decreased host resistance in juvenile rats following perinatal BPA exposure.

The strengths and weaknesses identified by the CEF Panel in this study are listed in Table 1.

Comments from the CEF Panel

The authors measured the cell populations, cytokines, immunoglobulins and MPO that provide a mechanistic framework underlying the immune-specific response to an antigen and parasitic infections using relevant models. There was some internal consistency in this study supporting the biological plausibility of the findings. Lack of enhancement of OVA-specific IgG in the plasma suggests no effect on antigen-specific tolerance in the GI tract, but in the disease-resistant model, the authors report an increase in the number of larvae in faeces and significant decrease in MPO following infection in BPA-exposed female offspring.

It is a significant limitation that the authors conducted these experiments using only one dose of BPA (5 μg/kg bw per day). The findings would have been considerably strengthened if all of the endpoints had been tested at multiple doses. The authors do not report on the allocation of the pups to the experimental groups and whether litter effects were controlled for. Also, the number of dams was not reported, and given the number of pups used per group, that of dams may have been as low as two or three per group. The study overall lacks details in the study design and report. Assessment of only one gender and use of outbred strain (not stated by the authors but normally the Wistar strain is outbred unless differently specified) limits interpretation of the findings. The lack of standard toxicological parameters (body and organ weights and histology of the spleen, thymus and intestine) is a limitation of the study.

Methods and statistics conducted for the flow cytometry study are unclear (i.e. number of gated events not reported, number of cells not reported and if the percentages reported reflect total number of cells or CD4+ cells). Given the small increase in the living larvae and the large variation in these data for the host resistance model, the biological significance of this result is considered questionable.

3.2. Weight of evidence of the immune effects of BPA

In the 2015 EFSA opinion, a WoE analysis was performed for each toxicological endpoint including the immune effects. In particular, whether BPA induces immune effects was considered using a tabular format for weighing different lines of evidence. The overall outcome of this WoE evaluation is presented in the conclusions on the immune effects taken from the 2015 EFSA opinion and reported below.

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

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

Using a WoE approach, the CEF Panel assigned a likelihood level of "-as likely as not- to likely" to immunotoxic effects of BPA. Since the likelihood level for this endpoint is less than "likely" (see Appendix A of EFSA CEF Panel, 2015), this endpoint was not taken forward for assessing the toxicological reference point, but was taken into account in the evaluation of uncertainty for hazard characterisation and risk characterisation (Section 4.3 of EFSA CEF Panel, 2015).'

The CEF Panel had already included the study by Bauer et al. (2012) in its 2015 WoE analysis of animal studies and reviewed it as follows:

'The CEF Panel notes that also the study by Bauer et al. (2012) indicated enhancement of ovalbumin-induced allergic responses, notably inflammation, by oral exposure to BPA, and that a dose-dependence was evident. The CEF Panel also noted that in this latter study the inflammation noted was seen in females but not males. It should be mentioned that elevated immune responses in female humans as well as female animals have been reported previously, including innate responses, cytokine responses and vaccine responses (Klein et al., 2010; McLelland and Smith, 2011; Hochstenbach et al.,2012).'

For the present statement, the CEF Panel took the 2015 WoE evaluation carried out on animal studies on the immune effects (EFSA CEF Panel, 2015) as the starting point and assessed whether the two Ménard et al. studies from 2014 have an impact on the overall outcome of the WoE analysis (see Table 1). No additional literature has been searched for in the public domain,[1] since a review of all the scientific evidence published after 2012 and relevant for BPA hazard assessment (including BPA immunotoxicity) will start next year. This will follow a protocol currently under development which will define a priori the strategy for collecting, appraising, analysing and integrating the relevant evidence.

The strengths and weaknesses identified by the Panel in the two new Ménard et al. studies are listed in the left hand side column of the WoE table (Table 1). The second column reports the (positive) answer to the question 'Is BPA immunotoxic in animals?' as reported by the study authors. Taking into consideration all the strengths and weaknesses of each study, the Panel assigned to the new evidence a low score for reliability (third column) and a *limited influence* (•/↑, see Table A.4 in Appendix A for an explanation of the symbols used for expressing the weight of each new line of evidence) on the likelihood of a positive answer to the question (fourth column).

After considering the individual influences of the two new lines of evidence and the starting point the Panel concluded that evidence from the new studies adds to the indications of immunotoxicity of BPA in animals reported in previous reviews. However, uncertainties in the dose–response, the study conduct and design, along with a high variability in the observed responses, lower the confidence in the data as presented.

