DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service
Food and Drug Administration

Memorandum

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From: Bisphenol A (BPA) Joint Emerging Science Working Group
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Subject: 2012 Updated Review of Literature and Data on Bisphenol A (CAS RN 80-05-7)

To: FDA Chemical and Environmental Science Council (CESC)
Office of the Commissioner
Attn: J Jesse L. Goodman, M.D., M.P.H.
Chief Scientist, Deputy Commissioner for Science and Public Health

EXECUTIVE SUMMARY

The BPA joint review working group (JRWG), composed of representatives from several FDA Centers, was formed in January 2011. The JRWG first interim review was completed May 24, 2011 and reviewed literature published since the last assessment performed by CFSAN in November 2009 through January 2011. The memorandum herein constitutes the second interim review of JRWG evaluating literature available February 1, 2011 to October 24, 2011 and the NCTR studies. The conclusions presented herein are those of the workgroup as of March 10, 2012.

The charge of this group is to periodically review the updated scientific literature and data for the purposes of informing the risk assessment on BPA. In addition, the group considers whether and how new scientific data (e.g., new pharmacokinetic data and models) may affect estimation of human exposure from regulated products, including modeling extrapolation/assessment of effects observed from *in vitro* or animal studies.

No new information was identified to suggest revision of the existing safety assessment level (NOAEL of 5 mg/kg bw/day; oral exposure). Reviewed pharmacokinetic studies have demonstrated that oral BPA

administration results in a much lower internal exposure of aglycone BPA (i.e., active form) than what occurs from parenteral exposures. Primates, including those of neonatal age, were also found to effectively metabolize BPA to its inactive form and excrete it more rapidly and efficiently than neonatal rodents. These pharmacokinetic differences reduce concerns about neonatal exposure in humans that have been raised based on some rodent studies using oral and particularly, non-oral exposures.

Previous JRWG and CFSAN reviews evaluated hazard identification endpoints related to developmental neurotoxicity, prostate and mammary carcinogenesis, glucose homeostasis, and sperm pathology but found mixed reported results and procedural limitations in many studies. The review herein does not change this conclusion. Additional information is needed to confirm the potential effects in these endpoints and clarify their relevance to a public health safety assessment. Extensively modified guideline-compliant studies are currently being conducted at the NCTR for the purpose of resolving these remaining issues.

BPA INTERIM REVIEW 2

INTRODUCTION

The U.S. Food and Drug Administration (FDA) previously convened a panel to begin work in December 2010 to review recent emerging scientific data on Bisphenol A (BPA) as part of the BPA Joint Emerging Science Working Group (of the FDA Chemical and Environmental Science Council, CESC) through the Office of the Commissioner. Expertise for this group has been drawn from many FDA Centers to assist in the review. Specifically, this group was tasked with addressing the following questions:

- what hazards should be added or removed from FDA's continuing review/research evaluation;
- what dose/response level for a specific effect/endpoint should be changed and to what level; and
- how should new exposure data or improved assessments be incorporated into risk assessment.

The function of the working group was strictly limited to performing a review of any new data for the purposes of informing the risk assessment on BPA. In addition, the group would consider whether and how new scientific data (e.g., new pharmacokinetic data and models) may affect estimation of exposure from regulated products, including modeling extrapolation or assessment of effects observed from *in vitro* or animal studies.

The first FDA BPA working group review[1] of literature included papers available in-press as of November 1, 2009 through January 31, 2011. Based on the literature reviewed, the working group provided the following conclusions in response to the charge questions and previous FDA assessments:

1) With regard to hazard identification, data available maintained the previously identified endpoints.
    a) Developmental neurotoxicity related to anxiety, learning and memory (between sexes), and molecular neuroanatomical endpoints with varying routes of administration.
    b) Developmental changes to rodent prostate with non-oral administration.

1 Bisphenol A (BPA) Joint Emerging Science Working Group to FDA Chemical and Environmental Science Council (CESC), May 24, 2011. *Updated Review of the 'Low-Dose' Literature (Data) on Bisphenol A (CAS RN 80-05-7) and Response to Charge Questions Regarding the Risk Assessment on Bisphenol A.*

c) Rodent mammary gland predisposition to cancer with non-oral administration.

In addition, the updated review expanded these endpoints to include:

d) Cardiovascular disease-related factors based on human epidemiology studies (*et al*., 2010), which continued the analysis of Lang *et al*. (2008) of NHANES data.

e) Perturbations in glucose homeostasis based on limited supporting evidence from a subcutaneous study in mice (Alonso-Magdalena *et al*., 2010). Data supporting this conclusion are based on exposure to adult females during pregnancy.

f) Sperm parameters based on human epidemiology data (Li *et al*., 2011) and some very limited recent supporting data from rodent studies utilizing multiple routes of exposure (Minamiyama *et al*., 2010; Salian *et al*., 2009b) were also considered for their impact on hazard identification. However, findings in large, multigenerational rodent studies have not demonstrated decreased reproductive function or effects on sperm parameters at low doses (Tyl *et al*., 2002; Tyl *et al*., 2008; Ema *et al*., 2001; Tinwell *et al*., 2002) and other reviewed epidemiology studies also do not provide clarity on this issue (Meeker *et al*., 2010a; Meeker *et al*., 2010b; Mendiola *et al*., 2010). Given the observations in humans and the mixed results in animals, these findings in human occupational exposure studies based on high exposure levels suggest that further evaluation in non-occupational (low exposure) populations is needed.

2) No new information was identified to inform the issue of dose-effect level. As such, the existing NOAEL identified in the previous review (5 mg/kg bw/day) is not altered.

3) No new studies were identified as useful for informing the BPA risk assessment. However, the hazard identification endpoints identified in this and previous reviews are currently being investigated by NIEHS and FDA/NCTR, and the results of those studies should directly address the risk assessment issue.

The memorandum herein constitutes the second review update from the BPA Joint Emerging Science Working Group as part of FDA's on-going assessment of BPA. The conclusions of the working group are meant to update the conclusions stated above from the first review memorandum dated May 24, 2011. The current conclusions to the charge are based on the second review of the scientific literature and data available between February 1, 2011 and October 24, 2011. The working group continued to adhere to the review methodology and criteria as defined previously.

METHODS

The methods employed by the group were identical to those used in the first BPA Joint Emerging Science Working Group memorandum dated May 24, 2011. This weight-of-evidence review was conducted in a three tiered fashion: (1) literature search inclusion criteria, (2) hazard identification inclusion criteria, and (3) risk assessment inclusion criteria. Hazard identification and risk assessment criteria used for review of toxicology/physiology studies were defined in the previous memorandum. Separate criteria for review of epidemiology and pharmacokinetic studies were also defined in the previous memorandum.

Literature Search

FDA's first review of the literature included papers available in-press through January 31, 2011. For the current review, PubMed was searched for publications (including those in-press) using the term

"bisphenol" from February 1, 2011 through October 24, 2011. Studies were limited to English language reports and included human epidemiology studies and animal studies with direct dosing to mammals (*in vivo,* direct dosing) with doses of ≤ 5 mg/kg bw/day. As a second search mechanism, a Dialog search was conducted for 2011 using MEDLINE, Embase SciSearch, and Pascal. The CAS RN (80-05-7) or Bisphenol A and various limiting terms[2] were used in this search. FDA also considers any non-published data submitted directly to FDA.

Risk Assessment (RA)

The criteria for risk assessment are briefly described below and were derived from guideline study foundations of Redbook, Organisation for Economic Co-operation and Development (OECD), and the Environmental Protection Agency (EPA). For expanded discussion, see previous workgroup memoranda and 'low dose' review memoranda.[3]

- Route of Administration: studies using direct dosing (oral, subcutaneous (SC), intraperitoneal (IP), intravenous (IV) as well as intramuscular (IM)) were considered.
- Sample Size and Statistical Analysis: n ≥ 10 for rodent studies; toxicological response and/or statistically significant result;
- End Point Measure (Validity): the endpoint measured is considered validated by the regulatory community or the experimental protocol utilized has scientific agreement as to its acceptability and there was confidence in the result; relevance of the finding to humans and how the finding relates to other data available in the scientific area of research were considered.
- Dose-Response: some knowledge of dose-response should be presented, with added weight given to studies that investigated a sufficiently wide range of doses;
- Sex: both sexes should be tested when appropriate for informing the validity or meaning of the data/endpoint measured;
- Repeatability: the results of the study were compared to findings in other laboratories or to complementary endpoints;
- Environmental Contamination: some characterization or consideration of the phytoestrogen content of the diet or any potential source of contamination (such as polycarbonate cages, etc.); these factors should be measured if possible to allow some insight into their contribution to findings.

As part of the weight-of–evidence assessment, findings from studies employing non-oral exposures were to be considered for their relevance to hazard identification and risk assessment based on the ability of pharmacokinetic data to inform dose translation. Data were also to be grouped among corroborating experiments to determine if they affected the strength or weakness of findings. Although studies were reviewed individually, collective findings were to be considered for their ability to indicate themes or identify potential hazards.

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2 Limiting terms included: not vitro, not fish or fishes; mammal? or animal? or human, epidemiology

3 Bisphenol A (BPA) Joint Emerging Science Working Group to FDA Chemical and Environmental Science Council (CESC), May 24, 2011. *Updated Review of the 'Low-Dose' Literature (Data) on Bisphenol A (CAS RN 80-05-7) and Response to Charge Questions Regarding the Risk Assessment on Bisphenol A.*
OFAS Review Memorandum, August 31, 2009, Aungst and Twaroski *Bisphenol A (CAS RN. 80-05-7): Review of Low Dose Studies.*
OFAS Review Memorandum, November 10, 2009, Aungst and Twaroski *Bisphenol A (CAS RN. 80-05-7): Response to reviewers of 'Review of Low Dose Studies' and update of the assessment.*

This updated literature review was carried out with the intent to identify new information that could inform the hazard identification (HI) and/or risk assessment of BPA. A number of studies identified in the updated literature review reported biological changes/observations that are currently of unknown toxicological relevance. As part of the multistep review process, these studies were assessed for quality although the impact of their findings is currently not known. As these were generally considered to be mechanistic studies, where links to adverse effects or pathways leading to toxicity are unknown, they were not considered as meeting the criteria for identification as "hazard". However, FDA scientists recognize that the importance and/or classification of these types of findings may change over time or may be informative with regard to potential modes of action of BPA. Thus, these studies were classified as relevant for mode of action (MOA) as opposed to HI.

Pharmacokinetic Criteria

Several key elements[4] were considered in the review of BPA pharmacokinetic (PK) studies. These included:

- Analytical methodology sufficiently validated and reported with respect to background (blank) levels; limit of detection/quantification; accuracy; and precision within the range of concentrations used for the study (i.e., intra- and inter-day variability).
- Measurement of both the conjugated and unconjugated (aglycone or "free") forms of BPA; quality control discussion of deconjugation enzymes (i.e., β-glucuronidase and sulfatase activities).
- Preferred dosing with isotopically labeled BPA to eliminate uncertainties surrounding use of native (i.e., unlabeled) BPA.
- Quality of methods used, with the highest weight given to mass spectrometric methods, particularly liquid-chromatography–tandem mass spectrometry (LC/MS/MS), as it provides best signal/noise performance and requires minimal sample preparation (i.e., derivatization reactions).
- Use of isotope dilution quantification (i.e., use of isotopically labeled internal standards) of at least 3 atomic mass units is preferred because of higher performance.
- Adequate demonstration of quality control in sample preparation and analysis (i.e., laboratory reagent and sample collection blanks, matrix spikes at relevant concentrations, authentic standards).
- For determination of pharmacokinetic parameters, samples obtained from individual animals (and humans) were considered more powerful statistically than those derived from pooled/averaged determinations.

Each study was reviewed for its ability to provide novel pharmacokinetic data or inform FDA's on-going efforts to develop physiologically based pharmacokinetic (PBPK) models for BPA. This initial PBPK has been published[5] and is included in this review.

Epidemiology Criteria

For epidemiology studies, several key elements were considered. These included:

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4 For expanded discussion, see Bisphenol A (BPA) Joint Emerging Science Working Group to FDA Chemical and Environmental Science Council (CESC), May 24, 2011. *Updated Review of the 'Low-Dose' Literature (Data) on Bisphenol A (CAS RN 80-05-7) and Response to Charge Questions Regarding the Risk Assessment on Bisphenol A.*

5 Fisher JW, Twaddle NC, Vanlandingham M, Doerge DR. (2011) Pharmacokinetic modeling: Prediction and evaluation of route dependent dosimetry of bisphenol A in monkeys with extrapolation to humans. *Toxicol Appl Pharmacol*. 257(1):122-36.

- Utility of study design [cross-sectional, case-control, cohort (prospective)], with more weight given to prospective studies; sufficiency of study size (consideration of uncertainty regarding size and representativeness of study sample and generalizability of results); use of multi-geographical approaches; adherence to proper statistical analyses.
- Measurement of BPA exposure and outcome metrics, with measurement uncertainty due to diurnal, seasonal, and individual variability, as well as possible environmental contamination, impact from lab plastics, interference by other biological compounds, and other factors being weighted in the analyses.
- Appropriate treatment of the data with regard to non-detectables [limit of detection values (LOD)]; adjustment of urinary concentrations (creatinine or specific gravity, with the use of specific gravity preferred); consideration given to the potential misclassification of LOD values in the confidence of the finding; and consideration of current state-of-the-science with regard to measurement of BPA in different biological matrices.
- Other factors: potential biologically plausible reverse causation; unconsidered confounders, risk factors and effect modifiers; and ascertainment of the correspondence between the measurement time and the relevant exposure window were also considered in interpretation of the results.

Each study was reviewed for its ability to inform the HI and RA process for BPA. Links to animal data were used to understand generalization to or justification of the use of specific endpoints.

UPDATED LITERATURE AND DATA REVIEW: February 1, 2011 through October 24, 2011

Based on the second updated literature search on BPA, six discrete areas were identified for review: neurotoxicity (including behavior); reproductive and developmental toxicity; carcinogenicity; pharmacokinetics; epidemiology; and other. A summary of the findings of the review and their impact on the current assessment of BPA and a summary of each publication reviewed are included below.

Pharmacokinetic (PK) Studies

Summary

Nine papers were reviewed. The reviews focused on the analytical methods provided in each paper and whether the paper was judged to be useful for hazard identification (HI) or risk assessment (RA). The 9 papers can be categorized as human biomonitoring for BPA exposure (4), clinical PK study for dietary intake of BPA (1), human and non-human primate PBPK model for BPA (1), animal studies examining the kinetics of BPA (2), and analytical method development for metabolites of BPA (1). The biomonitoring studies add to the growing number of papers documenting the exposure of humans to BPA. However, some papers (e.g., Olsen *et al*., 2011) clearly did not consider the potential for native BPA contamination by lab supplies, equipment, and in the processing of biological samples (serum and urine), perhaps introducing a systematic error in their reporting. Noteworthy were papers that did explicitly evaluate and document the impact of contamination on quantification of native BPA (e.g., Teeguarden *et al*., 2011; Vandentorren *et al*., 2011)

Experimental animal studies provided more pharmacokinetic data for discriminating between routes of administration of BPA, tissue levels of aglycone BPA, and the influence of maternal metabolism on fetal

dosimetry in rats (Doerge *et al*., 2011). Direct dosing of sheep with bisphenol A-glucuronide (BPA-G) showed that Phase II metabolites are not hydrolyzed to parental BPA *in vivo* or during sample preparation (LaCroix *et al*., 2011). The use of these studies in HI is not applicable. For RA purposes, the human PBPK model for BPA (Fisher *et al*., 2011) represents the most recent simulation model that has applications to interpreting human internal exposures to BPA at different ages. The animal PK studies provide pharmacokinetic information that can be included in the further development of PBPK models for laboratory animals and the human biomonitoring studies, if the studies have sufficient controls and monitoring for laboratory contamination, provide composite exposure information on BPA.

Individual Study Reviews
Human Data

INMA Project. Urinary concentrations of phthalates and phenols in a population of Spanish pregnant women and children (Casas *et al.*, 2011)
The study was designed to evaluate the extent of exposure to several phthalates and phenols in a sample of Spanish pregnant women. One spot urine sample was taken during the third trimester of pregnancy from 120 pregnant women and from 30 4-year old children belonging to 5 Spanish birth cohorts, and analyzed for 11 phthalate metabolites and 9 phenols, including BPA. The highest urinary concentrations were for the phthalate metabolite monoethyl phthalate and of methyl paraben. Urinary concentrations of all phthalate metabolites and of 2,4-dichlorophenol, 2,5-dichlorophenol, and bisphenol A were lower in the pregnant women than in the children. Among women, a positive relationship to social class and education was reported for most of the phthalate metabolites and phenols. While this study did employ LC/MS/MS for sample analysis, this study was deficient in other analytical methodology (e.g., lack of validation or reporting of methodology, and measurement of only total BPA). **The study provides evidence of environmental phthalate and phenol exposures in pregnant women and children but is not usable for HI or RA.**

Human biomonitoring of environmental chemicals-Early results of the 2007-2009 Canadian Health Measures Survey for males and females (Haines *et al.*, 2011)
This study reports biomonitoring of Canadians (aged 6-79) through urine analysis of environmental chemicals, including BPA (n = 2659 males and 2817 females). Geometric mean urinary levels of total BPA in males and females were reported as 1.29 µg/L (1.2-1.38, 5/95 CI) and 1.04 µg/L (0.94-1.16), respectively. Respective 95th percentile values were 6.77 and 7.04. These values are similar to BPA urinary concentrations measured in NHANES (median 2.7 µg/L, Calafat *et al*., 2008), a large recurring project of the CDC to evaluate chemical exposures in a sample statistically representative of the US population. **This study followed the criteria as stated in the Methods section above relevant to a biomonitoring study and is considered usable as a metric for aggregate human internal dosimetry of total BPA for purposes of margin of exposure estimates and international comparisons.**

Circulating levels of bisphenol A (BPA) and phthalates in an elderly population in Sweden, based on the Prospective Investigation of the Vasculature in Uppsala Seniors (PIVUS) (Olsén *et al.*, 2011)
This study reports on measured serum levels of total BPA in 1016 70-year old persons from Uppsala

Sweden. These participants were equipped with an arterial cannula for blood sampling and later for infusions of vasodilators. Male and female mean concentrations of total BPA were 3.6 and 3.89 ng/ml, respectively. The authors compare their study serum total BPA values to several other studies, which were lower (mean values range from 0.3 to 2.9 ng/ml), with the exception of a Korean study (9.0 ng/ml), but not comparable with the clinical BPA study of Teeguarden *et al*. (2011). It is difficult to determine what fraction of the measured serum samples represents contamination of the samples with BPA. The reported total serum BPA levels are considered quite high compared to the recent Teeguarden clinical study of PBA intake for 20 US volunteers. This may be due to larger BPA exposures, contamination of the samples, or differential clearance of BPA form the body in the elderly and young persons. **This study was deficient in analytical methodology (e.g., use of calf serum as a method blank, lack of aglycone measurement) for use in PBPK application and is not useful for HI or RA.**

Twenty-Four Hour Human Urine and Serum Profiles of Bisphenol A during High-Dietary Exposure (Teeguarden *et al.*, 2011)

Human volunteers (20) participated in a BPA kinetic study by ingesting food on a schedule, 3 times per day. Urine and blood samples were collected before eating breakfast at 7am and then hourly throughout the day until 10pm. Urinary excretion of total BPA provided the most useful data. BPA is considered to be completely excreted in urine as a phase II metabolite. Urine data were used to estimate total intake of BPA from each meal. Serum from 14% of blood samples had measureable levels of total BPA (1.3 nM up to 5.7 nM) and unconjugated BPA was not detected in serum (less than 1.3 nM). This is a very important study because samples were processed at two federal laboratories (CDC and FDA) to ensure quality control and the results of this human study help inform the serum kinetics of BPA and its metabolite after dietary intake of food containing BPA. There are many studies reporting on total BPA levels in serum from humans.

