Memorandum

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From: Bisphenol A (BPA) Joint Emerging Science Working Group
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Subject: 2014 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: Stephen M. Ostroff, M.D.
Acting Chief Scientist, Deputy Commissioner for Science and Public Health

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EXECUTIVE SUMMARY

The BPA joint review working group (JRWG), composed of representatives from several FDA Centers, was formed in January 2011. The JRWG performs systematic reviews of literature available on BPA using clearly defined criteria. The first interim report reviewed literature published from November 2009 through January 2011. The second interim report reviewed literature published from February 2011 through October 24, 2011. The memorandum herein constitutes the third interim review of JRWG evaluating literature available October 24, 2011 to July 23, 2013 and available NCTR studies.

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. This document is not a risk assessment. In addition, the group considers whether and how new scientific data (*e.g.*, new pharmacokinetic (PK) 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 (no observed adverse effect level (NOAEL) of 5 mg/kg body weight (bw)/day; oral exposure). Reviewed pharmacokinetic studies continue to provide information useful for age-dependent, interspecies, and route to route extrapolations. The characterized pharmacokinetic differences between primates and rodents reduce concerns about neonatal exposure that have been raised based on some rodent studies using oral and, particularly, non-oral exposures. Recent use of isotopically labeled BPA in pharmacokinetic studies and studies evaluating analytical methods and biomonitoring have provided a better understanding of the potential for and impact of contamination of study samples and inadvertent exposure of study subjects. The extent of potential contamination is much greater than previously realized and provides a possible explanation for inconsistencies in reported low-dose and nonmonotonic effects.

Previous JRWG and CFSAN reviews reported hazard identification endpoints related to developmental neurotoxicity, prostate and mammary carcinogenesis, glucose homeostasis, and sperm pathology. The information reviewed herein significantly reduces concern regarding these endpoints. A FDA/NIEHS chronic toxicity study is being conducted at the NCTR for the purpose of further resolving questions related to these endpoints.

BPA INTERIM REVIEW 3

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 new exposure data or improved assessments should be incorporated into risk assessment.

The function of the working group was strictly limited to performing a review of the updated data for the purposes of informing the risk assessment on BPA. In addition, the group considered whether and how new scientific data (*e.g.*, new PK 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.

Based on the literature reviewed in the previous working group memoranda, the group previously 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 or neuroanatomical endpoints with varying routes of administration.
    b) Developmental changes to prostate with non-oral administration.
    c) Mammary gland predisposition to cancer with non-oral administration.
    d) Cardiovascular disease-related factors based on human epidemiology studies.
    e) Perturbations in glucose homeostasis based on limited supporting evidence.
    f) Sperm/testicular/hormone related parameters based on occupational exposure epidemiology and very limited supporting data in rodents.
2) The available information supported maintaining the existing NOAEL (5 mg/kg bw/day).
3) Physiologically-based pharmacokinetic (PBPK) models were available and contributed 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.

The memorandum herein constitutes the third 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 previous review memoranda. The current conclusions to the charge are based on the third review of the scientific literature and data available between October 24, 2011 and July 23, 2013. 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. Each study was reviewed by an FDA expert followed by a secondary review by another FDA expert. FDA experts contributing to research studies reviewed herein and in previous memoranda were not involved with the review of their own studies for this Working Group.

Literature Search

For the current review, PubMed and Web of Science/Embase were searched for publications (including those in-press) using the term "bisphenol" from October 24, 2011 through July 23, 2013. Studies were limited to English language reports, human epidemiology and animal studies with direct dosing to mammals (*in vivo*, direct dosing), which included doses of ≤ 5 mg/kg bw/day. The CAS RN (80-05-7) or Bisphenol A and various limiting terms were used in this search. FDA also considered 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.[1]

- 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

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.*
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.*

contribution to findings.

As part of the weight-of-evidence assessment, findings from studies employing non-oral and 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.

This updated literature review was carried out with the intent to identify any 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 even though the impact of their findings is currently unknown. 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[2] were considered in the review of BPA PK studies. These included:

- Analytical methodology sufficiently validated and reported with respect to background (blank) levels; limit of detection/quantification (LOD/LOQ); 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.
- 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

2 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.*

pooled/averaged determinations.

Each study was reviewed for its ability to provide novel PK data or inform FDA's on-going efforts to develop PBPK models for BPA. This initial PBPK model has been published (Fisher *et al.*, 2011).

Epidemiology Criteria

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

- 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 [LOD values]; 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 to be 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: October 24, 2011 through July 23, 2013

Based on the third 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

Thirty four 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 34 papers can be categorized as human biomonitoring or source identification for BPA exposure (22 papers), with 4 of these also presenting new methods of analysis and 1 comparing PBPK modeling and biomonitoring. Eleven of the remaining papers

involved PK studies, and the remaining publication addressed analytical method characterization. As with past reviews, these biomonitoring studies continue to add to a large body of data regarding the population statistics of BPA exposure. In a number of instances, the focus is limited (Mahalingaiah *et al.*, 2012) and/or sample size (Krotz *et al.*, 2012) is so small that they offer little new information about BPA exposure. In many of the papers (Christensen *et al.*, 2012, Sathyanarayna *et al.*, 2013), the findings are consistent with and therefore increase confidence in estimates of aggregate daily BPA intake (<1 µg/kg bw/d) from several large international urinary biomonitoring studies. There are still studies (Geens *et al.*, 2012), where high concentrations in blood and tissues are reported that are inconsistent with controlled human absorption, distribution, metabolism, and excretion (ADME), PK studies in experimental animals, and PBPK modeling. It is likely that these high values are the result of sample contamination during collection, processing and/or analysis.