Overall, the CEF Panel reconfirmed the overall conclusion already expressed in 2015 of a likelihood level of 'from -as likely as not (ALAN)- to likely' (see Table A.2 for standard terms used for expressing the overall likelihood in the WoE tables) for BPA immunotoxic effects in animals.

[1] The CEF Panel was aware of the RIVM Expert Workshop report (in press) by Hessel EVS et al., Assessment of recent developmental immunotoxicity studies with bisphenol A in the context of the 2015 EFSA t-TDI, Repro Toxicol 2016; Available: http://dx.doi.org/10.1016/j.reprotox.2016.06.020. The present EFSA statement, however, addresses the RIVM report (RIVM, 2016) sent by the Ministry of Health that was specifically referred to in the Terms of Reference received by EFSA.

Table 1: Assessment of convincing associations between BPA exposure and immunotoxic effects in animals

Question 1: Is BPA immunotoxic in animals?	Answer to the question as reported by the study authors (positive, negative or uncertain)	Reliability of evidence (low, medium or high)	Influence on likelihood (see Table A.4 for key to symbols)
Starting point based on previous assessments (EFSA CEF Panel, 2015): Evidence from the new studies adds to the indications of immunotoxicity of BPA in animals reported in previous reviews.	**Some positive**		**ALAN to Likely[(a)]**
Line of Evidence: Ménard et al., 2014a	**Positive**	**Low**	•/↑
Strengths			
Relevant models are used			
Three dose levels tested (only for IgG)			
Phytoestrogen-free diet			
Use of non-polycarbonate cages and non-polycarbonate water bottles and negligible oestrogenicity for cages/water/bedding at E-screen			
Weaknesses			
Single dose-level study for all endpoints aside from antibody titres			
No positive control			
Tests performed in female offspring only			
Insufficient study reporting (information not given on: animal body weight, BPA source, BPA oral administration mode, number of dams/pups, endotoxin levels in OVA (this may drive food tolerance responses), IgG isotype, antibody levels for the oral challenge protocol; unclear methods and statistics for the flow cytometry study)			
Statistics: Lack of statistical evaluation of the non-monotonic dose response, no mentioning of power analyses and control for litter effect			
Line of Evidence: Ménard et al., 2014b	**Positive**	**Low**	•/↑
Strengths			
Relevant models are used			
Phytoestrogen-free diet			
Use of non-polycarbonate cages and non-polycarbonate water bottles and negligible oestrogenicity for cages/water/bedding at E-screen			
Weaknesses			
Single dose-level study			
No positive control			
Tests performed in female offspring only			

Question 1: Is BPA immunotoxic in animals?	Answer to the question as reported by the study authors (positive, negative or uncertain)	Reliability of evidence (low, medium or high)	Influence on likelihood (see Table A.4 for key to symbols)
Insufficient study reporting (information not given on: animal body weight, BPA source, BPA oral administration mode, number of dams/pups, endotoxin levels in OVA (this may drive food tolerance responses), IgG isotype, antibody levels for the oral challenge protocol; unclear methods and statistics for the flow cytometry study)			
Statistics: no mentioning of power analyses or control for litter effect			
Overall conclusion on the likelihood of immunotoxic effects of BPA in animals: Evidence from the new studies adds to the indications of immunotoxicity of BPA in animals reported in previous reviews. However, uncertainties in the dose–response, the study conduct and design, along with a high variability in the observed responses, lower the confidence in the data as presented.			***ALAN to* Likely[(a)]**

BPA: bisphenol A; EFSA CEF: EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids; IgG: immunoglobulin G; OVA: ovalbumin.
(a): See Table A.2 for legend.

3.3. Benchmark dose (BMD) analysis of the dose–response data by Ménard et al. (2014a)

In compliance with the draft update of the guidance of the Scientific Committee on the use of the benchmark dose approach in risk assessment (EFSA Scientific Committee, 2016, under public consultation until 20 September 2016), the results on OVA-specific IgG titres obtained in the food tolerance study with BPA (Ménard et al., 2014a) have been submitted to statistical dose–response modelling. The results obtained are reported in detail in Appendix B.

The data point within each treatment group for the tolerised mice shows high variability. As recommended by the EFSA Scientific Committee (2016), 'one might consider selecting a benchmark response (BMR) higher than 5% for endpoints that tend to show a relatively large within-group variation (EFSA Scientific Committee, 2016)'. The EFSA Scientific Committee also recommends to define the BMR as a per cent change in the mean response as compared to the background response. However, although a paper describing an approach to accomplish this has been submitted for publication (Slob, 2016), as yet no full reporting of this strategy is available, and appreciation of its consequences is not possible. Therefore, BMD modelling was only performed for the default BMR of 5% extra risk for IgG titres in both OVA-tolerised and OVA-immunised animals (for further details see Appendix B.1).