This study can be used as a 'standard' from which to gauge other biomonitoring studies for BPA. There are many reports in the literature for human serum total BPA concentrations. **The utility is very high because of the state-of-the-art analytical methods and the design of the study were based on mass balance principles.**

Bisphenol-A and phthalates contamination of urine samples by catheters in the Elfe pilot study: implications for large-scale biomonitoring studies (Vandentorren *et al.*, 2011)

This study evaluated the level of exposure of 250 pregnant females to BPA and DEHP by quantifying unconjugated ("free", i.e., measured in the absence of enzymatic hydrolysis) and conjugated BPA and DEHP metabolites in urine. The data were stratified by natural birth or by cesarean delivery. Compared to other epidemiology studies (National Health and Nutrition Survey), the median, 25th, and 75th percentile levels of free BPA in this study were similar to what has been reported (2.8, 1.3, 5.5 μg/L, respectively). However, the 95th percentile was higher in this study than in the national survey (115.4 vs 16 μg/L). This large difference was driven by values from women in the cesarean delivery group (95th percentile for cesarean group for unconjugated BPA was 273.9 vs 4.2 μg/L). Since the women were catheterized during the surgical delivery, it is very likely that the high values of unconjugated BPA in urine leached from the catheters. This was confirmed by a laboratory leachability study. This study demonstrates that urine sample collection can be used to monitor exposure of patients to total (i.e., conjugated) BPA but that such studies are prone to contamination from medical devices. The study also

highlights the potential to exposure of patients and infants to BPA and DEHP from urinary catheters.

Non-Human Primate Data

Pharmacokinetic modeling: Prediction and evaluation of route dependent dosimetry of bisphenol A in monkeys with extrapolation to humans (Fisher *et al.*, 2011)
This study reports the development and evaluation of a PBPK model of BPA metabolism in adult rhesus monkeys using intravenous (iv) and oral bolus doses of 100 μg deuterated BPA/kg. The model was further applied to evaluate against the published BPA metabolism data in monkeys (see citation for studies selected for comparison). A PK model was developed based on a primary study of oral and IV dosing of 4 female adult monkeys and blood sampling over 24 hr period. (Doerge *et al* 2011). Based on the collected data, a 7 compartment PK model was constructed for BPA and a single compartment PK model for BPA-c (BPA phase II metabolites). This PK model was then applied to 3 additional studies that utilized cynomolgus and rhesus monkeys. The results indicate that orally administered BPA in monkeys is affected by gut metabolism and by the stomach emptying time. The model suggests that 90% of orally administered BPA in adult and infant monkeys is metabolized in the small intestine. BPA clearance was adequately modeled in the adult and infant monkeys and also could be extrapolated to human data. With human data (Vogel *et al*. 2002), this PK model predicted that peak serum levels for free BPA values from monkeys and humans were similar for a 24 hr AUC. However, kinetics of BPA metabolites is difficult to extrapolate from monkey data to humans. A new human study of BPA metabolism is needed to compare the rates of metabolite generation/clearance with monkey data.

This study followed the criteria as stated in the Methods section above and has high utility for risk assessment. This study will be useful in evaluating future PK studies in primates and in predicting some aspects of BPA kinetics in humans. In comparison to the previous human BPA PBPK model, the new model successfully predicted lower aglycone levels in human serum using two parameter sets reflecting two adult monkey studies. A major difference between current PK models of human BPA metabolism and this study is that other models do not take into account gut metabolism, which appears to be extensive in the young and adult monkey data.

Other Mammalian Data

Distribution of bisphenol A into tissues of adult, neonatal, and fetal Sprague-Dawley rats (Doerge *et al.*, 2011)
This study used LC/MS/MS to measure placental transfer and concentrations of aglycone (receptor-active) and conjugated (inactive) BPA in tissues from Sprague–Dawley rats administered deuterated BPA (100 μg/kg bw) by oral and IV routes. The data presented here permit an examination of aglycone BPA distribution from pregnant rats to their fetuses at several gestational stages to evaluate critically the role of maternal and fetal Phase II metabolism which is responsible for detoxification and elimination of BPA.

In adult female rat tissues, the tissue/serum concentration ratios for aglycone BPA ranged from 0.7 in liver to 5 in adipose tissue, reflecting differences in tissue perfusion, composition, and metabolic capacity. Following IV administration to dams, placental transfer was observed for aglycone BPA into

fetuses at several gestational days (GD). The tissue/serum ratios were within the ranges observed in adult tissues and were not indicative of preferential accumulation of aglycone BPA or hydrolysis of conjugates in fetal tissue *in vivo*. Concentrations of aglycone BPA in GD 20 fetal brain were higher than in liver or serum. Oral administration of the same dose did not produce measurable levels of aglycone BPA in fetal tissues. Amniotic fluid consistently contained levels of BPA at or below those in maternal serum. Concentrations of aglycone BPA in tissues of neonatal rats decreased with age in a manner consistent with the corresponding circulating levels. Phase II metabolism of BPA increased with fetal age such that metabolism in the near-term fetus was similar to early post-natal rats. These results show that concentrations of aglycone BPA in fetal tissues are similar to those in other maternal and neonatal tissues and that maternal Phase II metabolism, especially following oral administration, and fetal age are critical in reducing exposures to the fetus. The results are consistent with literature precedent for placental transfer in rodents and do not support either preferential accumulation of aglycone BPA into the rat fetus or significant fetal hydrolysis of BPA-G *in vivo*.

Simultaneous quantification of bisphenol A and its glucuronide metabolite (BPA-G) in plasma and urine: applicability to toxicokinetic investigations (Lacroix *et al.*, 2011)
This study reports the collection and purification of BPA-G from sheep urine and subsequent validation of analytical methodology based on LC/MS/MS for the simultaneous quantification of parent and BPA-G in plasma and urine from sheep following intravenous and oral administration.

The study demonstrates the equivalence of direct quantification of BPA-G in plasma vs. using β-glucuronidase/arylsulfatase enzymatic hydrolysis to generate the parent BPA, albeit with 3- to 5-fold lower analytical sensitivity than for the parent compound. These results showed that BPA-G is the predominant Phase II metabolite in sheep plasma. Direct analysis of BPA-G in urine systematically underestimated values obtained through the total enzymatic hydrolysis procedure, suggesting that other metabolites are likely present in sheep urine. The study also reports that following intravenous infusion with BPA-G, analysis of sheep plasma samples contained no detectable parent BPA. This finding shows that hydrolysis of BPA-G does not occur *in vivo* or during sample handing and analysis. **These methodological data are not directly applicable for HI or RA but do reiterate PBPK modeling assumptions about BPA metabolites.**

Comparison of Serum Bisphenol A Concentrations in Mice Exposed to Bisphenol A through the Diet versus Oral Bolus Exposure (Sieli *et al.*, 2011)
This study compared the pharmacokinetics of BPA from bolus gavage (20 mg/kg bw) vs. ad libitum dietary administration (13 mg/kg bw estimated from food consumption over 24 h) to adult C57Bl/6J mice (10-12 weeks of age). Following gavage administration, maximal serum concentration (Cmax) was observed at the first time point (1 h) whereas after dietary administration the time to Cmax was delayed to 6 h. The relative bioavailability for unconjugated BPA (AUC0-24 h for diet vs. AUC0-24 h for gavage) was not significantly different for gavage vs. diet when compared on a dose-adjusted basis (10 ng x h/mL vs. 11 ng x h/mL, respectively). Serum concentrations of total and unconjugated BPA were reported to be higher after 7 d vs. 24 h of feeding the dosed diet (levels of unconjugated, but not total, BPA were reported to be statistically different. This finding was interpreted as a buildup of unconjugated BPA in serum from repeated feeding, although differences in the timing of food consumption with respect to serum collection were not apparently considered.

The study had a few limitations including the use of multiply-housed mice which yielded composite feed intake and PK parameters and required pooling of individuals at each time point. It is notable that the effects of extensive enterohepatic recirculation of BPA-G in mice were not considered by the authors. Speculation about BPA bioaccumulation is unwarranted based solely on the 24 hr unconjugated BPA measurement. These data confirm the complexities in serum pharmacokinetics typically associated with the absorption process following oral administration, particularly from the diet.

PBPK model application

The recently published human PBPK model for BPA (reviewed by this group) was used to simulate the FDA mean and 90th percentile BPA intake rates (μg/kg bw/d) for food in the infant (0-1 yr) and adult. The mean and 90th percentile BPA intake rates for the infant were 0.3 and 0.6 ug /kg bw/d and for the adult, 0.1 and 0.3 μg/kg/d, respectively.[6] Simulations were conducted for a 5 day period assuming a body weight of 3.4 kg for the infant and 70 kg for the adult. The infant was assumed to ingest the BPA during 6 meals per day and the adult was assumed to ingest BPA with 3 meals each day. The infant model physiological parameters were taken from Nong *et al*. 2006[7], who simulated a maturing child with a PBPK model and the BPA specific model parameters were taken from reports used to describe the kinetics of BPA in the 70 day old monkey (Fisher *et al*. 2011).

The model predicted that adult and infant serum aglycone BPA concentrations are extremely low and well below the LOD. For comparison, adult subjects in the Teeguarden *et al*., 2011 study consumed meals comprising canned foods and excreted BPA-G in urine at mean levels that exceeded the 95th percentile of aggregate BPA exposure from the NHANES U.S. population surveys. Total BPA levels (and 100% undetectable for aglycone BPA) ranged from undetectable in 83% of hourly serum samples (LOD 1.3 nM; 0.3 μg/L) to a maximum value of 5.7 nM (1.4 μg/L) in one subject who consumed dietary BPA equivalent to the 98th percentile from NHANES. No such controlled serum data exist in the infant for aglycone BPA or BPA-G. Our simulations of serum BPA-G in the adult using FDA daily dietary intake estimates for BPA (mean and 90th percentile) range from 0.4-1 nM (0.1 to 0.25 μg/L) with corresponding aglycone levels being approximately 1000-fold lower (see figures below). Similar food intake and urinary excretion values were estimated by international experts convened by the WHO/FAO JECFA Panel (WHO, 2011). The cited food intake estimates and biomonitoring studies from populations outside the U.S. provide worldwide context for comparisons of BPA exposure and expected serum levels of the active aglycone BPA and its metabolite. Using FDA/CFSAN daily dietary intake estimates for BPA in infants less than 1 year of age (mean and 90th percentile), the simulations of serum BPA-G range from 2-3 nM (0.5-1 μg/L) with the corresponding aglycone levels being approximately 1000-fold lower (see figures below).

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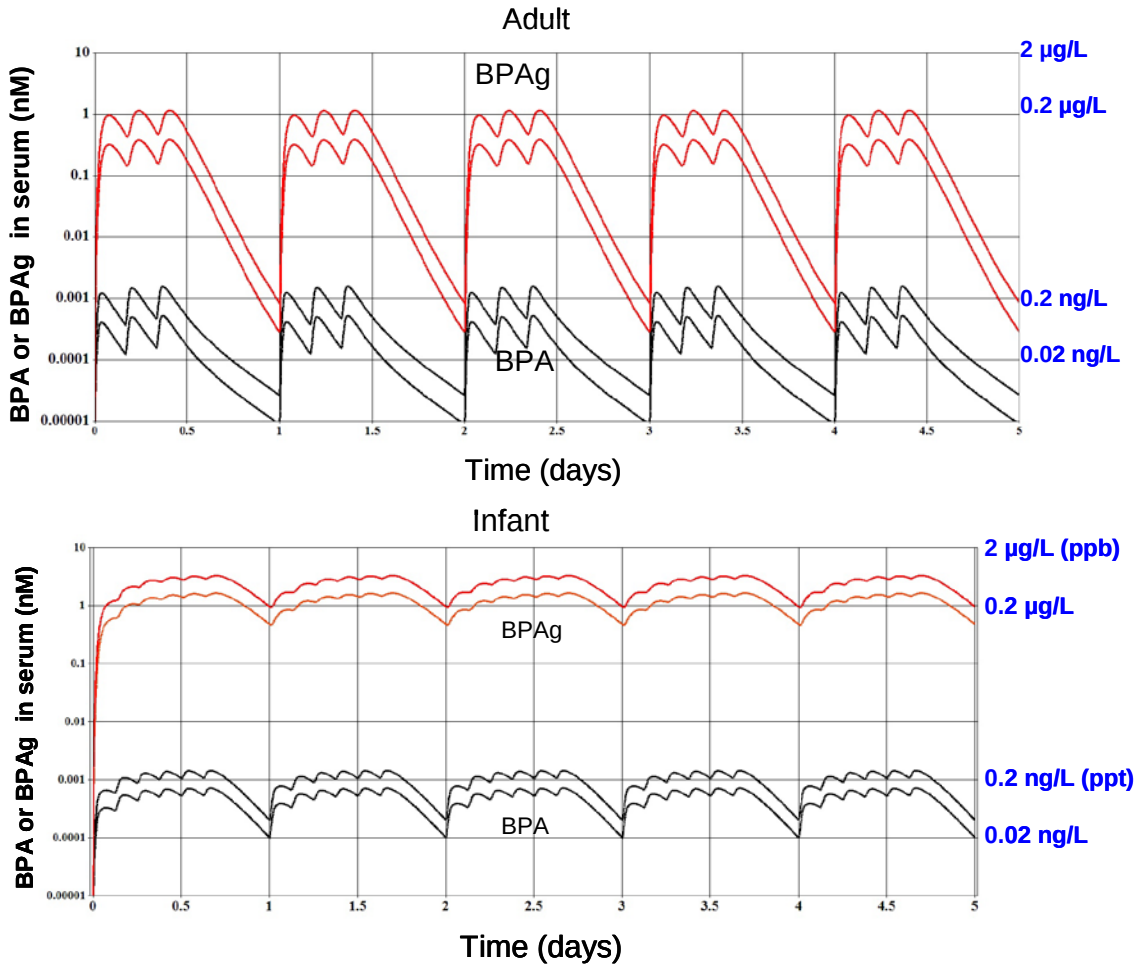
6 FDA Review Memorandum dated October 22, 2009, Division of Food Contact Notifications, Bailey, Hatwell, and Mihalov, *Exposure to Bisphenol A (BPA) for infants, toddlers and adults from the consumption of infant formula, toddler food and adult (canned) food*.

7 Nong A, McCarver DG, Hines RN, Krishnan K. (2006) Modeling interchild differences in pharmacokinetics on the basis of subject-specific data on physiology and hepatic CYP2E1 levels: a case study with toluene. *Toxicol Appl Pharmacol.* 214(1):78-87.



Neurobiology Studies

Summary

Of the 14 studies reviewed, eleven used rodent subjects, two were cohort studies of human children exposed to BPA pre- and/or post-natally, and one was a case report of an infant that appeared to have been prenatally exposed to a high level of BPA. Four studies treated rodents during adolescence or adulthood with BPA while the remainder used prenatal or early postnatal treatment. The majority of the studies focused on behavioral endpoints. Where supplementary online data was available for any study, those data were included in the overall review.

One rodent study (Ferguson *et al*., 2011) appeared to satisfy the criteria for hazard identification and/or risk assessment. This study examined preweaning endpoints during BPA treatment and showed that pre- and post-natal BPA exposure did not alter any of the developmental and behavioral endpoints examined. However, there are as yet no data from this lab on the potential long-term alterations resulting from the developmental treatment. Although the studies were of varying quality, there were common issues raised in the reviews of those studies categorized as not useful for hazard identification or risk assessment and

these issues were similar to those raised in our last review. In those studies that employed developmental BPA treatment, the most common issue was poor study design and/or statistical analyses with regard to litter of origin. Specifically, same-sex siblings were often counted as individual subjects in the analyses, a practice that has been repeatedly criticized by the experts in the field. Several studies did not report appropriate control for potential environmental exposure to estrogens or BPA. For example, caging and/or water bottle material was not described as low or BPA-free. While some studies describe the use of a low phytoestrogen diet, many gave no detail on the diet. Several studies evaluated only males or did not utilize a positive control group, although neither of these was as serious an issue as others. Some studies did not explicitly state that measurement of the nonautomated endpoints was done blind to treatment status.

One of the two cohort studies (Braun *et al*., 2011) described relatively long-lasting behavioral alterations that were more strongly correlated with gestational BPA exposure than childhood BPA exposure. The urinary BPA concentrations measured during pregnancy (median of 2.0 µg/L) appeared similar to those of other cohorts. The behavioral alterations measured at 3 years of age appeared more severe in girls ($p<0.10$) and this finding is similar to that reported from this same cohort when the subjects were 2 years of age (Braun *et al*., 2009). However, behavioral assessments of this cohort at 5 weeks of age indicated no BPA-associated alterations. Continued assessment of this cohort is vital to understanding the potential long-term behaviors associated with BPA exposure, but it is also crucial that the current findings be replicated in a separate cohort.

Individual Study Reviews

Oral Exposure

Several of these studies provided interesting observations on relatively non-traditional endpoints (e.g., partner preference) or the effects of BPA treatment during adolescence, a developmental period often overlooked in toxicological studies. These studies may motivate additional experiments examining unusual endpoints or treatment periods. However, only one study provided data that met the criteria for hazard identification and/or risk assessment.

Developmental treatment with bisphenol A or ethinyl estradiol causes few alterations on early preweaning measures (Ferguson *et al*., 2011).

This study determined the effects of oral BPA treatment on developmental markers and early preweaning behaviors in male and female Sprague-Dawley rats after pre- and post-natal treatment. The study included two doses of a reference estrogen, ethinyl estradiol (EE2), used a low phytoestrogen chow, and controlled for potential environmental BPA or estrogen exposures in caging and water bottle material. Postnatal day 21 (PND) rats were placed on the low phytoestrogen chow and were bred at adulthood. Pregnant dams were orally gavaged with 2.5 or 25.0 µg/kg/day BPA or 5.0 or 10.0 µg/kg/day EE2 from gestational day 6 through 21. From PNDs 1-21, offspring were orally treated with the same dose their dam had received. BPA treatment had no effects on gestational or lactational body weights, PND 1 anogenital distance, preweaning behaviors (righting or slant board behavior), PND 21 hormone levels, or PND 21 regional brain weights. EE2 treatment did alter gestational and lactational body weights, birth weights, and preweaning body weights. This study appears to have been robustly powered (number of litters/treatment group ranged 14-19 and n/sex/treatment group ranged 10-17) and the statistical analyses were appropriately conducted. Effects related to litter were accounted for in the design and statistical

analyses. **This study may be used for hazard identification or risk assessment.**

Protective effect of *N*-acetylcysteine on bisphenol A-induced cognitive dysfunction and oxidative stress in rats (Jain *et al*., 2011).

This study evaluated cognitive functions and oxidative stress markers in adult male Wistar rats treated orally with 2 or 20 µg/kg/day BPA for 28 days (n=8/group). Three additional groups were used to evaluate the potential protective effect of *N*-acetylcysteine (NAC) and were administered 100 mg/kg/day NAC only or prior to 2 or 20 µg/kg/day BPA for 28 days. Passive avoidance and water maze performance were evaluated near the end of the treatment period and, on the day after the last treatment, whole brain measurements of malondialdehyde (MDA) and reduced glutathione (GSH) were taken. Step-down latency in the passive avoidance test was decreased in a dose-response manner for both BPA dose groups during the initial acquisition and the first and second retention tests. However, in those groups that received NAC and BPA, step-down latencies were not different from control. Latency to locate the water maze platform was increased in a dose-response manner for both BPA dose groups during the initial acquisition and the first and second retention tests. Again, in those groups that received NAC and BPA, the latencies were not different from control. Swim speed was not measured in the water maze (or is not reported as measured) and this is essential in order to ascertain that the increased latency is indeed a memory deficit. Both BPA doses increased whole brain levels of MDA and decreased levels of GSH in a dose-response manner. In those groups that had received NAC and BPA, the MDA increase and the GSH decrease were attenuated. Details of BPA administration are missing. The reviewers assume that the oral treatment was via gavage but this is not directly stated and there is no mention of volume. Although data from other groups implicate BPA treatment in the generation of reactive oxygen species, it does so at much higher doses administered intraperitoneally. Further, that group did not find any change in brain levels of GSH. Use of a potent reactive oxygen species generator as a positive control would have helped to validate the measurements reported for BPA. **This study cannot be used for hazard identification or risk assessment** due to a potential lack of control for environmental exposure to estrogens or BPA (i.e., no description of water bottle material or of diet) and the evaluation of only one sex.

Disruption of adult expression of sexually selected traits by developmental exposure to bisphenol A (Jasarevic *et al*., 2011).

This study evaluated behavioral and hormonal effects of prenatal and lactational BPA (or ethinyl estradiol (EE2)) exposure in male and female deer mice (*Peromyscus maniculatus*). A low phytoestrogen diet, polypropylene cages and glass water bottles were used. Prior to mating, 30 female deer mice were assigned to: 1) control, 2) 50 mg BPA/kg diet, or 3) 0.1 ppb EE2 in the diet. Females remained on this diet until weaning of the offspring. Sensory and neuromuscular assessments of offspring at postnatal day (PND) 25 did not indicate any effects of BPA or EE2 treatment. PND 60 Barnes maze performance of BPA or EE2 exposed males was worse than same-sex controls on training days 2-7; however, BPA had no effects on female performance. Anxiety behavior of males exposed to BPA or EE2 was increased as measured by elevated plus maze behavior at PND 74; specifically, they exhibited increased durations of immobility, and decreased time in and entries into the open arms. Females exposed to BPA were not affected. A "mate choice" test indicated that control and BPA exposed females preferred control males more than BPA exposed males. Serum levels of testosterone and corticosterone were unaffected by BPA exposure in adult males. It is not clear if the breeder males

had access to the different diets (control, BPA and EE2) during breeding. If so, then those males should have remained in that same treatment group for subsequent breedings. There are questions regarding the "mate choice" methodology in that control and BPA females were exposed to a control male prior to mate choice assessment. **This study cannot be used for hazard identification or risk assessment** due to incomplete details that would allow a complete assessment. It is likely that effects related to litter of origin were not accounted for in the design or statistical procedures. Given 30 breeding pairs (30/sex were obtained) and three treatments, it is assumed that there were 10 breeding pairs/treatment group (although this is not stated). Each female appears to have been bred at least twice since number of litters/group ranged 24-25. There is no description of whether any of the subjects were littermates or if all were from different litters (but some still would have likely resulted from the same dam). Potential same-sex siblings from the same litter or from the same dam but different litters do not appear to have been accounted for the in the statistical analyses. It is not clear if this particular species behaves in a similar manner as typical laboratory mice which would allow some potential for comparisons of the results of this study with others. However, if this species is so unusual in its behavior, it is not clear how useful these data are. Finally, given the estimated high dose of BPA, any relevance to human risk assessment seems low. (NOTE: BPA dose was estimated as follows: Deer mice under standard housing conditions with 3 pups/litter consume ≈ 5.5 g food/day and the average body weight of an adult nonpregnant deer mouse is ≈ 19-20 g. Thus, the pregnant/lactating mice in this study likely consumed ≈ 13,750 µg BPA/kg/day. Further, it is likely that there was some direct pup exposure since deer mice began eating solid food at ≈ 15 days of age.)

<u>Exposure to bisphenol A appears to impair hippocampal neurogenesis and spatial learning and memory (Kim *et al*., 2011).</u>

This study evaluated the effects of oral BPA treatment in adolescent male C57Bl/C mice on water maze performance and hippocampal neurogenesis. Five-week-old male mice were orally gavaged for two weeks with 1, 5, or 20 mg/kg/day BPA (n=5-6/group). After the two week treatment period, mice were assessed for water maze performance. A separate group of mice were used for neurogenesis studies. On the 7th (last) day of water maze testing, the 20 mg/kg/day group had a longer latency to reach the platform than the control group. Latencies on days 1-6 did not differ and latencies for the 1 and 5 mg/kg/day groups were not different from control on any day. Swim speed did not differ among treatment groups. Hippocampal neuronal proliferation in the dentate gyrus was reduced by 20 mg/kg/day BPA treatment, but not by 1 or 5 mg/kg/day BPA. Surprisingly, hippocampal neuronal survival was increased by 1 mg/kg/day BPA treatment, but 5 and 20 mg/kg/day BPA had no effects. There were no treatment changes in brain-derived neurotrophic factor (BDNF) levels or any treatment-related reactive gliosis. **This study cannot be used for hazard identification or risk assessment** due to a potential lack of control for environmental exposure to estrogens or BPA (i.e., no description of caging or water bottle material or of diet) and small sample sizes. Further, the water maze behavioral data should have been analyzed using a repeated measures ANOVA. Measurement of BPA-induced alterations in hippocampal neurogenesis/survival, however, is a novel endpoint and the assessment of alterations after BPA treatment during adolescence is essential.

<u>Dose-dependent behavioral disturbances after a single neonatal Bisphenol A dose (Viberg *et al*., 2011).</u>

This study evaluated the effects of BPA (0.32, 3.2, or 4.8 mg/kg) administered orally on postnatal day 10

to male NMRI mice on body weight, adult activity (baseline and after a challenge of 80 µg/kg of nicotine), elevated plus maze behavior, and water maze performance (n=12-15/group). At four weeks of age, all BPA groups weighed less than controls but there were no differences at 10 weeks or 6 months of age. Activity of the 0.32 mg/kg BPA group was not altered at 2 or 5 months of age. However, locomotion, rearing, and total activity were decreased in a dose-response manner by 3.2 and 4.8 mg/kg BPA during the first 20-minute period of the 60-minute session at 2 and 5 months of age. Similarly, at both ages, mice of the 3.2 and 4.8 mg/kg BPA groups exhibited increased locomotion during the second and third 20-minute periods. Rearing and total activity exhibited a similar pattern at both ages in the 4.8 mg/kg BPA group. Nicotine caused similar effects in the control and 0.32 mg/kg BPA group (i.e., increased activity); however, nicotine had no effects on rearing (which was increased in control and 0.32 mg/kg BPA groups) in the 3.2 and 4.8 mg/kg BPA groups. Elevated plus maze behavior and water maze performance was not affected by BPA treatment. Use of automated assessments and measurement of habituation (activity was measured in three 20-minute periods for the 60-minute session) are advantages of this study. **This study cannot be used for hazard identification or risk assessment** due to a potential lack of control for environmental exposure to estrogens or BPA (i.e., no description of caging or water bottle material or of diet), lack of control for litter of origin in the design and statistical analyses, and the evaluation of only one sex. Although the total number of litters is not stated, as many as 4 or 5 littermates may have been tested for each behavioral assessment. The authors have been previously criticized for using this type of design. Litters were culled to 10-14 pups each but since litter size can have an effect on offspring body weight, all litters should have been culled to the same number. Body weight should have been analyzed using a repeated measures analysis. Finally, it is not clear why different post-hoc tests were used in different ANOVAs.

Gestational exposure to low dose bisphenol A alters social behavior in female mice (Wolstenholme *et al*., 2011).

This study evaluated the effects of prenatal BPA exposure on juvenile social and non-social behaviors in male and female C57Bl/6J mice. In addition, the embryonic expression of selected genes thought to regulate/influence behavior in a sexually dimorphic manner was evaluated at embryonic day 18.5. Adult female mice were placed on: 1) phytoestrogen-free chow (n=11) or 2) chow containing 1.25 mg BPA/kg (n=12). The estimated daily BPA dose was 5 µg/day. This is ≈ 200 µg/kg/day (assuming a 25 g pregnant mouse). One week later, they were mated with unexposed males and remained on the same chow until either embryo collection (5 litters/group) or parturition. At parturition, all pups (control and BPA-exposed) were fostered to control dams. Blood was collected from some of the dams used for embryo collection to measure serum levels of unconjugated BPA. When tested on postnatal day (PND) 20 for social behavior, BPA exposed females exhibited longer and/or more frequent nose-to-nose contact (as did BPA exposed males), side-by-side contact, and following behavior. BPA exposed females had shorter durations of self-grooming. Both BPA exposed sexes engaged in more approach behaviors. When tested on PND 24 with an adult male mouse for social preference, there were no treatment related differences. Elevated plus maze behavior at PND 24 was not affected by prior BPA exposure. Of the 11 gene expression levels measured, 4 were affected by BPA exposure: BPA exposed females had higher expression levels of *Slc1a1* (a glutamate transporter) and *Dnmt3a* (a DNA methyltransferase), but lower levels of *Dnmt1* (a DNA methyltransferase). BPA exposed males had lower expression levels of *Oxtr* (an oxytocin receptor). These changes were not directly associated with adverse effects. Serum levels of unconjugated BPA were 0.43 ng/ml (≈ 2 nM) and these were estimated by extrapolation of the standard

curve to zero since the limit of detection was 0.5 ng/ml. Measurement of serum BPA levels is a strength of this study as is the fostering procedure used to limit BPA exposure to the prenatal only period. **However, this study cannot be used for hazard identification or risk assessment** due to a potential lack of control for environmental exposure to estrogens or BPA (i.e., no description of caging or water bottle material) and it appears that litter was not appropriately accounted for in the design or statistical analyses. Given that 10 (5/group) of the 23 total litters were used for embryo collection, 13 litters (6 control and 7 BPA) were available for the behavioral assessments. However, the BPA behavioral group contained 18 females and 21 males so some of these must have been siblings. Similarly, the control behavioral group contained 13 females and 15 males.

Sex-specific influence of exposure to bisphenol-A between adolescence and young adulthood on mouse behaviors (Xu *et al*., 2011).

This study evaluated behavioral, hormonal, and reproductive organ weight effects in male and female ICR mice treated orally via gavage with 40 or 400 µg/kg/day during adolescence (postnatal days (PNDs) 32-87). A soy-free diet was used throughout the study, but it is not clear when mice were placed on this diet. The method of selecting the mice is not clear. Mice weighing 18-23 g were used and this could represent the range of subjects that were randomly selected. Alternatively, it could represent the range that was purposely selected and those not weighing within this range were not used. Body weight of males at the end of treatment was reduced by 40 or 400 µg/kg/day BPA. At the same time, female body weight was decreased by 40, but not 400, µg/kg/day BPA. Adult serum estradiol or testosterone levels were not affected nor were testis or uterine weights. PND 91 open field rearing of males was decreased by 40, but not 400, µg/kg/day BPA; rearing was unaffected in females. Overall locomotor activity was not altered. The sexually dimorphic behavior of grooming (males>females) in the open field was not apparent in either BPA group. Similarly, the sex difference in elevated plus maze behavior (increased entries into and duration in open arms in males) was not apparent in either BPA group. Males of both BPA groups exhibited fewer head dips in the EPM than did control males while females of the 400 µg/kg/day BPA group exhibited more head dips than female controls. Swim path length in the water maze was longer on days 3 and 4 in males treated with 40 µg/kg/day BPA than male controls. This was the only significant water maze effect. Males treated with 40 µg/kg/day BPA exhibited poorer retention in the step-down passive avoidance test relative to control males while there were no BPA effects in female performance. **This study cannot be used for hazard identification or risk assessment** due to a potential lack of control for environmental exposure to estrogens or BPA (i.e., no description of caging or water bottle material) and it is not clear that litter was appropriately accounted for in the design and statistical analyses. The total number of litters is not stated, however, the implication is that at least some of the subjects used for each endpoint were siblings. Another major concern is that each endpoint seems to have been subjected to three different statistical analyses: 1) the traditional two-way ANOVA (with treatment and sex as factors), 2) one-way ANOVAs with treatment as a factor and analyses conducted separately for each sex, and 3) a comparison between the sexes within each treatment group (the type of test is not specified). It is not clear in the Results section from which of these analyses the significant effects resulted. Two of the analyses should have used an analysis of covariance (body weight and organ weights).

Subcutaneous Exposure

These studies provided interesting information as to potential mechanisms of action for BPA and/or its

effects on nontraditional endpoints. However, none were categorized as useful for hazard identification or risk assessment, mainly due to the potential for estrogenic/BPA environmental exposures and lack of statistical control for litter effects. One (Bai *et al*., 2011) appears to describe BPA-induced effects opposite to those previously reported by the Patisaul lab; however, dose and route of administration differences may explain this.

Increase of anteroventral periventricular kisspeptin neurons and generation of E2-induced LH-surge system in male rats exposed to environmental dose of Bisphenol-A (Bai *et al*., 2011).

This study assessed kisspeptin and gonadotropin-releasing hormone (GnRH) levels in the hypothalamic anteroventral periventricular nucleus (AVPV) and preoptic area (POA) in intact and gonadectomized (GNX) male Sprague-Dawley rats after subcutaneously injecting their dams with 2 µg/kg/day BPA from gestational (GD) 10 to lactational day (LD) 7. Two sex/litters were gonadectomized on postnatal days (PND) 23, 43, or 83 and implanted with estradiol (E2). Regulation of E2-induced pituitary luteinizing hormone (LH) release in adult male offspring was also measured. Group sizes were 6-10/sex. Numbers of kisspeptin-containing cells in the AVPV were increased in GNX E2- and BPA-treated male offspring at PNDs 30, 50, and 90 relative to GNX male controls. Without E2 treatment, all groups exhibited very few kisspeptin-containing cells. E2- and BPA-treated intact male rats also exhibited increased numbers of kisspeptin-containing cells in the AVPV at PND 50. Numbers of GnRH-containing cells in the POA were decreased at PND 30, but increased at PNDs 50 and 90, in GNX E2- and BPA-treated male offspring. Without E2 treatment, GNX BPA-treated male offspring also exhibited increased numbers of GnRH-containing cells in the POA at PND 50. At PND 90, exogenous E2 produced an LH surge in GNX BPA-treated males but this was reversed by an antagonist of G-coupled protein receptor 54 (GPR54). At PNDs 30 and 50, basal levels of LH were increased and testosterone were decreased in intact BPA-treated males but levels at PND 90 were not different. At PNDs 50 and 90, but not PND 30, basal levels of E2 were increased in intact BPA-treated males. **This study cannot be used for hazard identification or risk assessment** due to a potential lack of control for environmental exposure to estrogens or BPA (i.e., no description of caging or water bottle material or of diet) and it is not clear if the litter was appropriately accounted for in the design or statistical analyses. Two/sex/group were used for each endpoint but it is not clear if those data were averaged prior to analyses as the total number of litters/group is not stated. There is a litter sex bias since litters were culled to retain "the maximal number of males per litter" which can influence later sexually dimorphic behavior and possibly the endpoints examined here. The relevance of the findings is unclear as many of the significant results occurred in males that were exogenously treated with E2.

Bisphenol-A impairs memory and reduces dendritic spine density in adult male rats (Eilam-Stock *et al*., 2011).

This study determined the effects of a single subcutaneous injection of 40 µg/kg BPA in adult male Sprague-Dawley rats on memory consolidation (object recognition (OR) and object placement (OP) tasks), hippocampal and medial prefrontal cortical dendritic spine density, and protein expression. BPA (or saline) was injected after the initial habituation trial (before the recognition trial) in the OR and OP tasks. BPA-treated rats exhibited decreased exploration of novel objects (in the OR task) or novelly placed objects (in the OP task); however, corticosterone levels were not altered by BPA treatment. BPA-treated rats had a small reduction in apical and basal spine density in the hippocampus and a somewhat greater reduction in apical and basal spine density in the medial prefrontal cortex. However, in rats that

were not behaviorally tested, all spine densities were lower and there were no BPA effects. Of the 6 proteins assessed, only two showed very slight changes in expression. BPA-treated rats had slightly lower hippocampal (but not mPFC) PSD-95 levels (synaptic fraction only) and slightly higher pCREB levels in the mPFC (cytosolic fraction only), but not in the hippocampus. **This study cannot be used for hazard identification or risk assessment** due to a potential lack of control for environmental exposure to estrogens or BPA (i.e., no description of caging or water bottle material or of diet). Sample sizes are relatively small (6-7/group) and only males were assessed. It is not stated that the behavioral or histological data were measured blind to treatment. It is not clear what the repeated measure is in the behavioral data ANOVAs or why a one-tailed t test was used for post-hoc tests when two-tailed t tests were used for other measures. Finally, rats treated with BPA in the OR task were then used as control subjects (and injected with saline) four days later in the OP task and vice-versa. Thus, the same rats were used as control and BPA subjects. Given the studies that describe effects of a single BPA treatment, there is the possibility that there were lingering BPA effects which may have interfered with behavior in the subsequent behavioral task.

Prenatal and lactational exposure to bisphenol A in mice alters expression of genes involved in cortical barrel development without morphological changes (Han *et al*., 2011).

This study determined the effects of prenatal and lactational BPA treatment on the development of the brain stem barrelettes, thalamic barreloids that project somatotopically into the cortical barrel fields of adult and preweanling ICR/Jcl male and female mice. Pregnant mice were subcutaneously injected with 20 µg/kg/day BPA from gestational day 0 through lactational day 21. At postnatal days (PND) 1, 4, 8 or at adulthood, offspring were perfused for measurement of the posterior medial barrel subfield (PMBSF), barreloid, and barrelette (adults), development of and gene expression in the barrel, barreloid, and barrelette (preweanlings). Morphologies of the PMBSF, barreloid, and barrelette in adults were not altered by BPA treatment nor were PND 1, 4, or 8 morphologies of the barrel, barreloid, and barrelette. There were several BPA gene expression changes in the cortex, thalamus and pons which were sometimes sex-specific. These changes were not particularly pronounced and they were not consistent across the three ages examined. **This study cannot be used for hazard identification or risk assessment** due to a potential lack of control for environmental exposure to estrogens or BPA (i.e., no description of caging or water bottle material or of diet) and it is not clear if the litter was appropriately accounted for in the design and statistical analyses. Further, some of the histological groups contained only 3 subjects/groups. The sensitivity of the barrel histology assay and the developmental time points selected (i.e., PNDs 1, 4, 8) may not have been optimized to detect BPA-mediated effects. A classic paper by Fox (1992)[8] describes the critical period for development during which thalamocortical afferents form barrel shaped clusters in the rat somatosensory cortex as occurring during the first 5 days, with P0 being largely undeveloped. Fox *et al*. found that input deprivation by pulling out selected whisker inputs at P0 had no, or only modest (barrel enlargement), effects on the large scale features of the barrels, at the same time when whisker disruption affected connectivity of whisker selective afferents within the barrel cortex. No correlations were drawn between the gene expression alterations and barrel morphology. Finally, with the exception of *Maoa*, the 6 genes analyzed here were not those noted to have major roles in barrel map formation in somatosensory cortex (Li and Crair, 2011).[9]

8 Fox K., A critical period for experience-dependent synaptic plasticity in rat barrel cortex. J Neurosci. 1992 May;12(5):1826-38.

9 Li H, Crair MC. How do barrels form in somatosensory cortex? Ann N Y Acad Sci. 2011 Apr;1225:119-29.

Bisphenol-A rapidly enhanced passive avoidance memory and phosphorylation of NMDA receptor subunits in hippocampus of young rats (Xu *et al*., 2011).

This study determined the effects of a single subcutaneous injection of BPA (50 or 500 µg/kg) administered at postnatal day (PND) 18 to male Sprague-Dawley rats on passive avoidance behavior and protein levels and phosphorylation states of the hippocampal NMDA receptor subunits NR1 and NR2B via Western blot analysis (n=4-10/group). A positive control group was injected subcutaneously with 10 µg/kg estradiol benzoate (EB), and an additional group was co-injected with 500 µg/kg BPA and 10 µg/kg EB. When BPA or EB was injected immediately after the behavioral training session, step-down latencies in the passive avoidance task were increased (normally indicating better memory). However, the combination (BPA+EB) had no effect. When injections occurred 23 hours prior to the behavioral training session, there were no effects of any treatment on step-down latencies. One hour after BPA or EB treatment, protein levels of NR1 and NR2B were not altered; however, phosphorylation levels were increased for NR1 and NR2B by both BPA doses and by the EB dose. The combination (BPA+EB) had no effect and there were no significant effects 24 hours after any treatment (BPA, EB, or BPA+EB). A separate group of PND 18 male rats were directly injected into the hippocampus with an estrogen antagonist (ICI 182,780) or ERK inhibitor (U0126) followed by a subcutaneous injection of 500 µg/kg BPA or 10 µg/kg EB. Both inhibitors blocked the phosphorylation increases in NR1 and NR2B and both blocked the phosphorylation increase in ERK that was produced by BPA or EB. Hippocampal ERß levels were not altered by any treatment. The authors did not clearly demonstrate that BPA acts directly via estrogen membrane receptors (as they claim). Further, ICI 182,780 is used as an estrogen antagonist, but this compound also acts as a high affinity agonist at the estrogen membrane receptor. **This study cannot be used for hazard identification or risk assessment** due to a potential lack of control for environmental exposure to estrogens or BPA (i.e., no description of caging or water bottle material or of diet) and it is not clear if the litter was appropriately accounted for in the design or statistical analyses. Further, the small sample size for some groups (e.g., phosphorylation levels) as well as the evaluation of one sex hinders the usefulness of this study. The relevance of a single subcutaneous injection of BPA is not clear.