Due to the high interanimal variability within the treatment groups and limited dose–response resulting in high confidence intervals, the CEF Panel concluded that the data was not suitable to derive a reference point for BPA on immunotoxicity. This conclusion was not altered when the Panel also tried to perform the BMD modelling using a much higher BMR (700%, derived on the basis of the strategy as outlined by Slob in 2016).

3.4. Discussion on the outcome of the Ménard et al. (2014a,b) studies

In the two Ménard et al. (2014a,b) studies, the authors reported potential age- and organ-specific effects of perinatal BPA exposure. The Panel noted that the results of the two papers do not support each other due to differences with regard to start of immune protocols and outcomes. Overall, the results further confirm that the immune system is a potential target for BPA. However, because of the limitations of the studies identified by the Panel, the findings do not call for a revision of the outcome of the previous WoE evaluation of the immune effects (from ALAN to likely) in the 2015 EFSA opinion (EFSA CEF Panel, 2015).

Our current understanding of the immune processes associated with the development of oral tolerance still has many gaps. However, the basic mechanisms are fairly well understood and are similar in the rodent and human. The authors have examined many endpoints associated with the known mechanisms that contribute to this response. Exposure to BPA in the perinatal period altered several immune processes that either regulate or are a manifestation of the immune response in the gastrointestinal tract. Food allergies in children have significantly increased in recent years and numerous authors have suggested that changes in the environment are more likely responsible for the enhanced allergy prevalence than genetic shifts.

The cells and soluble mediators that are involved in the immune response to parasitic infection are generally similar in rodents and humans, manifesting as a TH2 type response with an innate immune component. However, the specific aspects of the response that result in clearance are organism specific. The nematode *N. brasiliensis* is a rodent-specific pathogen. While the life cycle and immunological host response for *N. brasiliensis* is similar to that observed in humans following infection with helminthic pathogens, such as hookworms, the parallels are not exact. Although helminth diseases are well-controlled in developed countries, they remain a significant cause of morbidity in poorer countries where sanitation and access to healthcare are limited.

Thus, the indication that BPA at a perinatal exposure of 5 μg/kg bw per day may have the potential to alter the development of oral tolerance and susceptibility to parasitic infection in rodents is considered a cause for concern and warrants for further examinations.

4. Conclusions

Evidence from the new Ménard studies adds to the indications of potential immunotoxicity of BPA in animals already reported by EFSA in 2015.

Due to the high interanimal variability within the treatment groups resulting in high confidence intervals and limited dose response, the CEF Panel concludes that the data on anti-OVA IgG antibodies in the Ménard et al. study (2014a) are not suitable to derive a reference point for BPA on immunotoxicity. Furthermore, the limitations of both Ménard et al. studies observed by the Panel confound the interpretation of the study results and therefore prevent the assessment of the relevance to human health.

The CEF Panel overall considers that the results from the two Ménard et al. studies are not sufficient to call for a revision of the t-TDI set in EFSA's opinion on BPA from 2015.

EFSA will start a review of all the scientific evidence published after 2012 and relevant for BPA hazard assessment (including immunotoxicity) in 2017. The results of immunological studies, such as the two evaluated here, would form a useful contribution to this evaluation provided that the limitations identified herein were addressed.

Documentation provided to EFSA

1) The original data from the study by Ménard et al. (2014a) were kindly provided by Sandrine Ménard on 8 July 2016.

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Abbreviations

ALAN	as likely as not
BMD	benchmark dose
BMDL	95% lower confidence limit (single-sided) of the benchmark doses
BMDU	95% upper confidence limit (single-sided) of the benchmark doses
BMR	benchmark dose response
BPA	bisphenol A

bw	body weight
CEF	EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids
GI	gastrointestinal
GRO/KC	growth-regulated oncogene/keratinocyte chemoattractant
GUI-mode	graphical user interface mode
IFNγ	interferon gamma
IgG	immunoglobulin G
IL-4	interleukin 4
IL-13	interleukin 13
i.p.	intraperitoneal
MLN	mesenteric Lymph Node
MPO	myeloperoxidase
OECD	Organisation for Economic Co-operation and Development
OVA	ovalbumin
PND	postnatal day
RIVM	Dutch National Institute for Public Health and the Environment
s.c.	subcutaneous
t-TDI	temporary tolerable daily intake
TH	T helper
WoE	weight of evidence
WG	working group

Appendix A – Detailed methodology for the study review and the weight of evidence approach

A.1. Criteria and principles applied for assessing the strengths and weaknesses of animal studies

The criteria applied for reviewing the studies are listed in Table A.1. The appraisal of the strengths and weaknesses of the two studies was performed individually by three reviewers from the working group (WG) on BPA immunotoxicity and their evaluations were presented and discussed at a WG meeting.

Table A.1: Criteria applied to assess the strengths and weaknesses of animal studies

Criteria	Interpretation/assessment		Comments
	Strengths	Weaknesses	
Test substance identification			
Vehicle	–	Vehicle not reported	
Test organism characterisation			
Species and strain of the animal	–	Animal species and/or strain not reported	
Is the age and body weight of the test organisms given?	–	Animal age and/or body weight not reported	
Is the sex of the test organism given?	–	Sex of the animals tested not reported	
Study design description			
Use of *a priori* study protocol/ study plan	–	Lack of *a priori* study protocol or study plan	
Sample size – power of the study (number of animals)	Large sample size	Small sample size	This is based on expert judgement
Control procedures (were negative and/or positive controls included where required)?	Both naïve controls and vehicle controls available Adequate positive controls included (if appropriate)	No vehicle controls were tested	
Number of BPA doses	≥ 3 dose levels tested	Single dose level study	Not mentioned as a strength or weakness if two dose levels were tested
BPA dose levels		Too wide dose spacing *Too high dose levels tested	Wide dose spacing makes the study inadequate to study a dose–response relationship Testing of BPA at very high-dose levels is not informative of effects occurring at current human exposure levels
BPA exposure assessment	–	Feed consumption (BPA given by the diet) not measured BPA concentration and homogeneity in the feed mixture not guaranteed analytically (BPA given by the diet) Drinking water consumption (containing BPA) not measured	The exact BPA doses received by the animals cannot be established

Criteria	Interpretation/assessment		Comments
	Strengths	Weaknesses	
Route and type of administration/administration scheme	Oral administration via gavage (except for neurobehavioural studies)	Maternal administration via i.p. injection during pregnancy	Not mentioned if: BPA was given via diet or drinking water and food/water consumption was measured; BPA was given via s.c. injection; Maternal dosing via i.p. injection during pregnancy was considered as a weakness due to uncertain fetal dosing Oral administration via gavage was considered as a strength due to exact dosing: only exceptions were neurobehavioural studies addressing anxiety-like behaviours due to animal handling
Frequency and duration of exposure: Are frequency and duration of exposure as well as time-points of observations explained?	–	Single acute dose administration (depending on the endpoint)	Acute exposure is not representative of human exposure which is prolonged in time
BPA exposure assessment	BPA measurement in biological samples		The quality of the analysis is also checked
Test performance		Test performed in one sex only Low number of animals tested (in a test)	
Blind treatment	Blind treatment or Blind evaluation of samples. . ..	–	Blind treatment was considered as a strength if reported, and was not mentioned if not reported
Study results documentation/Study reporting			
Study reporting	–	Insufficient study reporting (give details)	Details, e.g. number of animals tested for each test unclear or not reported, time points unclear, dose levels, etc.
Statistical analysis	–	Inappropriate statistics (give details)	Details, e.g. litter effect not considered, inappropriate analysis
Plausibility of the study design and results			
Is the study design chosen appropriate for obtaining the substance-specific data aimed at?	–	Study design not appropriate to the scope	
Diet	Phytoestrogen-free diet (e.g. soy free diet) or feed content of oestrogens negligible at E-screen	Animal diet and phytoestrogen content not reported (or poorly described)	Confounding by diet

Criteria	Interpretation/assessment		Comments
	Strengths	Weaknesses	
Housing conditions/ environmental contamination	Use of non-polycarbonate cages, and of non plastic (e.g. glass) or BPA-free water bottles	Use of polycarbonate cages and plastic water bottles OR Type of cages and drinking bottles not reported	Confounding by environmental contamination
Quality assurance principles			
GLP/other quality assurance system	Study/analysis performed under GLP or XX quality assurance system	–	
Protocol according to existing guidelines, e.g. OECD guidelines or EU guidelines (or other e.g. national guidance)	Study/test performed according to XX guidelines	–	
Others	On a case-by-case basis	On a case-by-case basis	This is based on expert judgement

BPA: bisphenol A; GLP: good laboratory practice; OECD: Organisation for Economic Co-operation and Development.
*'Too high dose levels' is an exclusion criterion for the studies on reproductive and developmental toxicity, and therefore, this weakness is reported only for non-reproductive toxicity studies.