Human studies

Impact of early-life Bisphenol A exposure on behavior and executive function in children (Braun *et al*., 2011).

This is a continuation of a previous report (Braun *et al*., 2009) examining gestational and postnatal BPA exposure in young children. The subjects were 389 participants in the Health Outcomes and Measures of the Environment (HOME) Study sponsored by the NIEHS. Spot urine was collected from pregnant women at ≈ 16 and 26 weeks of gestation and at birth and from the infants/toddlers at 1, 2, and 3 years of age. Urine samples were analyzed by the CDC for BPA with correction for urinary output. Three-year-old children were rated by their parent on two scales: the Behavior Assessment System for Children 2 Parent Rating Scale (BASC-2) and the Behavior Rating Inventory of Executive Function-Preschool (BRIEF-P). The typical covariates were included in the statistical model as well those factors that could cause neurobehavioral alterations (e.g., tobacco smoke exposure). Childhood urinary BPA concentrations were less predictive of behavior scores than were gestational BPA concentrations. Gestational BPA concentrations were positively associated with anxiety, hyperactivity, and depression

scales of the BASC-2 and this association was greater in girls. Emotional control and inhibition scores on the BRIEF-P were positively associated with gestational BPA concentrations and again, the associations were larger in girls. This study was well-designed and well-conducted and the urinary BPA levels were determined using standardized methodology at the CDC. However, there is some missing information that would have been extremely helpful in assessing the utility of this study. The previous report (Braun *et al*., 2009) found that urinary BPA concentrations measured at 16 weeks of gestation were more associated with 2-year-old children's behavioral scores than were concentrations at 26 weeks of gestation or at birth. Here, gestational and birth urinary BPA levels were averaged so it is not known if the association between 3-year-old children's behavior was more related to early or late gestational urinary BPA. It is not clear that blood lead levels were included in the analyses as they were previously. The effects here must be replicated with a separate cohort; however, the continued association of gestational urinary BPA levels with altered behavior in girls could reflect long-term alterations resulting from developmental BPA exposure. **This study cannot be used for hazard identification or risk assessment** until there are further data.

Case report: High prenatal Bisphenol A exposure and infant neonatal neurobehavior (Sathyanarayana *et al*., 2011).

The case mother in this study was a participant in the Health Outcomes and Measures of the Environment (HOME) Study (see above). Spot urine was collected at approximately 16 and 26 weeks of gestation and at birth and analyzed by the CDC with correction for urinary output. Urine samples were obtained from the children at 1, 2, and 3 years of age. Urinary BPA concentrations for this case mother were 4.1, 583, and 1.9 µg/g creatinine at 16 and 27 weeks gestation and at birth, respectively. The level at 27 weeks of gestation (583 µg/g creatinine or 1250 µg/L) was higher than any value reported in the general population (median value for the entire HOME cohort at this gestational age was 2.0 µg/g creatinine). The sample from this case mother was reextracted and analyzed twice by the CDC with the same results. This sample was obtained in 2004 and during a follow-up phone interview in 2009, the mother reported using plastic food containers and utensils and recalled eating canned, generic ravioli daily during the time encompassing this gestational urine sample. At 14 hours of age and again at 27 days of age, the male infant was evaluated with the NICU Network Neurobehavioral Scale (NNNS). At 14 hours of age, the results were within normal ranges. However, at 27 days of age, the scores were above normal for the excitability, lethargy, and stress abstinence scales and below normal for the regulation and quality of movement scales. Subsequent annual test results using the Behavior Assessment System for Children 2 Parent Rating Scale (BASC-2) were within the normal range at 1-5 years of age. This case report was not intended to provide a quantitative or qualitative analysis of BPA exposure and its potential effects. The authors note that the adverse effects were transient. **This study cannot be used for hazard identification or risk assessment** as it presents a single subject in case report format.

Prenatal exposure to bisphenol A and phthalates and infant neurobehavior (Yolton *et al*., 2011).

This study evaluated associations between gestational concentrations of urinary BPA and scores on the NICU Network Neurobehavioral Scale (NNNS) of infants at 5 weeks of age in a cohort of 350 participants in the Health Outcomes and Measures of the Environment (HOME) Study sponsored by the NIEHS. Spot urine was collected from pregnant women at ≈ 16 and 26 weeks of gestation and at birth. Urine samples were analyzed by the CDC for BPA with correction for urinary output. There was no

evidence of non-linearity in any dose-response relationship. There was a bivariate association between urinary BPA concentrations measured at 16 gestational weeks and increased hypotonia in the infant. The multivariate analysis of this scale also indicated a trend toward increased hypotonia and increased BPA levels. No other NNNS subscale was associated with BPA levels in the bivariate analyses. However, the majority of the hypotonicity scores were zero (a good quality) and very few were more than one. The authors de-emphasize this single finding. This is a well-designed study and utilized rigorous statistical analyses. However, spot urine sampling does not give an accurate overall picture of BPA exposure, thus **this study cannot be used for hazard identification or risk assessment** until there are further data and/or replication in a separate cohort.

Reproductive and Developmental Studies

Summary
Ten published studies were reviewed in this area: five of which used rat models (Sprague-Dawley (1), Wistar (3), and Holtzman (1)), three used mice (C57BL/6 (2) and JF1♀xOG2 cross (1)), one used African green monkeys, and one used neonatal lambs. Two studies in rats and two studies in mice used the oral route of administration, while the remaining rodent studies used subcutaneous routes of administration, including a rat study that utilized a minipump. BPA was administered subcutaneously to lambs and by subcutaneous minipump to monkeys. Five studies evaluated females, four studies evaluated males, and one study evaluated unsexed fetuses. All three mouse studies and one rat study involved treatment of dams and evaluation of the effects on the offspring through indirect dosing. Three of these were by the oral route and one (a mouse study) was by subcutaneous administration. Two studies employed castration-based models; one used castrated male rats and the other used oophorectomized female monkeys.

In general, the findings reported in the literature were exploratory, mechanistic, or based on specific markers in previously identified target organs (gene expression was common). The main issues raised in discussion of the studies were similar to the last cycle of review. No literature study was guideline-compliant using common and validated endpoints for regulatory toxicology purposes. This does not mean the studies were inadequate, reflected poorly on the data quality, or potential importance of any finding; simply that a full regulatory risk assessment may not have been possible. Often, there was a lack of complete reporting of study details and the potential for selective reporting of positive study data because of limitations imposed by journals. In some instances, the authors discussed these constraints. Only three studies in intact animals were conducted with multiple doses of BPA that could be evaluated for dose response assessment, and no study confirmed systemic exposure. This is important for risk assessment to permit comparison of the relatively low level of aglycone produced following oral dosing to those predicted to result from human BPA exposures. BPA aglycone levels following subcutaneous administration are expected to be significantly greater than those produced from the oral route but again, this information was not supplied.

The overall conclusion of the review group was that while no literature study was useful for risk assessment, there were some findings identified as a potential hazard. Previously in nonclinical species,

effects for BPA have been described for the ovary, HPG axis and estrous cycle, testes/sperm, and prostate. Specifically in this review, effects on the testes (Cardoso *et al.*, 2011), mammary gland (Durando *et al*., 2011), and uterus/implantation (Varayoud *et al*., 2011) were noted. Another study (Xiao *et al*., 2011) had mechanistic findings related to and consistent with the uterus/implantation effects. These findings were not novel, but complement and extend previous published results in these organs. Of particular note, decreased implantation sites and uterine progesterone receptor expression in mated adult female mice and rats were noted when treated through subcutaneous administration during gestation or postnatally, respectively. This suggests that this finding is substantive since it occurred independently in two different species; Xiao *et al*., and Varayoud *et al*., have findings at 40 mg/kg bw/day and 20 µg/kg bw/day, respectively. However, the results of these studies do not affect the oral exposure NOAEL of 5 mg/kg bw/day given that they used subcutaneous dosing, showed a limited dose relationship, and did not provide internal dosimetry data. Available PK data cannot yet provide route to route or interspecies extrapolation. A study conducted in lambs (Rivera *et al*., 2011) was limited by measuring endpoints in juvenile animals that are not clearly associated with adverse effects in adults. Surgically altered animals with hormone re-supplementation provided some limited useful information (Aldad *et al.*, 2011; Wu *et al.*, 2011).

Below are the reviews of the individual studies and the comments relating to the value of the data and limitations and concerns are contained in those reviews. A number of general concerns were again raised during the discussion of the reviews that applied to most of the studies examined. These concerns included the possible effect of diet and vehicle on the endpoints examined, the limited detail in literature reports on study design, study conditions, and data analysis and reporting. There was variable reporting of details on the diet, water, vehicle, and other study materials to inform on background estrogenic activity. Even when provided, often there was not sufficient information or discussion of the potential effects of such activity on the endpoints measured (e.g., the phytoestrogen level in the diet or how that might influence the results of the study). The possible contribution of, or lack of consideration of, estrogenic activity from vegetable oils (such as sesame, corn, olive, and castor oils) was another concern. One study used DMSO at 50%, which was a concern raised last in the last review when used at 100%. Only two studies used a vehicle without some potential relevant physiological activity. A common comment in the papers was that all animals were identically subjected to the experimental conditions, so that background exposure was equivalent. The point that the background may be contributing to the total systemic exposure was not addressed. Another theme noted in discussion was the lack of consistency in the findings. Often, unexpected findings would be noted in the context of a series of related observations. When multiple doses or timepoints were used, the findings often did not have a discernable relationship. The authors usually addressed these observations as a context of the study limitations, such as sample size, measurements with known large variability (such as sex hormones), dose, or time dependency. All of these points could be readily addressed in future studies.

Individual Study Reviews

Oral exposure

Probable gamma-aminobutyric acid involvement in bisphenol A effect at the hypothalamic level in adult male rats (Cardoso *et al*., 2011)

This study evaluated the effect of BPA administered orally to the dam during gestation and lactation on the neuroendocrine control of the reproductive axis in adult male rats. Female Wistar rats (n = 10/group) were treated from GD 0 to PND 21, the time when litters were weaned, with either 0.1% ethanol in water or 25 mg/L BPA in the same vehicle. Based on water consumption estimates for the dams (water bottle weights), the ingested dose was 2.5 mg/kg body weight per day. Animals were housed in metal cages, maintained on a 12 hr light dark cycle, and fed a soybean meal-containing diet with unknown phytoestrogen levels. The authors suggest that this is not a factor since all animals had similar levels of food consumption. Male pups (n = 10/group, presumably from different litters) were held until PND 70 without further treatment. Animals were euthanized on PND 70 and the mediobasal and anterior preoptic areas of the hypothalamus dissected, preincubated for 30 minutes in media, and then incubated for 60 minutes in fresh media that was collected for GnRH and GABA analyses at the end of the incubation. Trunk blood, presumably from the same animals, was collected and assayed for LH, FSH, and testosterone. Testes were fixed with Bouin's solution for 24 hr, embedded in paraffin, and stained with H&E for histological evaluation. Leydig cell number and nuclear area of the Leydig cells were evaluated. Differences between the two groups were evaluated using t-tests.

GnRH release from the hypothalamic slices was reported as significantly reduced and GABA release significantly increased in the BPA-treated group. Serum FSH and testosterone were significantly reduced in BPA-treated animals while LH was unchanged. The numbers of Leydig cells in testes sections were unchanged, but the nuclear size was significantly reduced in the BPA-treated group to 46% of the controls. The qualitative histopathology assessment indicated that Leydig cells were not clustered in the BPA treated testes as they were in the controls and that the "typical organization of Sertoli cells was lost by reduction in their number and size resulting in a disorganized morphology of the germinal epithelium."

Strengths of the study include the use of an adequate group size and apparent attention to litter effects in the analysis, although the number of sperm-positive dams that delivered litters and the use of litter as the experimental unit are not explicitly stated. Limitations include the use of a single dose of BPA, single time point gonadotropin measurement, and use of a testicular stain that does not allow evaluation of stages of the seminiferous epithelium. Based on pharmacokinetic studies in rodents, the oral administration of this dose (2.5 mg/kg bw/day) of BPA to adult animals (dams) would be expected to result in very low exposures of the pups to the presumed active agent, BPA aglycone. The effects reported are internally consistent and for several endpoints (serum testosterone, GnRH release, Leydig cell nuclear area) of quite a large magnitude. The inclusion of additional doses, reporting of testicular weight, and a more thorough evaluation of the consequences of BPA exposure to spermatogenesis would have been useful. **The results of this study are of some utility for hazard identification and are not useful for risk assessment or dose response assessment since only a single dose of BPA was used.**

Effects of endocrine disruptors on imprinted gene expression in the mouse embryo (Kang *et al*., 2011)
The objective of this study was to screen for epigenetic perturbations caused by endocrine active chemicals at imprinted loci. The manuscript investigated the effects of BPA, vinclozolin, and DEHP. This review will focus on BPA. JF1 female mice mated with OG2 male mice were gavaged daily from 8.5 days post coitum (dpc) to 12.5 dpc. The JF1xOG2 cross allowed for allele specific analysis of imprinted genes. Mice were housed in polypropylene (potentially BPA-free) cages, received 5K96 diet,

and charcoal filtered water in glass water bottles. Mice were fed for several generations before the study on the 5K96 diet. Mice were dosed with BPA (0.2 mg/kg/day) in tocopherol-stripped corn oil. Mice were euthanized on 13.5 dpc and fetuses were collected. RNA was isolated from specific fetal tissues for quantitative RT-PCR and multiplex Sequenom SNuPE allelotype assays. Three biological replicates of head, embryo body (minus organs), heart, liver, lung, stomach, intestines, placenta, amnion, and yolk sac were analyzed. The parental expression of 38 imprinted loci was measured and compared with a baseline vehicle only treated control. A paired t-test was used for comparing between control and treated groups.

The compounds tested generally were reported not to have more than a 5% effect on the imprinted genes investigated. BPA dosing of the dam led to a 10% change in the imprinted expression of Slc22a18 in the eviscerated embryo body and for maternal allele-specific expression of Rtl1/Rtl1as transcripts in liver, lung, and intestines. Transcript levels for both Rtl1 and Rtl1as were similarly reduced by BPA and DEHP in head, embryo, liver, lung, stomach, intestines, amnion and yolk sac. The authors propose that these changes may have biological significance, because either deletion or overexpression of the Rtl1 transcripts results in late fetal or early neonatal lethality. The authors conclude that maintenance of strongly biased monoallelic expression of imprinted genes is generally insensitive to disruption by endocrine disruptors, including BPA.

This study provides little utility for hazard identification and no utility for risk assessment. No toxicological relationships to apparent changes in imprinting were provided. The authors have not considered that the treated dam is the biological unit and this raises some question regarding statistics. Only a single dose of BPA is studied, preventing determination of a dose-response relationship. The relevance of the potential imprinted gene changes (significant or not) to any toxicity is not considered.

Effects of in utero exposure to Bisphenol A or diethylstilbestrol on the adult male reproductive system (Larocca *et al*., 2011)

This study investigated the effects of in utero exposure to BPA and DES on the reproductive system of sexually mature male mice. Pregnant C57/B16 mice (N = 11 – 14) were dosed by gavage with sesame oil, 50 µg/kg BPA (>99%), 1000 µg/kg BPA, or 2 µg/kg diethylstilbestrol (DES, 97.5%) from gestational days (GD) 10 to 16. Pups (1/litter) were weaned at postnatal day (PND) 21. Selected males were sacrificed on PND 56. All animals were housed in polycarbonate cages, fed with Purina 5010 (a soy-based chow) and water in a central pipe system ad libitum. The parameters examined include viability, litter size at birth, weaning and PND 56 body weight, testis weight, spermatid head counts, anogenital distance (AGD), testis histopathology including measurement of seminiferous tubule diameter, serum testosterone (T) levels, germ cell apoptosis by the terminal deoxynucleotidyl transferase mediated dUTP nick and labeling (TUNEL) method, and mRNA levels of genes associated with sexual differentiation and maturation of the testis by quantitative RT-PCR including WT1, GATA4, 3βHSD, Inhibin B, Cyp11a1, FasL, Fas, ID2, StAR, HPRT, and Cypl7al. One-way ANOVA with Tukey HSD and student t-test were used for statistical analysis.

No significant changes, statistically or biologically, were observed in the BPA-exposed groups (n = 4-10) in any parameter examined in the study. However, some statistically significant changes were observed in DES-treated animals (n = 4-6), which include decreased litter size at weaning, increased AGD at PND 56, and decreased mRNA levels of GATA4 and ID2 in PND 56 testis. While the mean serum

testosterone level was approximately 50% of control in the DES group, the difference was not significantly different.

The authors have demonstrated that in utero exposure of C57/Bl6 mice to BPA from GD 10 to 16 at 50 or 1000 µg/kg did not induce statistically significant changes in the parameters examined in this study. DES at 2 µg/kg did affect some endpoints. Limitations of this study include small sample sizes for most measured end-points (n = 4-10), lack of control for potential environmental (PC cages) and dietary estrogen exposure (soy-based chow), one time point measurements (hormone, mRNA, etc), uncertainty of the biological and functional interpretation of the measurements/changes at the molecular levels, i.e. relationship of change in mRNA levels to expected functional changes in the reproductive system. **The data in this study are in themselves not useful for either HI or RA.**

Oral exposure to low-dose bisphenol A aggravates testosterone-induced benign hyperplasia prostate in rats (Wu *et al*., 2011)

The objective of this study was to evaluate if BPA had an effect on prostatic changes induced by testosterone (T) supplementation in castrated male rats comparable to a reference estrogen, 17b-estradiol (E2). Male castrated Sprague-Dawley rats of 9 weeks were housed with sawdust bedding in plastic cages; water and pellet diet were ad libitum. Animals were randomized to 6 groups (n=10) based on weight and, except for the negative control group, received T at 1 mg/kg/day (s.c. in olive oil). T-treated animals received nothing (model control), 10, 30, or 90 µg/kg/day of BPA (p.o., in carboxymethylcellulose), or 50 µg/kg/day of E2 (s.c., in olive oil), for 4 weeks. After 4 weeks, the animals were weighed and then sacrificed under anesthesia for gross and histopathological evaluation of the prostate. The ventral (VP) and dorsolateral (DLP) lobes of the prostate were excised and weighed, then divided into 3 parts with 1 part fixed in 10% formalin, processed, and stained with H&E. Hormone measurements (T, Dihydrotestosterone, E2, Prolactin (PRL)) plus PSA were obtained at an unspecified time. Statistical analysis was one-factor ANOVA, with significant results tested by least-squares means test.

There was a reported significant increase in prostate volume and weight relative to body weight in all groups receiving T compared to the negative control, with E2 and 10 µg/kg BPA significantly increased compared to the model control (T only). The VP and DLP relative weights were increased in the E2 and 10 µg/kg BPA groups compared to the model control. The VP and DLP epithelium were increased and glandular cavities were enlarged in all groups compared to negative control and the E2 and all BPA groups had a significant increase in prostatic epithelium compared to the model control. There was a dose-dependent decrease in acinar luminal area for BPA that was significant at 90 µg/kg. Height of the VP and DLP epithelium was significantly increased in the E2 and 10 µg/kg BPA groups compared to the model control. Compared to the model control, there was significantly decreased T at 90 µg/kg BPA and increased PSA with E2 and 30 µg/kg BPA.