A.2. WoE approach

The CEF Panel applied a WoE approach to assess the overall likelihood (using the terms as in Table A.2) of the association between BPA exposure and the immunotoxic effects in animals.

Table A.2: Set of standard terms used for expressing the overall likelihood in the WoE tables (adapted from Mastrandrea et al., 2010)

Likelihood
Very likely
Likely
From -as likely as not (ALAN)- to likely
As likely as not (ALAN)
From unlikely to -as likely as not (ALAN)-
Unlikely
Very unlikely

WoE: weight of evidence.

A tabular format (Table A.3) was used to facilitate the consistent treatment of the whole body of evidence and transparently document the WoE analysis.

Table A.3: Example of table used in the WoE approach

Question 1: Is BPA……..?	**Answer to the question as reported by the study authors**	**Reliability of evidence**	**Influence on likelihood**
Starting point based on previous assessments (EFSA, 2006; 2010; 2015): (summarise conclusions of previous assessments relating to this question)	**Positive, negative or uncertain**	**Low, medium or high**	**See Table A.4 for key to symbols**
Line of Evidence 1: new evidence on …….. *Strengths:* *Weaknesses:*			
Line of Evidence 2: increased effect on…… *Strengths:* *Weaknesses:*			
Overall conclusion on likelihood:			**Chosen likelihood level (see Table A.2)**

BPA: bisphenol A; WoE: weight of evidence.

The conclusions of earlier assessments of BPA by EFSA in 2015 were taken as the starting point for the new evaluation.

To draw its conclusion for each association question, the CEF Panel first summarised the strengths and weaknesses of each line of evidence and 2015 assessments in an overall reliability assessment (expressed qualitatively on a scale of low, medium or high) and expressed it in terms of *weight* or *influence* on the likelihood of a positive answer to each question, when considered independently of the other lines of evidence (see Table A.4).

Taking into account the individual influences of all the lines of evidence and the starting point, the CEF Panel expressed its conclusions in terms of the overall likelihood of a positive answer to the question on the causal association between BPA exposure and the selected immunotoxic endpoint using a scale of likelihood categories spanning from 'Very unlikely' to 'Very likely' (Table A.2). On this scale 'As likely as not' means a level of likelihood between 'Unlikely' and 'Likely', where it is about equally likely that BPA causes, or does not cause, the effect.

The approach described above is generically summarised in Table A.3.

The CEF Panel found it helpful to include separate columns in Table A.3 summarising steps in the evaluation of each line of evidence.

The second column indicates the answer to the question as reported *by the study authors* (e.g. a positive, negative or uncertain answer to the question), i.e. before the CEF Panel assessed strengths and weaknesses.

The third column gives the CEF Panel's assessment of the *reliability* based on the evaluation of the strengths and weaknesses of each line of evidence, expressed qualitatively on a scale of low, medium or high. A low score for reliability does not necessarily imply a poor quality study: e.g. it may relate to a well-conducted study with results not reaching statistical significance, but the treatment groups are not large enough to be statistically confident there is no effect. The CEF Panel did not use a fixed formula to assess reliability of a study from its number of strengths and weaknesses, because this would not take appropriate account of the varying weights of different strengths and weaknesses. Instead, the reliability of the evidence as well as the influence on likelihood were agreed based on collective expert judgement at the WG and CEF Panel meetings.

The evaluation of the weight or influence of each line of evidence was then recorded in the right hand column using a defined set of symbols (see Table A.4).

Table A.4: Definition of symbols used for expressing the influence on likelihood of each line of evidence in the WoE tables

Symbols	Interpretation
↑	Minor contribution to increasing likelihood
↑↑	Moderate contribution to increasing likelihood
↑↑↑	Major contribution to increasing likelihood
↓	Minor contribution to decreasing likelihood
↓↓	Moderate contribution to decreasing likelihood
↓↓↓	Major contribution to decreasing likelihood
•	Negligible influence on likelihood

WoE: weight of evidence.
Pairs of symbols indicate uncertainty about the influence, e.g. •/↑ = between negligible and minor positive influence on likelihood.

The number (from one to three) of upward and downward arrows indicates the degree (small, medium and high) of the impact of the new evidence to increase or decrease, respectively, the likelihood of a positive answer to the question. In developing its judgment on the influence or weight of each line of evidence, the CEF Panel took into account all the strengths and weaknesses it identified in the left hand column of the WoE table.

The 'dot' is used when the reliability of the new line of evidence is considered as insufficient as to have an impact on the likelihood of a positive answer to a question. When the evidence base was too weak to make a firm judgement about the influence, a range of symbols was given to reflect that additional uncertainty. For example, •/↑ indicates between negligible and minor positive influence on likelihood.

A conclusion on the overall likelihood that BPA exposure was associated with a particular effect in animals was expressed in the bottom row of the WoE table (Table A.3) both as a narrative statement and using the likelihood terms as in Table A.2. Such conclusion was drawn after considering the individual influences of all the lines of evidence and the starting point by expert judgement after a thorough discussion at a WG and/or CEF Panel meeting level, and not by any standardised combination of scores for reliability and influence, which would be simplistic and preclude the consideration of other factors.

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

Appendix B – Dose–response modelling of bisphenol A on immunotoxicity

In compliance with the draft update of the guidance of the Scientific Committee on the use of the benchmark dose approach in risk assessment (EFSA Scientific Committee, 2016, under public consultation until 20 September 2016), the results on OVA-specific IgG titres obtained in the food tolerance study with BPA (Ménard et al., 2014a) have been submitted to statistical dose–response modelling. This study has been summarised in Section 3.3 of the main document.

For all modelling the R version 3.2.2 and the statistical package PROAST (version 61.6) has been used in graphical user interface mode (GUI-mode). This package is available via: http://www.rivm.nl/en/foodnutritionandwater/foodsafety/proast.jsp; the version mentioned can be requested directly from the authors. Using this statistical package, 95% lower confidence limit (single-sided) of the benchmark doses (BMDLs) were calculated (see EFSA Scientific Committee, 2016) for the various effects. For the evaluation, the statistical models available in PROAST for continuous data (i.e. the Exponential and Hill families of models) were used.

B.1. Consideration of the use of the default BMR of 5% for continuous data

The data point within each treatment group for the tolerised mice shows high variability (see Table B.1 below). As recommended by the EFSA Scientific Committee of EFSA, 'one might consider selecting a BMR higher than 5% for endpoints that tend to show a relatively large within-group variation (EFSA Scientific Committee, 2016)'. The EFSA Scientific Committee also recommends to define the benchmark response (BMR) as a per cent change in the mean response as compared to the background response. However, although a paper describing an approach to accomplish this has been submitted for publication (Slob, 2016), as yet no full reporting of this strategy is available and appreciation of its consequences is not possible. Therefore, BMD modelling was only performed for the default BMR of 5% extra risk for IgG titres in both OVA-tolerised and OVA-immunised animals.

The dose–response modelling was carried out using individual data on OVA-specific IgG titres in OVA-tolerised or OVA-immunised rats as provided by the study author. The data used are presented in Table B.1.

B.2. Parameters used for BMD calculation (settings within PROAST)

The evaluations were carried out for female rats (the only sex used in the experiment) with the following settings:

- BMR of 5% extra risk for continuous data on both immunised and tolerised animals.
- No restrictions for model parameters to limit, e.g. steepness of the fitted dose–response curves (default option).
- For all evaluations, the following criteria were used to decide on acceptability of modelling output.
- For continuous variables, the model selected from the Exponential and Hill nested model families was the model with the lowest loglikelihood from either the minimal or maximal model.

It is noted that although the data in this table reflect measurements on individual animals no litter data was provided. Therefore, the BMDL modelling could not take the possible litter effect into account.

Table B.1: Experimental data (OVA-specific IgG titres × 10^3) provided by Ménard et al. (2014a) used for dose–response modelling and calculation of a BMDL for BPA