The authors identified a series of hormonally dependent changes in body and prostate weight, serum parameters and prostate histological endpoints that E2 and BPA also affected. This model is more correctly stated as a model of testosterone-induced effects on the prostate. The BPA dose-response showed an inverse relationship that could be interpreted as estrogenic at the low dose, and then apparently anti-hormonal compared to T+E2 as dose increased. E2 demonstrated a significant effect on

all endpoints compared to T alone without affecting PRL levels. This contrasts with the literature that suggests the action of E2 on the rat prostate is mediated through PRL. The authors stated they measured PSA, but rodents lack a homologous gene product.

This study has limited utility for hazard identification and no utility for risk assessment. Castration/supplementation methods are useful for mechanism of action information, but this study does not have any notable new findings. The castrated/supplemented rodent is not an appropriate model for evaluating human BPH. Systemic levels of BPA and potential sources of contamination in caging, water and food were not evaluated. The amounts of phytoestrogens in the food were not reported.

Subcutaneous exposure

Bisphenol-A exposure alters endometrial progesterone receptor expression in the nonhuman primate. (Aldad *et al*., 2011)

This study investigated the effect of BPA on 17b-estradiol benzoate (EB)-induced endometrial progesterone receptor (PR) expression in oophorectomized female monkeys and human Ishikawa cells. Oophorectomized (OVX) female African green monkeys of an unstated but reproductive age were used. Start of treatment following OVX was unclear, but appears to be immediately while still under anesthesia. Animals were divided into 4 groups (n=3). The vehicle control was cholesterol administered via Alzet minipump and a 4-cm silastic implant containing cholesterol. The estrogen group was given EB (crystalline, no dose provided) administered in a silastic implant plus a vehicle control minipump. The BPA group received a control implant and BPA at 50 µg/kg/day in cholesterol via minipump. The EB+BPA group received BPA via minipump plus the EB-containing implant. Both implants and minipumps were implanted below the skin of the back. Animals were treated for 28 days then euthanized under anesthesia that concluded with perfusion of 4% paraformaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). The uterus was dissected and post fixed in the same fixative, but lacking glutaraldehyde. Immunohistochemistry and analysis of endometrial PR was performed. Human Ishikawa endometrial adenocarcinoma cells were treated with vehicle, 17b-estradiol (E2) at 1 µM, BPA at 1 µM, or E2+BPA for 24 h, then RNA was extracted and subject to real time PCR. The vehicles were ethanol for E2 and methanol for BPA. Source and purity of BPA was not identified. Statistical analysis was not identified, but was performed.

PR Expression in the endometrial glands and stroma reportedly significantly increased 49-fold and 11-fold, respectively compared to vehicle control. BPA had no significant effect alone, but repressed 38% and 50% of glandular and stromal expression, respectively. In Ishikawa cells, total PR and PR-B expression was increased 5.2-fold and 4.1-fold, respectively, by E2 compared to control. Unlike the *in vivo* experiment, BPA alone increased expression by total PR and PR-B by 1.7-fold and 1.9 fold, respectively compared to control. BPA repressed 40% and 30% of total E2-induced PR and PR-B expression.

The authors demonstrated that BPA could inhibit estrogen-induced PR gene expression in the endometrium of the African green monkey. This is the first report of suppression of the PR gene's expression in an adult primate, albeit surgically altered. This would be consistent with the known estrogen-dependent regulation of PR and suppression by many functional anti-estrogens. Other than this

basic observation, little can be concluded since the methods section was inadequate for an evaluation of the system's validity. Oophorectomy/supplementation methods are useful for mechanism of action information, but only a single dose of BPA was used, EB treatment used silastic implants and BPA was administered through an Alzet minipump. The dose of EB, BPA source, and animal husbandry information were not provided. Systemic levels of BPA and potential sources of contamination in caging, water and food were not evaluated. **This study has limited utility for hazard identification and no utility for risk assessment.**

Hypermethylation of estrogen receptor promoter region in adult testis of rats exposed neonatally to bisphenol A (Doshi *et al*., 2011)

This study examined the effect of neonatal exposure to BPA on methylation of the ERα and ERβ promoter regions in adult testis. The authors hypothesize that exposure might alter ERα and ERβ through an epigenetic mechanism. This hypothesis was based on a previous report from this lab of neonatal exposure of mice to BPA impairing fertility in adulthood as well as altered ERα and ERβ gene expression in the adult testis. An in-house bred strain of Holzman rats was dosed subcutaneously on postnatal days 1 through 5 with 2.4 µg BPA/pup/day (n=8). Exact dosing (mg/kg bw) was not determined. BPA was dissolved in ethanol and diluted in sesame oil (phytoestrogen content not determined). Adult rats were sacrificed on PND 125. Testicular DNA was extracted and subject to bisulfite genomic sequencing PCR. Expression of DNA methyltransferases was examined by quantitative RT-PCR and Western Blotting.

Total testicular RNA transcript levels of ERα and ERβ were reportedly reduced on PND 125. Bisulfite sequencing revealed hypermethylation of ERα and ERβ promoters to varying extents. An increase in Dnmt3a and Dnmt3b RNA and protein was also observed. The authors concluded that methylation mediated epigenetic changes in the testis may be one possible mechanism by which BPA affects fertility and spermatogenesis.

This study has limited utility for hazard identification and no utility for risk assessment. An in-house bred strain of rats was used, preventing replication in another lab. Exact dosing of BPA was not determined on a mg/kg bw basis. A dose response was not demonstrated. While protein levels were not determined, the study did show a decrease in whole testicular ERα and ERβ mRNA on PND 125 after dosing from PND 1 – 5 and provided a potential mode of action (induction of Dnmt3a and Dnmt3b). Unfortunately, the exact cell types were not examined, so it cannot be determined if ER mRNA levels were reduced in the same cell types that expressed increased Dnmt.

Prenatal exposure to bisphenol A promotes angiogenesis and alters steroid-mediated responses in the mammary glands of cycling rats (Durando *et al*., 2011)

This study investigated the effects of prenatal exposure to BPA on angiogenesis and steroid hormone-mediated responses in the mammary glands of virgin cycling rats. Sexually matured Wistar-derived females were mated with fertile males. On gestational day (GD) 8, miniature osmotic pumps, delivering 50% DMSO, 25 µg or 250 µg BPA (99%)/kg bw/day at a rate of 0.25 µl/h, were implanted for 14 days. Animals were kept on a phytoestrogen undefined chow and housed in stainless steel cages with sterile pine wood shaving and ad libitum tap water in glass bottles. Retained female offspring (one animal per litter per time point per treatment group, N = 8-10) were sacrificed and autopsied on postnatal day (PND)

50 or 110. All samples were collected at diestrus I phase confirmed by vaginal smear. The serum levels of steroid hormones, expression of steroid receptors and their co-regulators in the mammary gland, and angiogenesis were evaluated. Serum levels of estradiol (E2) and progesterone (P4) were measured by RIA. PR, ERα, ERβ, SMRT (co-repressor), SRC-3 (co-activator), VEGF, and vWF in mammary gland were evaluated by immunohistochemistry. Angiogenesis and protein expression were measured and quantified by morphometry and integrated optical density (IOD) methods, respectively. Kruskal-Wallis analysis and Dunn's posttest were used for statistical analysis.

Reported significant changes include: the increased percentage of hyperplastic ducts in 25 µg-BPA groups at PND 110, but not in 250 µg-BPA group at PND 110 nor both BPA groups at PND 50; increased ERα expression in 250 µg-BPA groups at PND 50 and 110; decreased SRC-3 expression in 250 µg-BPA group at PND 110; decreased SMRT expression in 250 µg-BPA group at PND 110; decreased serum P4 levels in both BPA groups at PND 50, but not at PND 110. Increased relative vascular area in mammary gland was observed at PND 50 and 110, in concomitant with elevated VEGF expression in 250 µg-BPA group at PND 50, but not at PND 110. The authors concluded that prenatal BPA exposure induced modifications in the mammary gland endocrine environment and on angiogenesis that are dose- and time-specific.

Based on the findings in their early studies, the authors in this study attempt to demonstrate the mechanism involved in BPA-altered histoarchitecture and carcinogenic susceptibility in rodent mammary gland. The study did observe some changes at hormonal, cellular, and molecular levels in mammary gland, but not conclusively. The authors tried to control the environmental BPA exposure in many aspects, but a phytoestrogen undefined chow was used. That may confound observed low dose effects. That only two BPA doses used in the study without a positive control severely limits the result interpretation. No mammary gland was examined from male offspring. The subcutaneous implant administration route is a concern for the data interpretation and extrapolation to oral exposures. **This study has only limited utility for HI and no utility for RA.**

Neonatal exposure to bisphenol A or diethylstilbesterol alters the ovarian follicular dynamics in the lamb (Rivera *et al*., 2011)

This study evaluated the effect of neonatal exposure to BPA on follicle development in neonatal female lambs. Corriedale ewes (2-4 years old) were mated to Hampshire Down rams. Female lambs born during August and September were randomly assigned to one of three treatment groups. Female lambs were dosed by sc injection on Postnatal Days 1 to 14 with DES at 5 µg/kg/day (n=6) or BPA at 50 µg/kg/day (n=6). The controls were dosed with the vehicle, corn oil (n=10). The experiments were conducted in an experimental farm belonging to a university in Argentina. The ewes grazed pasture with low rate of clover but the phytoestrogen content in the pasture was not analyzed. All control and treated lambs were sacrificed on PND 30. Female lambs were weighed and ovaries were removed, weighed, and further processed. Lambs were observed for clinical signs of acute or chronic toxicity. Ovaries were serially sectioned (5 µm thick) and one slide out of every 40 sections was stained with picosirius-hematoxylin. Follicular dynamics were evaluated by morphometry for the percentage of primordial and/or recruited follicles and the incidence of multioocyte follicles (MOFs). Ovarian sections were used to evaluate protein expression of ERα, ERβ, AR, Ki67, and p27 by immunohistochemistry. Hormone assays were done by RIA for serum levels of E2 or T. The results were analyzed statistically by the

Kruskall-Wallis and Dunn post hoc test.

Reported findings in the BPA and DES groups included significantly decreased ovarian weights, decreased primordial follicle reserve, and increased growing follicles (transitional and primary follicles). The increased incidence of MOFs which were mostly in the primordial stage was statistically significant only in the BPA group and non-statistically significant in the DES group. There was a higher proliferation rate in the large preantral cells (granulosa cells) and small antral follicles (granulosa and theca cells) as determined by immunoreactivity for Ki67. Both BPA and DES groups showed a significantly higher expression of p27 in granulosa cells of small antral follicles. Both groups showed an increased number of small antral atretic follicles. There were no effects in the BPA or DES groups on the expression of ERα, ERβ, and AR or the serum levels of E2 or T.

This study is related to a previously reviewed study by Rodriguez *et al*, 2010 which was conducted in female rats. **Like the study by Rodriquez, this study is basically a mechanistic discovery study and is not useful for hazard identification or risk assessment.** The endpoints measured such as receptor protein expression, Ki67 and p27 biomarkers, and morphological counting of MOFs are at the cellular and molecular levels. These events are not clearly associated with adverse effects. The biological significance of these endpoints on follicular development and fertility in adult sheep needs to be determined. The study included only one dose of BPA and thus there was no dose-response measurement. It is difficult to translate dose by sc to the oral route.

Neonatal exposure to Bisphenol A alters rat uterine implantation-associated gene expression and reduces the number of implantation sites (Varayoud *et al*., 2011)

The objective of this study was to evaluate if early postnatal exposure to BPA has an effect on female reproductive performance, uterine homeobox A10 (Hoxa 10) and Hoxa10-target gene expression, or ovarian steroid levels and uterine estrogen receptor alpha and progesterone receptor expression. Inbred Wistar-derived rats were singly housed in stainless steel cages with wood bedding and tap water in glass bottles, and with a diet not evaluated for phytoestrogen. Newborn rats were administered test chemicals by SC with vehicle (corn oil that was charcoal stripped). Pups of singly housed pregnant rats were cross-fostered. Female offspring were randomly assigned to one of 5 groups (n=10-17): corn oil, 0.2 μg/kg or 20 μg/kg DES, or 0.05 mg/kg or 20 mg/kg BPA. Chemicals were injected sc on pnd1, 3, 5, and 7 at the nape of the neck. On pnd21 animals were weaned, housed 4 per cage, and held without further treatment. On pnd80, the exposed rats were assigned to the different studies: a) female pregnancy rates and number of corpora lutea, implantation and resorption sites on pnd18 of pregnancy with 10-13 females per dose group; b) assessment of gene expression; c) ovarian steroid levels and uterine estrogen receptor alpha and progesterone receptor expression during preimplantation period (pnd5).

The number of corpora lutea was reported as similar across groups. Implantation sites were significantly reduced for the DES0.2 and BPA20 group. The DES20 group was infertile. No difference in serum concentration of ovarian steroid hormone levels for any of the dosed groups was reported. Uterine estrogen receptor alpha and progesterone receptor expression was significantly lower for the DES0.2 and both BPA groups. Expression of downstream genes EMX-2 and ITGB3 were changed in the direction expected given down-regulated Hoxa10 in the DES 0.2 and high BPA groups only.

This study has some utility for hazard ID (a long-lasting reduction of the number of implantation sites but only at a neonatal dose of 20 mg/kg BPA). A possible mode of action is suggested (through Hox signalling). The uterine environment of the rats exposed neonatally to 20 mg/kg BPA is not normal.

Preimplantation exposure to bisphenol A (BPA) affects embryo transport preimplantation embryo development, and uterine receptivity in mice. (Xiao *et al*., 2011)

The objective of the study was to investigate the effects of preimplantation exposure to BPA on preimplantation embryo development, embryo transport, and uterine receptivity in C57BL6 mice using subcutaneous (sc) exposure of BPA and four study designs. Sesame oil was the vehicle. Mice were housed in polypropylene cages and given free access to rodent diet 5053 by Purina Mills and water. The study data were analyzed by one-way ANOVA with Dunnett's t test or X2 with Fischer's exact test.

Study Design 1. Detection of implantation sites: Daily doses of 0 (n=14), 0.025 (n=7), 0.5 (n=7), 10 (n=10), 40 (n=9), and 100 (n=9) mg BPA/kg bw/day or 0.01 (n=5) mg/kg/day of estradiol (E2) were given sc from GD 0.5 to 3.5. GD 0.5 is the day a vaginal plug was observed after mating virginal females (2-3 months old). At sacrifice on GD day 4.5 or 5.5 and females were given an i.v. injection of Evans blue dye to visualize the number and position of implantation sites. Uterine tissues were frozen for immunohistochemistry to determine the presence and location of the progesterone receptor (PR). There were no implantation sites in the females in the highest BPA dose or the E2 group. There were no effects on number of implantation sites in the 0.025, 0.5, or 10 mg BPA/kg/day dose groups but in the 40 mg BPA/kg group there was a nonstatistically significant decrease. Loss of PR in the uterine epithelium is associated with the establishment of uterine receptivity in mammals. Sustained PR expression in the luminal epithelium on GD 4.5 uteri exposed to 40 and 100 mg BPA/kg bw/day confirmed delayed implantation according to the authors.

Study Design 2. Embryo transplant and development: Daily doses of 0 (n=8) and 100 mg BPA/kg bw/day (n=5) were given sc from GD 0.5 to 3.5. After sacrifice on gestation day 3.5, uteri and oviducts were flushed with PBS to detect the presence of embryos and the stages of embryo development. Four BPA treated females had embryos in the oviduct vs. all embryos in the control were detected in the uterus. All control group embryos were blastocysts as compared to only 27% of the embryos in the BPA group which were at an earlier stage in development.

Study Design 3. Uterine receptivity: Pseudopregnant females were given sc injections of 0 (n=5) or 100 mg/kg bw/day (n=4) of BPA from GD 0.5 -3.5 at which time blastocysts were harvested from superovulated females and transferred to the GD 3.5 uteri of the pseudopregnant females. On GD 4.5 four females in the control group had detectable implantation sites vs. none of the females in the BPA dose group. The article concluded defective uterine receptivity.

Study Design 4. Study to determine consequences of delayed implantation: Daily doses of 0 or 40 mg/kg bw/day were administered sc on GD 0.5-3.5 (n=4-7). The authors reported statistically significant increase in gestation length, reduced litter size, and reduced postnatal survival rate. Comparable gender ratios were observed.

The authors concluded that high doses of BPA (40 or 100 mg/kg bw/day) by the sc route have adverse

effects on processes critical for embryo implantation. **This study is a mechanistic study which is not useful for hazard identification and for risk assessment.** Study limitations for risk assessment: no doses less than 40 mg/kg or 100 mg/kg were used (except study design 1), low and uneven n values in the study groups, rodent chow which contains phytoestrogens, non oral route (sc), study designs with only one or two treatment groups, no information on type of water bottle, possible effects of sesame oil vehicle, lack of adequate controls, and publication did not report analytical work on drinking water for contaminants or dosing solutions for added BPA level.

Carcinogenesis Studies

Summary

Several papers were reviewed that attempted to address the potential carcinogenic properties of BPA with regard to mammary cancer in various mouse models. Of the studies reviewed, two administered BPA via drinking water (Ayyanan *et al*., 2011 and Jenkins *et al*., 2011), and one used gavage (Lozada and Keri, 2011).

The Ayyanan *et al*. study used C57Bl6 mice and looked at mammary gland progesterone response and cell number after prenatal exposure to BPA, but did not have a neoplasm endpoint. Furthermore, the paper was difficult to follow and had many internal inconsistencies. The Lozada and Keri study used FVB/N mice, which have persistent mammary hyperplasia, and which were exposed prenatally and initiated with DMBA postnatally. The latency for mammary squamous cell carcinoma was reduced, although no morphologic effects on mammary gland development were seen. The Jenkins *et al*., study used adult MMTV-erbB2/neu transgenic mice, a mouse with an increased susceptibility to mammary neoplasms because of the erbB2 being linked to a strong viral promoter. All measures of tumor development (multiplicity, time to tumor, tumor volume, lung metastases) were reported to be increased in a nonmonotonic way (effects at two lower doses, but not at the highest dose). Likewise, the low BPA dose, but not the high dose, was reported to increase phosphorylation of erbB2 and erbB3 and increase expression and/or phosphorylation of downstream targets. Nonmonotonic dose effects have been reported in other studies with BPA. The Jenkins *et al*, 2011 study could conceivably be used for a hazard ID. However, all studies had design limitations that severely limit their ability to influence the risk assessment of BPA.

Individual Study Reviews

Perinatal Exposure to Bisphenol A Increases Adult Mammary Gland Progesterone Response and Cell Number (Ayyanan *et al*., 2011)

The objective of this study was to determine whether perinatal exposure to a range of low doses of BPA is sufficient to alter mammary gland hormone response later in life, with a possible impact on breast cancer risk. BPA was added to the drinking water of C57B1/6 breeding pairs of mice at 2.5µg/liter to 5 mg/liter (4 dams/dose). Based upon average water intake and average weight, BPA intake was calculated at 0.6 µg to 1.2 mg/kg bw/d. DES was used as a positive control at 0.12 or 1.2 µg/kg bw/d. Female offspring were exposed in utero and postnatally through milk. One or two females per litter were killed at 30 $\pm$ d of age. The rest were kept for later analysis. Mammary cell preparations were performed by the Ministry of Health (MOH) in Kenya, and cells were counted using Casy TT Cell Counter

Analyzer. The mRNA expression levels of two target genes, the progesterone receptor (PR) and the amphiregulin and the mRNA specifying secretory leukoprotease inhibitor (SLPI) were assessed. The number of terminal end bubs (TEBs) was counted in treated animals and compared to a cohort of mice. Cell counts were looked at in adult (3 month) female treated mice and compared to control mice.

Exposure to low doses of BPA had no significant effect on litter size, sex ratio, or body weight at weaning. Parental daily uptake of 0.12 ug DES/kg-bw resulted in increased PR and decreased SLPI mRNA but did not significantly affect amphiregulin or ERα mRNA expression. Daily uptake of 3, 120, and 1200 ug/kg-bw BPA resulted in dose-dependent effects on PR and SLPI mRNA expression that was statistically comparable to DES. Perinatal exposure to BPA resulted in statistically significant increase in adjusted TEB numbers at a dose of 3 mg/kg-bw. In glands from adult (3 month) BPA-exposed female mice, cell numbers were on average 50% higher than in the controls. DES mice had a 70% increase in cell counts. Immunohistochemistry for PR revealed a pronounced increase in PR-positive cells within the lumen. Wnt-4 mRNA levels was increased in BPA exposed animals.

This study suffered from a number of limitations and other inconsistencies such as the following: article not well written or well organized; methods difficult to interpret; number of experimental groups not mentioned; dose groups get pooled and picked apart; plots are inconsistent; no information on concordance of mouse with human hormonal regulation of cancer induction; cohort of untreated mice used in the TEBs comparison not described; no information on control group for analysis of mammary gland cell number in adult females; information on DES treated mice for TEBs count was not given; and methods for immunochemistry and Wnt-4 analysis not stated. **These and other inconsistencies make it difficult to draw any reliable conclusions from this research and preclude use of this study for HI or RA.**

Chronic Oral Exposure to Bisphenol A Results in a Non-Monotonic Dose Response in Mammary Carcinogenesis and Metastasis in MMTV-erbB2 Mice (Jenkins *et al.*, 2011)

The purpose of this study was to determine if BPA administered orally to adults enhanced development of mammary tumors in virgin MMTV-erbB2/neu transgenic mice. The model was selected based on rat studies that implicated the erbB family of receptor tyrosine kinases in BPA-induced alterations in cell proliferation and apoptosis and acceleration of mammary carcinogenesis. The underlying hypothesis is that women with breast cancers over expressing HER2/erbB2 could be susceptible to exposure to BPA as adults. Four doses (2.5, 25, 250, and 2,500 µg BPA/L) were administered in the drinking water (with 0.05% ethanol, also used as control) from postnatal day 56 to the end of the study (PND 112 or 252). Estimated intakes were 0.5, 5, 50, and 500 µg BPA/kg body weight/day. Group size varied from 36 to 94. At PND 112, 5- 17 mice per dose group were sacrificed for evaluation of cell proliferation and apoptosis and 6– 8 mice from control, 25, and 2,500 µg BPA/L were used for evaluation of molecular endpoints (protein levels by Western blots). Remaining animals were maintained for evaluation of tumor development. Only invasive mammary adenocarcinomas were considered in the data evaluation.

Body weight (decrease) and uterine weight (increase, absolute and relative to body weight) were reported to be affected only by the 250 dose. All measures of tumor development (multiplicity, time to tumor, tumor volume, lung metastases) were reported to be increased at the 25 dose group, and all but tumor volume increased by the lowest dose. The higher doses were reported to have no statistically significant

effects. Cell proliferation index was significantly increased relative to control in the 25, 250, and 2,500 doses while the apoptotic index was significantly increased in the 2,500 dose group. The ratio of proliferation to apoptosis differed significantly from control only at 25. Molecular analyses were conducted in young mice prior to the appearance of preneoplastic or neoplastic lesions and consisted of assessment of protein and phosphorylated proteins in signaling pathways associated with erbB. Only the 25 and 2,500 dose groups were evaluated. The expression of the transgene, erbB2, was not altered, but the low BPA dose, but not the high dose, was reported to increase phosphorylation of erbB2 and erbB3 and increase expression and/or phosphorylation of downstream targets.

The findings of this study are of interest, although the mechanistic basis of the reported nonmonotonic dose response is unclear given the low levels of the presumed active agent, BPA aglycone, which would be expected after oral administration of BPA to adult animals. Strengths include an adequate number of animals (although see below), use of multiple BPA doses, blinded reading of the samples, attention to background estrogen/BPA contamination, and evaluation of multiple endpoints. Limitations include lack of certification of the dosing solutions, no measurement of water consumption (although previous average values were used), no discussion of allocation of animals to dose groups or tissues to the various evaluations (e.g. the reasons for the unequal sample numbers in the various groups, lack of detail as to the number of samples in each group and how samples were selected for proliferation/apoptosis and protein assessments), and no evaluation of the possible influence of stage of estrous cycle on the endpoints evaluated in PND 112 animals. In some cases, results that are not statistically significant are discussed as if they are significant when the results are in the direction that fit the hypothesis, but not when they do not. The authors argue that the trend for the ratio of proliferation to apoptosis agrees with the tumor results. However, this is not clear given the similar magnitude of effects for tumor endpoints in the lowest two doses but the lesser, and non-significant, effect of the low BPA dose on cell proliferation and apoptosis. With regard to the protein measurements, the use of the full dose range and inclusion of internal control proteins would have improved the study. Still, the data do indicate differential effects of the low and high doses of BPA, with low doses accelerating tumor development in this model.

While the use of mice with erbB2 linked to a strong viral promoter may not be generally directly relevant to human carcinogenesis or to risk assessment because of the exaggerated sensitivity of these mice, the model used seems appropriate to address the authors' hypothesis. A plausible and testable hypothesis regarding the mechanistic basis for the observed dose response needs to be formulated. **The results of this study with regard to acceleration of mammary tumorigenesis in the MMTV-erbB2 mouse may be useful for consideration in hazard identification and provide information relevant to dose response assessment.**

Bisphenol A increases mammary cancer risk in two distinct mouse models of breast cancer (Lozada Weber *et al.*, 2011)

The purpose of this study was to determine if in utero exposure to BPA would cause effects on mammary development, ductal invasion or other morphologic effects, predispose the adult mouse to mammary carcinogenesis after carcinogenic insult, or if BPA promotes established tumor growth. FVB/N mice were gavaged with vehicle (100 µl mineral oil, 25 µg/kg BPA, or 250 µg/kg; materials and methods says 250 µg/kg but some results only report 25 µg/kg bisphenol A). BPA was dissolved in 50% PBS/DMSO

and the appropriate amount was added to mineral oil. Female offspring were randomly assigned to experimental groups, containing a minimum of 5 mice from at least 3 litters. Offspring were observed for vaginal opening. Mammary glands were collected at 3, 5, or 8 weeks of age. Ductal length (from midpoint of lymph node to terminal bud) was measured at 3 and 5 weeks. Mice exposed to 25 μg/kg had a slightly earlier vaginal opening (d21-22 vs d 22-24 for controls). Results for 250 μg/kg were not reported. No morphologic effects were seen for mammary gland development at various time points. Using the same fetal exposure, groups of 10 female offspring from 3 litters were exposed to a dose of DMBA (1 mg/mouse) at age of 5 wk and another dose of DMBA at age of 6 wk. The latency of mammary tumors was assessed by palpation. The latency for mammary squamous cell carcinoma was 111 weeks for controls; 69 weeks for the low dose, and 51 weeks for the high dose.

Some limitations of the study included: consideration of the number of mice as independent units in the statistical analysis rather than considering the dam as the independent unit; no dose-response data for vaginal opening were given; multiparous FVB/N mice have persistent mammary hyperplasia; the relevance of the DMBA carcinogenesis model to humans is not apparent. **This study has minimal utility for hazard ID, much less for risk assessment.**

Other Endpoint Studies

Summary

The group reviewed two papers that primarily addressed the effects of BPA on glucose and lipid metabolism. The primary objective of the Marmugi *et al*. study was to examine changes in gene expression related to lipid synthesis in mice exposed to BPA. Typically, changes in gene expression alone are insufficient to serve as the basis for regulatory decision making; however, this group also assessed other endpoints as well, including the accumulation and composition of lipids in the liver and changes in plasma insulin in BPA-exposed mice. These investigators reported that plasma insulin levels were significantly increased with a maximal effect at the lowest dose (5 μg BPA/kg/day in feed); however, there was no corresponding change in plasma glucose levels, which raises questions about the physiological significance of the changes in insulin. BPA was also reported in this study to alter lipid metabolism, with a significant increase in plasma triglyceride levels (500 μg BPA/kg/day), accumulation of lipids in the liver, and changes in expression of genes related to hepatic lipid metabolism; but it is not clear whether these physiological changes can be considered to be adverse. In the paper by Wei *et al*. (2011), the primary objective was to determine "whether perinatal BPA exposure would contribute to metabolic syndrome in rat offspring and whether metabolic disrupt *(sic)* effects of BPA was exacerbated under high-fat feeding condition." They reported that adult offspring on normal diet had increased body weight, elevated serum insulin, and impaired glucose tolerance, but only following perinatal exposure to BPA at the lowest dose (50 μg/kg/day). Offspring on a high-fat diet reportedly developed obesity, dyslipidemia, hyperleptindemia, hyperglycemia, hyperinsulinemia, and glucose intolerance but again, only following perinatal exposure at the lowest dose. No adverse effects were observed in adult rats fed either diet following perinatal BPA exposure at 250 or 1250 μg/kg/day.

The results of these studies may be useful to suggest that exposure to BPA represents a potential hazard, assuming the results in rodents are reproducible and applicable to humans. It is curious that changes in

plasma insulin are not associated with compensatory changes in plasma glucose, which limits the physiological significance of these findings. **Consequently, the results of these studies can be used for hazard identification, but suggest minimal use for risk assessment.**

Individual Study Reviews

Low doses of bisphenol A induce gene expression related to lipid synthesis and trigger triglyceride accumulation in adult mouse liver (Marmugi *et al*., 2011)

The objective of this study was to examine changes in gene expression related to lipid synthesis in mice exposed to BPA. This study also assessed other endpoints as well, including hepatic lipid content and fatty acid composition and plasma levels of triglycerides, glucose, total cholesterol, LDL- and HDL cholesterol and insulin. Six-week-old male CD1 mice were exposed to different doses of BPA (0, 0.05, 0.5, 5, and 50ppm in the feed; dose estimated by authors as 0, 5, 50, 500 and 5000 μg/kg/day) through food for 28 days (n=6/dose). Reported results included no effect on body weight gain and relative liver weight; significant increase in perigonadic white adipose tissue weight (50 μg/kg/day); plasma insulin levels were significantly increased (5, 50 and 500 μg BPA/kg/day) with a maximal effect at the lowest dose; no significant effect on plasma glucose and total, LDL- or HDL-cholesterol levels; significant increase in plasma triglyceride levels (500 μg BPA/kg/day); accumulation of lipids in the liver; significant increase in liver triglycerides (50 and 500 μg BPA/kg/day); and changes in expression of genes related to hepatic lipid metabolism.

It is interesting to note that the dose-response relationship for plasma insulin response and for many of the findings reported in the paper are monotonic, but with the highest effects seen at the lowest BPA dose. It is also curious that changes in plasma insulin are not associated with compensatory changes in plasma glucose which limits the physiological significance of these findings.

The reported changes in gene expression following exposure of mice to BPA have limited utility as supporting data, but the physiological changes observed in BPA-exposed animals (significant increases in perigonadic white adipose tissue weight, plasma insulin and triglycerides, and lipids in the liver) are relevant for their utility for hazard assessment. It is also important to consider whether these effects are "adverse" from a hazard identification perspective and the implications of the non-monotonic dose-response relationships. **This study may be used for hazard identification.**

Perinatal exposure to bisphenol A at reference dose predisposes offspring to metabolic syndrome in adult rats on a high-fat diet (Wei *et al*., 2011)

The objective of this study was to determine "whether perinatal BPA exposure would contribute to metabolic syndrome in rat offspring and whether metabolic disrupt (sic) effects of BPA was exacerbated under high-fat feeding condition." Pregnant rats were assigned to four groups and were subjected to the following treatments from GD 0 to the weaning at postnatal day (PND) 21 by oral gavage: 1) corn oil; 2) BPA 50 ug/kg/day, 3) BPA 250 ug/kg/day and 4) BPA 1250 ug/kg/day. At delivery, offspring were culled to 10 per lactating dam (five male and five female when possible) and kept with their mothers until PND21. Offspring were weighed on PND 1, 5, 10, 15, and 21. On PND 21, litters with less than five male pups or five female pups were excluded from the following study. The remaining pups were sexed and weaned. Thirty-two male and 32 female pups (four pups per litter) from each group (eight

litters per group) were chosen randomly and received either a normal diet or a high-fat diet (two pups per litter per group). Measurements included blood glucose, serum triglycerides, total cholesterol, high-density lipoprotein (HDL), and low-density lipoprotein (LDL), insulin and leptin. Glucose tolerance tests and insulin tolerance tests were administered. Body weight, pancreas morphology and changes in gene expression were also measured. The investigators state that they attempted to control for litter effects by including offspring from different litters in each experimental condition (except for the measurement of body weight, fasting blood glucose and serum insulin in which all animals are represented) and by including differences in litter size as a cofactor in all statistical analyses. Additional lack of detail prevents clear identification of sample size tested or analyzed per experimental condition.

Reported results in adult offspring on normal diet following perinatal exposure included: increased body weight, elevated serum insulin, and impaired glucose tolerance in adult offspring (BPA - 50 ug/kg/day). On a high-fat diet, reported results included: dyslipidemia, hyperleptindemia, hyperglycemia, hyperinsulinemia, and glucose intolerance (BPA - 50 ug/kg/day). No adverse effects were reported in adult rats fed either diet following perinatal BPA exposure at 250 or 1250 ug/kg/day. One of the concerns with this study was the use of corn oil as vehicle. This is based on uncertainties associated with the potential effects of the corn oil on glucose homeostasis and reproduction (as noted by Sato *et al*., 2000). Limitations prevent clear interpretation of this study; however, the results of this study may be useful to suggest that exposure to BPA represents a potential hazard, assuming the results in rodents are reproducible and applicable to humans. **Consequently, the results of this study may be considered for hazard identification.**

Epidemiology Studies

Summary

Twelve published epidemiologic studies utilizing a cross-sectional or cohort designs tested putative associations between BPA exposure (variably defined as serum, urinary, or past occupational) and individual endpoints in small samples of human subjects from around the world. Assessed outcomes spanned the gamut and included hormone levels, diabetes, obesity, vascular disease, developmental endpoints, or gene expression. No two studies measured endpoints in an identical fashion. No associations were found between BPA exposure and the following parameters: 1) embryo quality indicators in US women undergoing *in vitro* fertilization (IVF) procedures, 2) thyroid hormone levels in French boys born with or without cryptorchidism, 3) thyroid hormone levels in a sample of US adolescents; 4) glucose regulation/diabetes in Chinese adults; 5) estrogen/androgen signaling pathway-related gene expression in Italian adult males; 6) thyroid and various reproductive hormone levels in US male partners of subfertile couples; and 7) peak estradiol levels or oocyte counts in US women undergoing *in vitro* fertilization (IVF) procedures.

A few studies reported an association between BPA exposure and measured endpoints, including birth weight or anogenital distance in male offspring, estradiol level normalized to the number of mature-sized follicles in women undergoing IVF, diabetes mellitus, sperm damage, or obesity. However, critical review of these studies indicated significant limitations in study design that made the claims of association questionable or unsupportable. Limitations included absence of control for possible

confounders, lack of reporting of statistical tests utilized, poor or absent reporting of BPA exposure levels, discrepancies in reporting time of measurement of BPA exposure or health outcome, measurement of BPA in biological media at a single point in time only, lack of clinical significance of measured outcome, inability to separate contribution of BPA from association with other measured compounds in a mixture, as well as many other limitations. Our review indicated that no single study was able to make a defining contribution to hazard identification or risk assessment at present, since no study demonstrated the causal relationship between exposure and outcome that is required by the hazard identification step within risk assessment[10].

While we have concluded that the studies evaluated as part of this literature review do not have current utility for hazard identification or risk assessment, information from these studies may have ancillary or supportive utility when viewed in the context of similar findings from previous and future epidemiological studies, or in the context of the results from the animal toxicity studies. One study[11] was considered to have demonstrated a preliminary or suggestive positive association (trend) between paraben + BPA co-exposures and sperm damage in males. However, associations of BPA with serum hormone levels, semen quality parameters, and sperm DNA damage measures were not specifically assessed in the study. The possible association reported in this study was examined more closely in the context of adverse male reproductive effects, if any, reported in the mammalian toxicology database for BPA. The preliminary sperm findings from the NCTR 90-day subchronic rat toxicity study of BPA[12] show no significant effect on any sperm parameters at BPA doses of concern to human health. However, sperm DNA damage was not directly assessed. While a correlation between sperm DNA damage and infertility exists in humans and animals, the understanding of the causal relationships is generally poor and likely multifactoral. Overall from human studies, no consistent relation between sperm DNA damage and fertilization rates or embryo quality during IVF or IVF/ICSI procedures has been demonstrated. Evaluation of sperm DNA damage currently has only a modest predictive value.

Individual Study Reviews

Serum unconjugated bisphenol A concentrations in men may influence embryo quality indicators during *in vitro* fertilization. (Bloom *et al.*, 2011).

This study examined the relationship between BPA serum concentrations in couples and the quality of embryos produced through *in vivo* fertilization (IVF). It used samples and data collected as part of a larger on-going study of IVF outcomes and exposures to trace minerals (Study of Metals and Assisted Reproductive Technologies – SMART). The SMART study included 58 female patients and 37 male partners recruited between September, 2007 and August, 2008 in San Francisco (Bloom *et al*., 2011). The current study used measurements of 186 embryos and serum BPA from 27 couples from this group. BPA was measured in serum samples collected on the day of oocyte and sperm collection using HPLC with Coularray detection, with a lower limit of detection (LOD) of 0.3 ng/mL. BPA concentration

10 National Research Council. 1983. Risk assessment in the federal government. Managing the process. National Academy Press, Washington, DC.

11 Meeker JD, Yang T, Ye X, Calafat AM, Hauser R. 2011. Urinary concentrations of parabens and serum hormone levels, semen quality parameters, and sperm DNA damage. Environ Health Perspect119(2):252-7.

12 NCTR Project No. E02176.01. NTP Project C10034. http://ntp.niehs.nih.gov/?objectid=BC9825E3-123F-7908-7BF465F9E25681B0

medians were 3.3 ng/mL for women (range 0.0-67.4; 14.8% or 4 <LOD) and 0.48 ng/mL for men (range 0.0-22.7; 48.1% or 13 <LOD). Embryo quality indicators, embryo cell number (ECN) and embryo fragmentation score (EFS) were measured on the day of embryo transfer (usually day 2 post-fertilization). Couples produced a median of 6 embryos (range 1-14) with a median ECN per couple of 6.4 (range 3.0-9.3) and median EFS per couple of 2.3 (range 1.2-4.0). BPA levels were log-transformed and logistic regression used to predict the risk for having an embryo score in a higher tertile. Separate models were created for ECN and EFS, including maternal BPA, age, and race (Asian/not) and paternal BPA, age, and race. Day of embryo transfer was included for ECN.

There was no association between maternal BPA level and ECN or EFS, with OR's for both models close to 1.0. Paternal BPA had a significant beneficial effect on EFS. ECN decreased with increasing BPA, however this is difficult to interpret as ECN does not have a linear relationship with embryo quality and an ECN of 4 is considered ideal at day 2 (Racowsky *et al*., 2009 and 2011). The models compared ECN tertiles (6-12 cells versus 1-5 cells and 8-12 cells versus 1-7 cells); thus, an inverse association between BPA and ECN could reflect a negative effect on embryo cleavage rate or improved embryo quality through a decrease in embryos with overly high ECN values. Major limitations in the study included the small size and the limited ability to control for known confounders of ECN and EFS. Further, couples must have produced viable embryos to be included in the study, perhaps already selecting for those without effects due to BPA, and no interactions between BPA levels for male and female partners were considered, although levels for both were included in the model. **This study does not provide new information for hazard identification or risk assessment that should be incorporated into the FDA assessment.**

<u>Cord blood thyroid tests in boys born with and without cryptorchidism: correlations with birth parameters and in utero exposures. (Brucker-Davis *et al.*, 2011)</u>
This study was designed to examine cord blood (CB) thyroid tests and their correlation with CB and milk xenobiotic concentrations in boys born in Nice and Grasse, France. All boys born alive at 34 weeks of gestational age were eligible. The initial study included 95 cryptorchid cases and 190 matched controls (gestational age, birth weight and parental geographical origin where possible) born between April, 2002 and April, 2005 in Nice and Grasse, France. The results reported in the current study were based on 84 cord blood samples and mothers' milk from control male infants without cryptorchidism. For the analysis of BPA, serum volume permitting, cord blood from 53 boys and no milk samples from mothers were used. All cord blood samples were positive for BPA (n=53; median = 0.9 ng/mL; range = 0.2-3.3 ng/mL) measured by radioimmunoassay. Thyroid function was assessed by measuring thyroid stimulating hormone (TSH), free thyroxine (fT4) and free triiodothyronine (fT3) using ADVIA Centaur chemiluminescence assays. There was no table reporting complete results of analysis for BPA or the 10 other potential xenobiotics with TSH, fT4 and fT3. The correlation coefficients and p values were reported for those comparisons where $p<0.08$. The only reported result for BPA was a correlation with TSH where the Spearman $r = -0.25$, $p=0.077$.