Treatment μg/kg bw per day		Individual data on OVA-specific IgG titres[(a)]												
Control	Tolerised	0.16	0.32	0.08	0.16	20.48	0.16	0.16	20.48	20.48	10.24	0.16	20.48	1.28
	Tolerised	0.08	2.56	0.16	20.48	10.24	2.56	0.16	0.08	0.64	0.64	40.96		
	Immunised	163.8	81.92	81.92	81.92	163.8	40.96	81.92	163.8	327.7	327.7	163.8	655.4	327.7
	Immunised	655.4	1311	655.4	327.7	1311	327.7	655.4	655.4	655.4	327.7	655.4	163.8	327.7
BPA 0.5	Tolerised	10.24	0.16	0.08	5.12	5.12	0.64	1.28	327.7	2.56	5.12	2.56	20.48	163.8
	Immunised	327.7	655.4	81.92	81.92	163.8	655.4	327.7	327.7	163.8	327.7	327.7		
BPA 5	Tolerised	5.12	5.12	0.32	0.64	0.08	327.7	10.24	327.7	10.24	327.7	10.24	1311	5.12
	Tolerised	10.24	81.92	0.64	0.32	0.16	5.12	0.08	81.92	40.96				
	Immunised	81.92	163.8	655.4	1311	327.7	1311	327.7	1311	163.8	327.7	1311	1311	1311
	Immunised	1311	655.4	655.4	81.92	163.8	81.92	163.8	327.7	655.4	327.7	655.4	655.4	
BPA 50	Tolerised	20.48	0.32	20.48	5.12	5.12	163.8	163.8	327.7	0.64	81.92	163.8	81.92	
	Immunised	655.4	163.8	1311	1311	163.8	327.7	81.92	655.4	655.4	327.7	327.7	655.4	655.4

OVA: ovalbumin; BMDL: 95% lower confidence limit (single-sided) of the Benchmark doses; bw: body weight; BPA: bisphenol A; IgG: immunoglobulin G.
(a): Expressed by the study authors as × 10^3 of the highest plasma dilution giving an optical density at least twice the blank value.

B.3. BMD modelling of the IgG response in OVA-tolerised and OVA-immunised rats

For continuous responses, the Hill and Exponential nested model families are applicable. In GUI-mode, PROAST has two options for dose–response modelling: either selection of the minimal or maximal model. The minimal model is used as recommended in the updated EFSA draft guidance on BMD modelling (EFSA Scientific Committee, 2016). Note that the BMDL and 95% upper confidence limit (single-sided) of the benchmark doses (BMDU) values may come from different models.

BMD modelling in OVA-tolerised rats

Figure B.1 gives the graphical representation of the fitted dose–response curves for the IgG titres in OVA-tolerised rats using a BMR of 5%. The BMD modelling with minimal and maximal model was similar. The outcome from the BMD modelling is shown below (Table B.2).

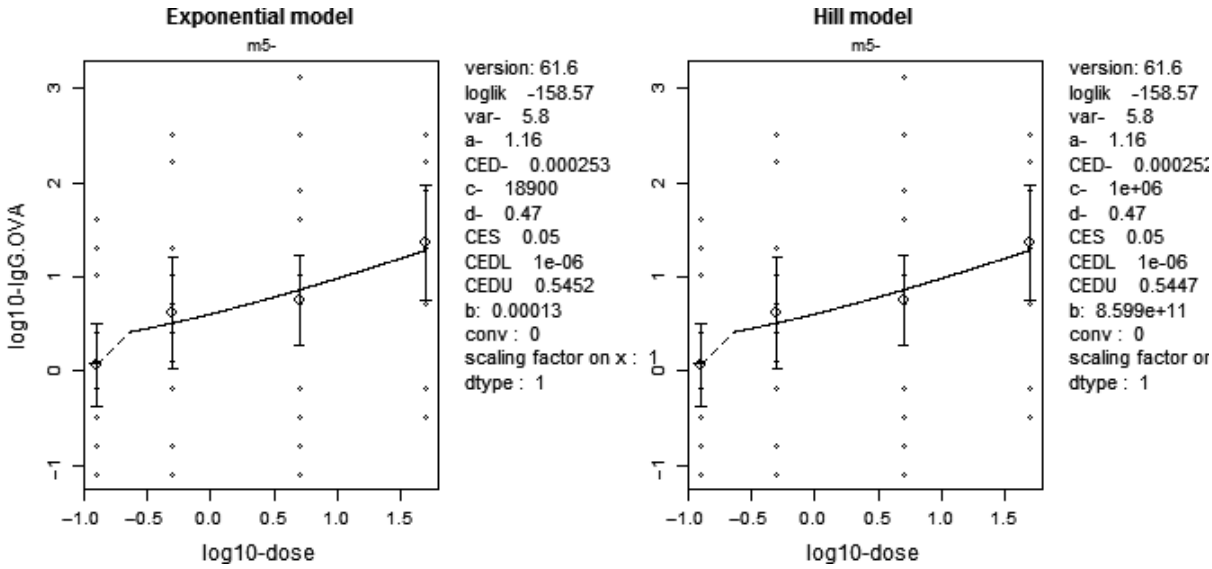

Figure B.1: Dose–response modelling on IgG titres in OVA-Tolerised female rats perinatally exposed to various doses of BPA using a BMR of 5%

Table B.2: Outcome of the BMD modelling on IgG titres in OVA-Tolerised female rats

Model	No of parameters	loglik		$BMDL_{05}$ (μg/kg bw per day)		$BMDU_{05}$ (μg/kg bw per day)	
		Exponential	Hill	Exponential	Hill	Exponential	Hill
Model 3	4	−158.47	−158.38				
Model 5	5	−158.57		0.000001	0.000001	0.5452	0.5447
Full model	5	−158.36					

BMD: benchmark dose; BMDL05: Benchmark dose lower confidence interval when the percent change in mean response is 5%; BMDU05: Benchmark dose upper confidence interval when the percent change in mean response is 5%; IgG: immunoglobulin G; OVA: ovalbumin.