This study did not find an association for BPA levels with thyroid function (TSH, fT4 and fT3), although associations with fT3 and fT4 were found for other xenobiotics. The authors noted literature that reports TSH in cord blood may show a physiologic surge at birth and that levels may also be higher for children born via C section. Such physiologic variability, in addition to biases such as measuring BPA levels and

thyroid function at the same time point, may have made it difficult to adequately assess potential associations of BPA and thyroid function, especially in such a small study. The results provide the concentrations of BPA exposure (as measured with cord blood) in a small group of newborns in a geographically focused area in France. **This study does not provide new information for hazard identification or risk assessment that should be incorporated into the FDA assessment.**

Urinary bisphenol A and obesity: NHANES 2003-2006. (Carwile *et al.*, 2011)
The authors in this Harvard University cross-sectional study hypothesized that urinary BPA concentration would be positively associated with general and central obesity. This is a cross-sectional analysis of 2,747 adults from the US National Health and Nutrition Survey (NHANES): subjects were (2003/04) and (2005/06) adults aged 18–74 years (researchers excluded pregnant subjects or those with missing data). A one-third random subset of subjects was generated from NHANES 2003/04 and 2005/06 responders. The selected subjects supplied urine samples, and were asked questions relating to medical conditions before the physical examination in the participants' home. Self-reported information relating to race, education, income and behaviors were abstracted from NHANES survey. Trained health technicians measured participants' weight, height, and waist circumference. Waist circumference was measured to the nearest 0.1cm. The primary outcomes were general and central obesity, measured using BMI and waist circumference, respectively. Elevated waist circumference was defined according to the National Cholesterol Education Program Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment PanelIII) criteria as >=102cm in men and >=88 cm in women. BMI was calculated as weight in kilograms divided by height in meters squared and used to define overweight [25.0<=BMI<=29.9] and obesity [BMI>=30.0]. The level of BPA (free and conjugated) were analyzed by the Division of Environmental Health Laboratory Sciences using online solid-phase extraction coupled to high-performance liquid chromatography–isotope dilution tandem mass spectrometry with peak focusing. Multivariable models were adjusted for race, education, and smoking status. The lower limit of detection (LLOD) for BPA concentrations was 0.36 ng/ml in 2003/04 and 0.4 ng/ml in 2005/06. BPA results below the LLOD (8% in 2003/04 and 8% in 2005/06) aged 18/74 years with measured BPA and urinary creatinine levels, were replaced with a value equal to the LLOD divided by the square root of two in order to distinguish between a non-detectable laboratory test result from a measured laboratory test result. The creatinine-adjusted geometric mean urinary BPA concentration was 2.05 µg /g creatinine (25th percentile: 1.18, 75% percentile: 3.33). Relative to those in the lowest BPA quartile, participants in the upper BPA quartiles were more likely to be classified as obese (quartile 2 odds ratio (OR): 1.85, 95% confidence interval (CI): 1.22, 2.79; quartile 3 OR: 1.60, 95% CI: 1.05–2.44; quartile 4 OR: 1.76, 95% CI: 1.06–2.94). However, there was not a statistically significant increase in the prevalence odds of overweight compared to subjects in the lowest BPA quartile. Higher BPA concentration was also associated with abdominal obesity (quartile 2 OR: 1.62, 95% CI: 1.11, 2.36; quartile 3 OR: 1.39, 95% CI: 1.02–1.90; quartile 4 OR: 1.58, 95% CI: 1.03–2.42). A non-linear association was found between 2nd, (1.48 kg/m2) 3rd (1.69 kg/m2) and 4th (1.56 kg/m2) quartile BPA exposure levels and BMI. The upper three quartiles also had 3.64-3.89 cm waist circumference and had 39-63% higher odds of an abdominally obese classification than 1st quartile subjects.

This was a population-based study with a nationally representative sample and a relatively large sample size for a cross-sectional study. BPA was measured as a spot urine that may not reflect the actual BPA

exposure. 24- hour urine samples are preferable for less measurement error of BPA exposure. In addition, BPA urine levels may not reflect free concentration of BPA in the circulating system (bloodstream) that has more direct access to the human organs stem. The strength of this study is using NHANES data, which are a good representative of general population. There are several limitations to the current study. Firstly, concentrations of BPA are only measured at one point in time with assumption that this is representative of long term exposure. Secondly, it is questionable whether or not the data from 2003/2004 cohort and 2005/2006 cohort should be pooled. From previous NHANES BPA publications it has been noted that the population distribution of BPA concentrations varies substantially between two cohorts and no explanation for this variation in the exposure was presented. Thirdly, the multivariable models do not account for total caloric intake, yet the authors state the difference in reported total calories between groups was small; normal weight individuals reported consuming approximately 50 fewer calories per day than overweight individuals and 90 fewer calories per day than those classified as obese. The authors also do not state how total caloric intake was estimated nor explain why it was not included in the model. Fourthly, the authors do not account for the confounding associated with the relationship between canned food consumption and BMI category, citing that it is difficult to disentangle, as some canned foods are considered healthy (e.g., canned beans and vegetables) and might therefore be more commonly consumed by normal weight individuals, while others (e.g., canned soda) have been associated with increased risk of obesity. Finally, some demographics like low income persons, Hispanic Americans and non-Hispanic blacks are oversampled in the cohort. Urinary BPA was measured as spot BPA, a technique that is not optimal since BPA is known to vacillate widely at individually sampled time points based on consumption[13], although the authors claim that this is moderately predictive[14].
This study has limited utility for hazard identification and no use for risk assessment.

<u>Circulating levels of bisphenol A and phthalates are related to carotid atherosclerosis in the elderly. (Lind and Lind, 2011)</u>

This cross-sectional study of a randomly chosen subset from the register of subjects aged 70 living in the community of Uppsala, Sweden. A total of 1016 subjects participated, giving a participation rate of 50.1%. The carotid artery was assessed by external B-mode ultrasound. The common carotid artery, the bulb and the internal carotid artery at both sides were visually investigated for the presence of plaque. A plaque was judged to be present in a particular carotid artery if a local thickening of the IMT was seen that was more than 50% thicker than the surrounding IMT in any part of the carotid artery investigated, and also if the atherosclerosis was extensive (IMT > 1.2 mm in all carotid segments) without focally thickened parts. Human serum was analyzed for levels of bisphenol A (BPA) and ten phthalate metabolites by an API 4000 liquid chromatograph/tandem mass spectrometer at ALS Canada following the general procedures presented by the Centers for Disease Control and Prevention. BPA and four of ten phthalate metabolites (mono-isobutyl phthalate (MiBP), mono-methyl phthalate (MMP), mono-ethyl phthalate (MEP), mono-(2-ethylhexyl) phthalate (MEHP)) were detectable in all but 5–12 subjects. Gender adjustment was performed in the first set of models. The second set of models adjusted for multiple cardiovascular risk factors (body mass index, fasting blood glucose, systolic and diastolic blood pressure, HDL and LDL-cholesterol, serum triglycerides, smoking, antihypertensive treatment and statin

13 Carwile JL, Ye X, Zhou X, Calafat AM, Michels KB. (2011) Canned soup consumption and urinary bisphenol A: a randomized crossover trial. JAMA 306: 2218-20.

14 Mahalingaiah S, Meeker JD, Pearson KR, Calafat AM, Ye X, Petrozza J, Hauser R. (2008) Temporal variability and predictors of urinary bisphenol A concentrations in men and women. Environ Health Perspect. 116(2):173-8.

use). High levels of BPA, mono-isobutyl phthalate (MiBP) and MMP were associated with an echogenic intima-medial thickness – grey scale media (IM-GSM) and plaque GSM.

This is a cross-sectional study of an entirely Caucasian population aged 70 or above. BPA were also related to the echogenicity of the plaque, this study has substantial limitations. The cross-sectional nature of this study requires many assumptions when interpreting the data. The authors assume that the serum BPA levels observed during one draw are representative of the individual's life long exposure to BPA. This approach does not consider the hourly, daily, or otherwise variability in the assessment of BPA levels. Also, this a large assumption given that substantial sources of BPA are diet and occupational exposure each of which could have reasonably changed throughout the course of a septuagenarians' life. None of the adjustment factors considered the confounding effects of diet or education. The study measured echogenic IM-GSM and plaque GSM, which are possible biomarkers of CV. How these measurements correlated with clinical outcomes of CV needs further investigation. **This study has limited utility for hazard identification and no use for risk assessment.**

Relationship between urinary phthalate and bisphenol A concentrations and serum thyroid measures in U.S. adults and adolescents from the National Health and Nutrition Examination Survey (NHANES) 2007-2008. (Meeker and Ferguson, 2011)

This study used cross-sectional data from the nationally representative National Health and Nutrition Examination Survey (NHANES) 2007-2008 to evaluate associations between urinary bisphenol A (BPA) concentrations and serum thyroid measures among 1,346 adults (>19 years old) and 329 adolescents (≥12 and ≤19 years old). Serum thyroid measures were used as the outcome (free and total T3 and T4, TSH, and thyroglobulin), and urinary BPA concentration was used as a main predictor/exposure variable [from specimens collected on same participant visit to mobile exam centers and analyzed using, respectively, immunoenzymatic assays (www.cdc.gov/nchs/nhanes/nhanes2007-2008/THYROD_E.htm), and online solid-phase extraction (SPE), isotope dilution, and high-performance liquid chromatography (HPLC) separation, followed by electrospray ionization and tandem mass spectrometry (MS/MS) (www.cdc.gov/nchs/nhanes/nhanes2007-2008/EPH_E.htm]. The study also examined phthalate metabolites as main predictor/exposure variables. Because significant differences were observed in thyroid hormone levels in adolescents compared to adults, the data were analyzed for each group. Weighted multivariable linear regression models were used to account for the NHANES sampling method and study design; unweighted models were also used for comparison because of weight variability concerns and inclusion of variables used to derive the weights in the models. Age, sex, race (gathered by questionnaire), body mass index (BMI), serum continine (as a measure of tobacco smoke exposure), urinary iodine (collected by physical examination and lab analyses) and urinary creatinine (to standardize for urine dilution) were included as covariates.

Among adults, there was no significant relationship between BPA and T_3, TSH, or thyroglobulin; however a significant inverse relationship was observed with total T_4, where an interquartile range increase in BPA was associated with a 1.7% (95% CI: -3.5, -0.01) decline in total T_4. This relationship weakened when sample weights were included. An initial inverse relationship was described for BPA and TSH that disappeared when one influential outlier was removed. These findings were consistent when modeling was stratified by sex. Among adolescents, there were no significant associations between urinary BPA and serum thyroid measures.

The study used well-defined outcome and exposure variables, applied appropriate statistical methods, and included some but not other potentially important covariates. Of the six thyroid measurements, only one was statistically significantly associated with urinary BPA concentration, and only in the unweighted analysis. Much stronger associations were found for phthalate metabolites measured in the same samples. The cross-sectional design, with exposure and outcome measurements each based on only one sample taken at only one time, is a major limitation; whether or not exposure based on a single sample represents long-term average body burden is questionable. **This study may be useful for hypothesis-generation and future studies but not for hazard identification or risk assessment.**

Relationship of urinary bisphenol A concentration to risk for prevalent type 2 diabetes in Chinese adults: a cross-sectional analysis. (Ning *et al.*, 2011)

The goal of this cross-sectional study was 'to confirm the association between bisphenol A and diabetes in a community-based investigation in China'. In 2008, the researchers invited all registered permanent residents over age 40 years in Songnan Community, Baoshan District, Shanghai, China to participate in fasting plasma glucose screening. They tested 10,185 respondents and divided them into 3 groups: normal glucose regulation (NGR) with fasting plasma glucose < 101 mg/dL and no history of diabetes; impaired glucose regulation (IGR) with fasting plasma glucose ≥ 101 but < 126 mg/dL and no history of diabetes; and diabetes, defined as a fasting plasma glucose ≥ 126 mg/dL or a history of diabetes. Then, one year later, they randomly selected participants from each of the groups (NGR, n=1588; IGR, n=748; Diabetes, n=1087) and collected: sociodemographic and lifestyle characteristics, and medical and family history using an oral questionnaire; anthropometric measurements (weight, height, waist circumference, and blood pressure); a 75-g oral glucose tolerance test (with a 10 hour fast and glucose and insulin measured at 0 and 2 hours); blood (ALT, GGT, albumin, total bilirubin, serum creatinine, total cholesterol, HDL, LDL, triglycerides, and high sensitivity C-reactive protein); and a morning spot urine (total free and conjugated bisphenol A measured by liquid chromatography-mass spectrometry).

The authors presented the median and interquartile range of urinary BPA, based on glucose metabolic status as 0.79 (0.76-0.84) for NGR; 0.79 (0.74-0.85) for IGR and 0.82 (0.78-0.87) for Type 2 Diabetes. Although there was not a pattern with BPA for these groups, other risk factors, such as age, BMI, waist circumference, and glucose and insulin measurements followed the increasing patterns that would be expected from other studies. The authors then used logistic regression to determine odds ratios (OR) for Type 2 Diabetes and found no significant effects when considering BPA alone in the model. In the multivariable analysis, "adjusted ORs were slightly increased for participants in the second bisphenol A quartile (0.48 to 0.81 ng/mL) (adjusted OR, 1.30 [95% CI, 1.03 to 1.64]) and the fourth quartile (≥1.43 ng/mL) (adjusted OR, 1.37 [CI, 1.08 to 1.74]) but not the third quartile (0.82 to 1.43 ng/mL) (adjusted OR, 1.09 [CI, 0.86 to 1.39]), and a test of the trend of the association was not statistically significant."

This study did not find consistent patterns of association between BPA levels and glucose regulation/diabetes. Urine BPA was measured at a single point in time, one year after the diagnosis of diabetes and simultaneously with the measurement of the other risk factors, including fasting glucose levels. Absorbed BPA is cleared from the body in less than 24 hours, making urinary BPA measurements collected one year after diagnosis and many years after the period of development of diabetes of questionable value. Exposure measured on a single random day may or may not be

representative of typical past daily exposures. Additionally, diet and lifestyle changes resulting from the diagnosis of diabetes could change BPA levels post-diagnosis. Although some of these biases would be toward the null, it is not appropriate to presume an effect in a study that did not find one based on such potential biases. **This study does not provide new information for hazard identification or risk assessment that should be incorporated into the FDA assessment.**

Bisphenol A Exposure is Associated with In-Vivo Estrogenic Gene Expression in Adults. (Melzer, 2011)
This cross-sectional study assessed the relationship between spot urinary BPA concentrations in a subsample of adult males (n=96, 32-76 years old) enrolled in the unrelated InCHIANTI study in Italy in 2008-09, and the expression of 6 genes involved in estrogen or androgen receptor signaling pathways. Single spot urine and peripheral blood samples were collected from participants in the morning on the day of study. Total (free + conjugated) BPA levels were measured in urine samples using HPLC-MS, while total RNA was extracted from peripheral blood samples and reverse transcribed using quantitative real-time PCR. Candidate gene expression levels (ESR1, ESR2, ESRRA, ESRRB, ESRRG, AR) were determined relative to control genes (GUSB, ACTB). The relationship between spot urine levels of BPA and gene expression was tested using multivariable linear regression in STATA or "R". Models were adjusted to account for potential confounders, including age, BMI, education level, LDL cholesterol, % neutrophils, and % monocytes, among others. It was stated that gene expression levels were log-transformed, since they were not normally distributed.

Mean gene expression levels were from 1-1.2-fold higher than control for all genes, except ESRRB, which was approximately 3-fold higher. A commonly accepted cut-off for gene expression is a 2-fold difference from (either lower or higher than) controls; gene expression levels below this cut off are considered to be background noise. Therefore, a very small degree of gene expression, relative to controls, was measured in only 1 out of 6 candidate genes in this study. Even after adjusting for age and all other confounders (described above), an association was not observed between ESRRB expression and urinary BPA concentration ($p=0.617$). Despite the <2-fold expression of the other 5 out of 6 candidate genes, the authors of the study proceeded to test for associations between these other gene expression levels and urinary BPA concentrations. An association was observed between urinary BPA and ESR2 ($p=0.048$) or ESRRA ($p=0.019$) gene expression after adjusting for all potential confounders. However, as pointed out previously, the expression of these two genes was similar to control levels and therefore is not considered to be biologically meaningful. Similarly, a statistically significant association was observed between ESSRA gene expression and secondary school as highest level of education ($p=0.015$); however, the biological significance of this association would also be considered to be lacking.

Although the materials and methods of this study were well-described, it yielded no clinically relevant results and no apparent adverse effects were observed in the subjects. Only 1 candidate gene, ESRRB, was expressed in circulating leukocytes at levels >2-fold control levels. However, an association between ESRRB gene expression and urinary BPA concentration was not observed. The positive associations reported between ESR2 and ESRRA are considered to be without biological significance, since the expression levels of these genes were within background noise. In addition, the rationale for measuring changes in estrogen- or androgen-responsive genes in peripheral blood cells was unreported. Therefore, it remains unclear how measures in this particular cell type would be relevant to changes in the endocrine

organs that are clearly under the control of estrogen or androgen signaling pathways. Because of limitations in experimental design (use of leukocytes and not the relevant target tissues) and a lack of clinically relevant results, **there is no utility of this study for hazard identification and risk assessment.**

In utero exposure to bisphenol-A and anogenital distance of male offspring. (Miao *et al.*, 2011b).
This cross-sectional study assessed the relationship between reconstructed estimates of occupational BPA exposures in either pregnant mothers or fathers (during mother's pregnancy) from China, and anogenital distance (AGD; distance between center of anus to anterior base of penis) in male offspring (n=153, 0-17 years old). Past exposure levels were estimated based on a combination of employment history, work environment, and use of protective measures reported in an interview and current airborne exposure levels averaged for job classes (8-hour time-weighted average, TWA_8). However, none of these "estimated" past exposures was reported in the paper. Parental exposures to BPA were analyzed as "exposed"/"not exposed" and also categorized into "low" or "high" for father, mother, or both parents, but specific values for the TWA_8 corresponding to each category were not provided. Geometric mean urine BPA levels were measured for a subgroup of the parents but these samples did not correspond to the period of exposure and were only used to support the gradient of low or high exposure categories and were not used in the analysis. AGD was measured by a single physician who was blind to the putative exposure status of the parents. The relationship between putative occupational exposure of parents to BPA and AGD in male offspring was tested using "multiple linear regression models". However, the paper provided no details on how these models were constructed, nor did it report which statistical tests were utilized within the models.

A negative association was reported between AGD among all male offspring and maternal exposure to BPA ($p=0.003$), and among male offspring <8 years of age and maternal exposure to BPA ($p=0.002$). No statistically significant association ($p<0.05$) was observed between paternal BPA exposure and AGD in male offspring. It was assumed that a correlation coefficient formed the basis for the reported P-value; however, this information was unreported. It was also stated that an inverse correlation was observed between "low" or "high" TWA_8 values in parents and AGD in male offspring and that the overall trend for this relationship was statistically significant ($p=0.0008$). However, since neither the ranges of TWA_8 values that correspond to the authors' categories of "low" or "high" TWA_8 exposures, nor the statistical test used to test for significance (trend or pairwise), were reported, this relationship cannot be verified.

The major weaknesses of this study are 1) a lack of quantitative information or exposure ranges for exposure classes for past parental exposures to BPA in the study sample; 2) no consideration of co-exposures to other chemicals used in epoxy resin manufacture, such as phthalates; and 3) failure to report the statistical methods utilized to test the reported association between parental BPA exposure and anogenital distance in male offspring. As a result, the reported inverse dose-response relationship between parental exposures to BPA and anogenital distance in male offspring is difficult to interpret. As pointed out by the study authors, "these findings need to be confirmed by other studies." Based on the lack of transparency in both the data and their statistical analysis, and the consequent uncertainty inherent in the reporting of the dose-response data, **the utility of this study for hazard identification and risk assessment is low.**

Urinary Concentrations of Parabens and Serum Hormone Levels, Semen Quality Parameters, and Sperm DNA Damage. (Meeker *et al.*, 2011)

This is a cohort study. Participants were male partners in subfertile couples seeking treatment between 2000 and 2004 from the Vincent Memorial Obstetrics and Gynecology Service. The eligible participants were 18 to 55 years of age without postvasectomy status. On the day of clinic visit, a single spot urine sample was collected, and second and third urine samples were collected from a subset of men at subsequent clinic visits. Urine samples were analyzed for parabens, methyl paraben (MP), propyl paraben (PP), butyl paraben (BP), and bisphenol A (BPA). One nonfasting blood sample was drawn on the same day and time that the first urine sample was collected. Serum testosterone, estradiol (E2), sex-hormone-binding globulin (SHBG), inhibin B, follicle-stimulating hormone (FSH), luteinizing hormone (LH), prolactin, free thyroxine (T4), total triiodothyronine (T3), and thyroid-stimulating hormone (TSH) were measured using immunoassay methods. A semen sample was collected from each subject after a 48-hour abstinence period and was analyzed for sperm concentration, motility, and motion parameters by a computer-aided semen analyzer. Associations of MP, PP and BP with serum hormone levels, semen quality parameters, and sperm DNA damage measures and interaction of parabens with BPA were assessed using multivariable linear regression. When urinary BPA quartiles were added to the model, BP and BPA were both positively associated with sperm DNA damage (p for trend = 0.03). No other positive associations were observed.

This study focused on parabens, with BPA results and discussion limited to the following: "when urinary BPA quartiles were added to the model, BP and BPA were both positively associated with sperm DNA damage." The associations of BPA alone with serum hormone levels, semen quality parameters, and sperm DNA damage measures are not clearly addressed. This posed a major limitation on the utility of this study for BPA risk identification and assessment. Additional issues concern study bias. First, there is no clear explanation for the difference in number of samples used for analyses of hormone levels (n = 167), semen quality parameters (n = 190), and sperm DNA damage measures (n=132). Second, the time of semen collection in relation to the time of urine and blood collection is not specified. Third, the blood specimens were nonfasting. However, the study was otherwise well designed and used a reasonable sample size. Exposures and adverse health outcomes were well defined. Analysis methods for the urine, blood, and semen samples are clearly referenced. Quality control for measurements of MP, PP, BP, and BPA were conducted and corrected using SG; the limit of detection is provided. To consider the potential temporal effect, second and third urine samples were collected from a subset of participants. Age, body mass index, abstinence period, race, smoking status, and timing of the clinic visit were included in the models as covariates and potential confounders. The relationship between BPA and sperm DNA damage observed by this study has limited utility in hazard identification. Putative associations between BPA and potential sperm damage were evaluated in the context of animal databases as discussed in the Epidemiological studies summary section above. **This study has limited utility for hazard identification, and has no utility for risk assessment.**

Relationship between urinary bisphenol A levels and diabetes mellitus. (Shankar and Teppala, 2011)

This is a cross-sectional study using the cohort of National Health and Nutrition Surveys (NHANES) 2003-2008. This analysis was executed at West Virginia University School of Medicine with a primary endpoint of diabetes mellitus (type 2). The researchers hypothesized a link between diabetes and high levels of urinary BPA. The sample was of participants >20 years old with available urinary BPA samples

(4,792). Subject with cardiac disease and those missing data on covariates were excluded from analysis. Additional variables assessed were age, gender, race, smoking status, alcohol intake, education, history of diabetes, oral hypoglycemic index intake and insulin administration. 3,967 participants remained after exclusions, of whom 467 had diabetes. Diabetes was diagnosed by the guidelines of the American Diabetes Association, and serum glucose was measured by modified hexokinase method. BPA was measured using solid phase extraction coupled with HPLC and tandem mass spectrometry. This method has a LOD 0.1-2 ng/mL in 100 µL. Subjects' BPA measure was stratified into quartiles. The authors state that they identified a positive association between urinary BPA levels and diabetes independent of cofounders (e.g., age, gender, race). Their analysis claims that the association *p-trends* were significant for BPA level and diabetes (multivariate adjusted = 0.002). Authors further cite a positive association for BPA and diabetes with a p-interaction for the cross-product BPA X smoking status term of 0.6530.

This study has no utility in predicting safety or effectiveness of BPA, especially given that it, "...is cross-sectional in nature making it impossible to draw cause and effect in the observed associations." Additionally, the cross-sectional nature of the NHANES allows for the potential for reverse causation. Additionally, the authors provide insufficient information on the urine sampling protocol to assess the methodology. Based on previous NHANES publications regarding BPA, the following concerns exist regarding the BPA exposure assessment. Firstly, concentrations of BPA are only measured at one point in time with an assumption that this is representative of long term exposure. Secondly, it is questionable whether or not the data from 2003/2004 cohort and 2005/2006 cohort should be pooled, as the population distribution of BPA concentrations varies substantially between two cohorts and no explanation for this variation in the exposure has ever been presented. **This study has limited utility for hazard identification or no utility for risk assessment.**

<u>Bisphenol A exposure reduces the estradiol response to gonadotropin stimulation during *in vitro* fertilization (Bloom *et al.*, 2011)</u>

The study population consisted of 58 women and 37 male partners completing a first *in vitro* fertility (IVF) cycle at the University of California at San Francisco Center for Reproductive Health between March 12, 2007 and April 29, 2008. The women participants completed questionnaires collecting covariate data, including age, smoking, race/ethnicity, and body mass index (BMI). Included in this study were 44 women with serum unconjugated BPA concentration data and peak estradiol (E_2) level or number of oocytes available. No differences on the covariates were found between women with or without BPA concentrations. Basal follicle stimulating hormone (FSH) concentrations were taken from medical records; antral follicle count (AFC) was done using transvaginal ultrasonography (TVU) at the initial consult and before the ovarian stimulation, which entailed human chorionic gonadotropin (5000-10,000 IU hCG) administered subcutaneously after observing at least two follicles of 16 to 18 mm in diameter. Follicle maturation and E_2 levels were assessed throughout stimulation by TVU and chemiluminescent enzyme immunoassay, respectively. Oocytes were retrieved 36 hours after the procedure; on the same day, fasting (at least 8 h) blood specimens were collected and processed in polyethylene terephthalate (PET) vacutainers and polypropyylene cryovials. Unconjugated BPA levels were measured by high-performance liquid chromatography (ESA Coularray 5600 detector); empty serum collection tubes and diluent and extraction blanks were tested but found not to contain detectable BPA. Serum BPA concentration was found unrelated (R^2=0.002) to measured serum volume. The limit of detection (LOD) was 0.3 ng/mL. Machine-read values were reported for samples below the LOD to

avoid bias when censoring such values. Multivariable linear regression was used to estimate the association between unconjugated BPA concentration and peak E_2, controlling for selected covariates in 42 women (excluded: one each missing E_2 and AFC). A significant inverse association was not observed between unconjugated BPA concentrations and peak E_2 levels in women having IVF [beta= -0.16, 95% confidence interval (CI): -0.32, -0.01, p = 0.07]. A significant inverse association was observed between E_2 normalized to the number of mature-sized follicles at time of hCG trigger and BPA (beta = -0.14, 95% CI: -0.24, -0.03), although a significant inverse association was also observed between normalized E_2 and smoking [beta= -0.34, 95% confidence interval (CI): -0.65, -0.03, p = 0.03]. An association was not observed between BPA and number of oocytes retrieved (adjusted risk ratio = 0.95, 95% CI: 0.82, 1.10, p = 0.49).

The authors explain in their Discussion section that they intended this study to provide preliminary data assessing support for the hypothesized association between BPA exposure and reduced oocyte counts. They acknowledge the study used a very small sample and was inadequately powered. Other limitations were as follows: (1) despite no differences between women with and without unconjugated BPA concentrations on collected covariate data, potential differences in endocrine factors beyond E_2 levels may have confounded results but were not addressed; and (2) the BPA content of containers and equipment used to store and/or process samples was not ascertained and could not be ruled out. Strengths included (1) measurement of unconjugated BPA serum levels, and (2) steps to avoid bias for samples below the LOD. This study provides preliminary data suggesting an inverse association between unconjugated BPA serum and peak estradiol levels that future work may or may not confirm; the possibility of this association warrants monitoring in the future, but **the current study is too small and underpowered for the findings to be of use for current hazard identification or risk assessment.**

In utero exposure to bisphenol-A and its effect on birth weight of offspring (Miao *et al.*, 2011a)

This retrospective cohort study assessed the effect of parental occupational BPA exposure during pregnancy on the birth weight of newborns, using participants from a 2004-2008 investigation in China (Li *et al*. 2010) examining potential reproductive effects of BPA. Same-city eligible exposed participants included parents recruited from employees at one BPA and three epoxy resin factories, and eligible unexposed participants included parents recruited from employees at factories without BPA exposure during the index pregnancy, along with their spouses and offspring. Personal air sampling monitors were used to measure and calculate 8-hour time-weighted average (TWA_8) parental exposure. Past exposure levels during the index pregnancy were estimated based on a combination of employment history, work environment, and use of protective measures reported in an interview and current airborne exposure levels averaged for job classes (TWA_8). However, none of these "estimated" past or current exposure values were reported and, therefore, they cannot be verified. Of 587 offspring, 444 and 143, respectively, had occupationally BPA unexposed and exposed parents (fathers: 93, mothers: 50). Participant rates were 67.3% for the exposed and 60.7% for the unexposed parents. Eligibility criteria for offspring included live-born singleton status and at least one occupationally exposed parent during the pregnancy. Urinary BPA for those who provided samples was used as an additional exposure measure. Trained staff administered in-person interviews to gather additional socio-demographic, reproductive and medical, health-related behavioral, hazardous environmental exposure, employment history, and maternal and newborn data. BPA exposure was categorized into doses, using paternal BPA workplace exposure as an

indirect maternal exposure in a lower dose category than direct maternal exposure, and using TWA_8 measurements. However, specific values for the TWA_8 corresponding to each exposure category (i.e., maternal or paternal "low" or "high") were not provided. In multiple linear regression modeling, newborn birth weight as a continuous variable was compared between the occupationally BPA-exposed and -unexposed during pregnancy, controlling for maternal age at birth, body weight before pregnancy, education, and gravidity, as well as family income and birth calendar year. However, the paper provided no details on how these models were constructed, nor did it report which statistical tests were utilized within the models. Birth weights of infants with paternal workplace BPA exposure averaged 90.5 g less (P=0.10) and those with maternal exposure averaged 168.4 g less (P=0.02) than did those with unexposed parents. The association held when pre-term births were excluded from the analysis and strengthened when offspring older than 15 years were excluded to reduce the potential effect of recall error. The dose-response trend (P=0.003) showed average birth weight decreased, respectively, by 57.5 g, 153.0 g, 196.1 g, and 234.7 g at paternal low, paternal high, maternal low, and maternal high TWA_8 workplace BPA exposures.

Weaknesses: (1) small sample sizes for exposed parents; (2) lack of validation studies for individual exposure by ambient personal air sampling method; (3) no other measurement of individual BPA levels, except urinary BPA for a limited number of participants acknowledged as not reflective of level during pregnancy and used only as support for TWA_8 categorization; (4) potential differential misclassification because of exposure mismeasurement and unmeasured non-workplace exposures; (5) all mothers worked at least 3 months during pregnancy, but effect of differences in total months worked on BPA exposure during pregnancy was not addressed; (6) analyses controlled for some but not other potentially important factors, such as residential and neighborhood BPA exposures, and dietary intake; (7) potential effects of pre-pregnancy parental exposures were not considered; (8) reasonable analytical steps addressed potential effect of pre-term births and recall bias, but inaccurate recall of birth weight could also have affected the results differentially; and (9) failure to report the statistical methods utilized to test the reported associations between parental BPA exposure and birth weight in offspring.

Despite a significant association between maternal BPA exposure and reduced newborn birth weight, and despite a significant dose-response trend, uncertainty regarding birth weight recall and individual exposure measurement and classification, as well as several other significant weaknesses listed above, **renders this study of questionable use for hazard identification and of no use for risk assessment.**

JRWG OVERALL CONCLUSIONS

The function of this group was limited to reviewing the new data for the purposes of informing the risk assessment on BPA. In addition, the group would consider whether and how new scientific data (e.g., new PK data and models) may affect estimation of exposure from regulated products, including modeling extrapolation/assessment of effects observed from *in vitro* or animal studies. The workgroup was tasked with addressing the following questions:

1) what hazards should be added or removed from FDA's continuing review/research evaluation;
2) what dose/response level for a specific effect/endpoint should be changed and to what level; and
3) how should new exposure data or improved assessments be incorporated into risk assessment.

PK conclusions and PBPK model application

Review of published studies and NCTR work has contributed to a more complete understanding of pharmacokinetic properties following BPA exposure. Conclusions relevant to human risk assessments are categorized and summarized below:

Cross-species and age-related differences

- The pharmacokinetics of BPA have been directly compared in adult rodents, non-human primates, and humans. The remarkable similarities between adults of all species reinforce the predominant effect of presystemic metabolism in the GI tract and liver that attenuates internal exposures to aglycone BPA following oral administration at below 1% of total.
- Pharmacokinetic studies in neonatal rodents show a regular development of metabolic and excretory capacities that serve to reduce serum and tissue levels of aglycone BPA. Adult levels are reached at or near weaning. Similar studies in neonatal monkeys show much smaller developmental changes to the extent that internal exposures from the same oral BPA dose would produce approximately 10-fold higher aglycone BPA levels in a newborn rodent vs. a newborn monkey.
- The kinetics of Phase II metabolite formation and tissue time course for aglycone BPA suggest rapid elimination of the aglycone BPA and do not support sequestration or accumulation of BPA in serum or tissues.
- Preferential accumulation of aglycone BPA in fetal tissues does not occur because of the prominent effect of maternal metabolism augmented by fetal metabolism that increases throughout gestation.
- Tissue concentrations of aglycone BPA are related to the blood perfusion rate in the tissue, cellular composition of the tissue, the metabolic capacity of the tissue manifested by the thermodynamic estimate of the tissue/serum partition coefficient. *In vivo* distribution ratios for the aglycone BPA range from 5-0.7 in the order: adipose > mammary > brain, muscle, ovary > uterus > liver.

Administration route comparison

- Oral exposure results in substantial presystemic Phase II metabolism in gut and additional metabolism in liver that attenuates internal exposures to aglycone BPA to <1%. Therefore, internal exposures to BPA aglycone following parenteral administration (IV and subcutaneous injection) are substantially greater than following oral exposure.
- BPA aglycone levels in rat milk are approximately 300-fold lower than in maternal serum and lactational exposure to rat pups produced BPA aglycone levels that were approximately 500-fold lower than that produced by equivalent gavage dosing.

Data from these experimental animal models and the available human studies were used to develop a PBPK model of human internal exposures at different ages. Simulations of internal exposures were calculated based on FDA dietary estimates[15] for mean and 90th percentile BPA intake rates for infants of

15 FDA Review Memorandum dated October 22, 2009, Division of Food Contact Notifications, Bailey, Hatwell, and Mihalov, *Exposure to Bisphenol A (BPA) for infants, toddlers and adults from the consumption of infant formula, toddler food and adult (canned) food*. http://www.regulations.gov/#!documentDetail;D=FDA-2010-N-0100-0009

0.3 and 0.6 μg/kg bw/d and 0.1 and 0.3 μg/kg bw/d for the adults. Simulation results:

- Serum BPA-G levels in the adult range from 0.4-1 nM (0.1 to 0.25 μg/L) with corresponding aglycone levels being approximately 1000-fold lower (0.4-1 pM).
- Serum BPA-G levels in infants range from 2-4 nM (0.5-1 μg/L) with the corresponding aglycone levels being approximately 1000-fold lower (2-4 pM).

With additional data for the model and relevant exposure estimates, this model could provide internal serum level estimates of BPA based on alternative routes of administration relevant to other FDA regulated products (e.g., medical devices).

Charge questions

Based on the literature reviewed, this WG concludes the following in response to the charge questions:

1) What hazards should be added or removed from FDA's continuing review/research evaluation?
   No new endpoints were identified for hazard identification.[16] The data reviewed herein do not change conclusions on previously identified endpoints.

   a) Mammary gland predisposition to cancer with non-oral administration and oral administration in a transgenic mouse line with increased carcinogenic susceptibility although with significant limitations.

   b) Perturbations in glucose homeostasis supported by limited animal studies. To determine if perturbations in glucose homeostasis should be maintained as an endpoint for hazard identification and relevant to human health, the JRWG will examine the collective data concerning possible metabolic effects of BPA, specifically glucose/insulin regulation for the next review update. Obesity and thyroid data are relevant to this topic and may be included in the next assessment.

   c) Testicular/hormone related parameters based on gestational exposure in Cardoso *et al*., 2011. However, testosterone levels were not affected in Ferguson *et al*., 2011 with gestational and neonatal low dose exposure. Also, findings in large, multigenerational rodent studies have not demonstrated decreased reproductive function or effects on sperm parameters at low doses. Given the observations in humans and the mixed results in animals, the JRWG will compare/examine epidemiological and animal data concerning the endpoint of sperm parameters for the next review update. The preliminary sperm findings from the NCTR 90-day subchronic rat toxicity study of BPA show no significant effect on any sperm parameters at BPA doses of concern to human health. However, sperm DNA damage was not directly assessed. While a correlation between sperm DNA damage and infertility exists in humans and animals, the understanding of the causal relationships is generally poor and likely multifactoral. Overall from human studies, no consistent relationships between sperm DNA damage and fertilization rates or embryo quality during IVF or IVF/ICSI procedures have been demonstrated. Evaluation of sperm DNA damage currently has only a modest predictive value.

16 Effect on number of implantation sites (Varayoud *et al*., 2011) was noted for hazard ID although the effect level (20 mg/kg bw/d BPA through subcutaneous injection into neonatal rats) was greater than the 5 mg/kg bw/d cutoff level identified in the literature search methods.

2) What dose/response level for a specific effect/endpoint should be changed and to what level?

a) The NCTR 90-day rodent study will be reviewed and incorporated into the assessment during the next review cycle. The results of this study are expected to address some of the hazard endpoints identified and will be directly relevant to the risk assessment.

b) One rodent study appeared to satisfy the criteria for risk assessment (Ferguson *et al*., 2011); BPA treatment to dam and neonates (oral, 2.5 or 25.0 µg/kg/day) had no effects on gestational or lactational body weights, PND 1 anogenital distance, preweaning behaviors (righting or slant board behavior), PND 21 hormone levels, or PND 21 regional brain weights. However, this study examined preweaning endpoints during BPA treatment and there are, as yet, no data from this lab on the potential long-term alterations resulting from the developmental treatment.

These new developmental toxicity data along with the studies reviewed herein do not affect the dose-effect level and the existing NOAEL (5 mg/kg bw/day; oral exposure).

c) No new information was identified to inform the issue of dose-effect level for non-oral exposures.

3) How should new exposure data or improved assessments be incorporated into risk assessment?

a) The reviewed PK data and PBPK model contribute to the improvement of the BPA risk assessment by clarifying the PK activities related to BPA metabolism and distribution and by estimating internal exposures of both the conjugated and unconjugated forms of BPA through adult and infant oral dietary exposures. This model has improved estimates of internal exposure to administered oral dose.

b) The reviewed PK data and PBPK model also contribute to the improvement of BPA risk assessments concerning non-oral exposures by providing characterization of expected internal mechanisms and potentially route-to-route comparisons. This model will also better inform species-to-species comparisons.

c) Hazard identification endpoints identified in this and previous reviews are currently being investigated by NIEHS and FDA/NCTR, and the results of those studies should further address risk assessment issues.

d) Problems with measurement of exposure in epidemiological studies remain a significant limitation (e.g., appropriate timing of BPA exposure, BPA measurement, and endpoint measurement).

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