BMD modelling in OVA-immunised rats

Figure B.2 gives the graphical representation of the fitted dose–response curves for the IgG titres in OVA-immunised rats using a BMR of 5%. Only the maximal model gave an estimate on BMDL as shown below (Table B.3).

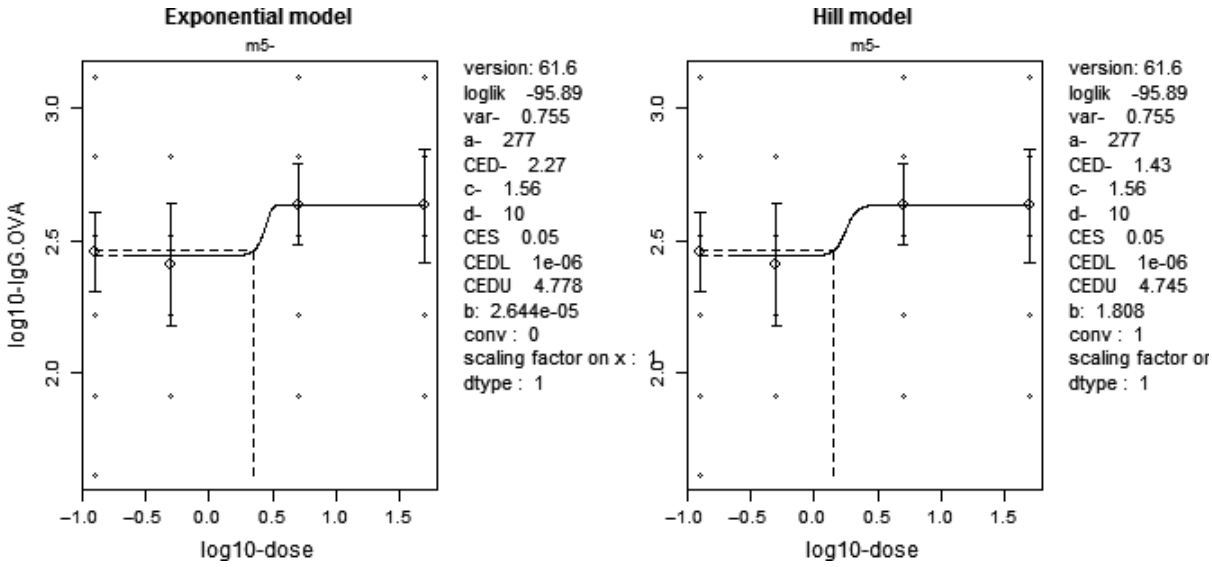

Figure B.2: Dose–response modelling on IgG titres in OVA-immunised female rats perinatally exposed to various doses of BPA using a BMR of 5%

Table B.3: Outcome of the BMD modelling on IgG titres in OVA-immunised female rats

Model	No of parameters	loglik		$BMDL_{05}$ (µg/kg bw per day)		$BMDU_{05}$ (µg/kg bw per day)	
		Exponential	Hill	Exponential	Hill	Exponential	Hill
Model 3	4	−96.72	−96.75				
Model 5	5	−95.89		0.000001	0.000001	4.778	4.745
Full model	5	−95.82					

BMD: benchmark dose; BMDL05: Benchmark dose lower confidence interval when the percent change in mean response is 5%; BMDU05: Benchmark dose upper confidence interval when the percent change in mean response is 5%; IgG: immunoglobulin G; OVA: ovalbumin.

B.4. Conclusions on the BMD modelling of the IgG response

Due to the high interanimal variability within the treatment groups and limited dose–response resulting in high confidence intervals, the CEF Panel concluded that the data was not suitable to derive a reference point for BPA on immunotoxicity. This conclusion was not altered when the Panel also tried to perform the BMD modelling using a much higher BMR (700%, derived on the basis of the strategy as outlined by Slob in 2016).