The potential for contamination by native aglycone BPA remains an important consideration in performing laboratory and/or clinical studies, the collection and analysis of samples, and in the acceptance and interpretation of data. A number of papers address the issue of contamination. Ye *et al.* (2013) discuss the propensity for contamination by BPA and other environmental chemicals, pointing out that even in a laboratory that has been performing trace analysis of environmental contaminants for decades, background contamination may still occur. The researchers discuss steps that can be taken to reduce background contamination and the critical quality assurance/quality control procedures that should be implemented and monitored to ensure data accuracy to the greatest extent possible. Teeguarden *et al.* (2013) address the issue of potential contamination in a number of historical biomonitoring studies by comparing the contrasting views of BPA exposure based on serum and urine versus PBPK models and human/nonhuman primate ADME studies. The introduction of this paper serves as a comprehensive review of the BPA literature and findings, and the discussion provides context for inconsistencies between some biomonitoring results and recent laboratory human and nonhuman primate ADME results. Teeguarden and coauthors discussed data from two defined dosing studies that demonstrated the relationship between the administered dose and the maximal resultant serum concentrations of total and aglycone BPA. Each µg of BPA ingested per kg of body weight produced peak serum concentrations of total BPA (*i.e.*, after complete enzymatic hydrolysis of conjugates) that were 13 -17 nM. In both studies, aglycone BPA was below the limit of detection for all of the serum samples collected. Teeguarden and coauthors also discussed concurrent measurements of total BPA concentrations in serum and urine, which were approximately 22-fold lower in serum. This analysis emphasizes the quantitative relationships between ingested amounts of BPA and the resultant concentrations of total BPA in serum and urine that make possible a clear interpretation of BPA urine and serum biomonitoring data often used in epidemiological investigations.

Churchwell *et al.* (2014) also address the issue of post-exposure sample contamination with native aglycone BPA in a rat oral administration (gavage) study. Given the large range of dosing, it was impractical to utilize labeled BPA for this rodent study, so great care was taken to minimize BPA contamination of serum samples, although ultimately unsuccessfully. The authors spend considerable effort discussing and characterizing the source and magnitude of unintended

exposure within the animal rooms and utilize this information in their experimental approach and interpretation of results. The findings show that the serum BPA-glucuronide concentrations produced by low dosing levels (2.5 and 8 µg/kg bw/d) were not statistically different from the naïve and vehicle controls. Given the effort to minimize unintended exposure to BPA (*e.g.*, through analysis of the diet, water, cage materials, etc.) and develop a reproducible dose with precision, these results emphasize that efforts to dose at or below these concentrations would be difficult to interpret without similarly extensive measurements of animal input parameters and internal dosimetry.

A number of the PK (nonhuman primate, animal, and modeling) publications have addressed the data gaps in understanding BPA exposure and have direct application to HI or RA. Corbel *et al.* (2013), Doerge *et al.* (2013), and Patterson *et al.* (2013) all provide data on the behavior of BPA and metabolites that should assist in understanding the metabolism, distribution and elimination of BPA in animal models. The work presented by Mazur *et al.* (2013) and Yang *et al.* (2013) are useful in understanding on-going refinements and assessing the applicability of current PBPK models to interspecies comparisons and estimates of human internal exposures through the diet. These also are useful for understanding interspecies differences that should be considered in the interpretation of animal studies during RA. Two publications, one a PBPK modeling paper (Partosch *et al.*, 2013) and the other an *in vitro* PK study (Lusin *et al.*, 2012), focused on the impact of polymorphisms on enzyme-mediated BPA metabolism that could help explain interindividual differences in PK; however, given the many different uridine 5'-diphospho-glucuronosyltransferases (UGTs) that catalyze the glucuronidation of BPA, results that focus on a single isoform probably have limited applicability.

Individual Study Reviews
Human Data

Interpreting variability in population biomonitoring data: Role of elimination kinetics (Aylward *et al.*, 2012)
The authors used a simple compartmental model to analyze urinary data collected by the CDC from 8 individuals, comparing spot urine samples (*i.e.*, those collected without knowledge of prior exposure timing), 24 hr voids, and weekly average daily voids of three chemicals, one of which was BPA. Following enzymatic deconjugation, BPA was detected in 91% of the samples. Using reverse dosimetry principles and assumptions about urinary excretion of BPA as a marker of exposure, intake calculations were performed. This work shows that spot urine samples are useful for calculating daily urinary excretion of BPA for the purposes of calculating intake of BPA.

All criteria for PK evaluation were met by this study. The methods used by the CDC are highly regarded in this field. Native BPA measurements were made in urine (no dosing). Extensively validated methodology was used. Individual urine data were collected. This study helps verify the use of spot urine BPA measurements for calculating intake of BPA by all routes of exposure.

Variability of Urinary Phthalate Metabolite and Bisphenol A Concentrations before and during

Pregnancy (Braun *et al.*, 2012)

This study characterized the variability of urinary phthalate metabolite and BPA concentrations before and during pregnancy and the ability of a single spot urine sample to classify average gestational exposure. 1,001 urine samples from 137 women were collected before and during pregnancy. The analyses were restricted to women who delivered a liveborn infant and provided two or more pregnancy urine samples and two or more urine samples before that pregnancy. Urinary concentrations of monoethyl phthalate (MEP), mono-n-butyl phthalate (MBP), mono-iso-butyl phthalate, monobenzyl phthalate (MBzP), four metabolites of di-(2-ethylhexyl) phthalate (DEHP), and BPA were measured. Biomarker variability was characterized using intraclass correlation coefficients (ICCs) and conducted several surrogate category analyses to determine whether a single spot urine sample could adequately classify average gestational exposure. The study authors' major conclusions are: (1) Absolute concentrations of phthalate metabolites and BPA were similar before and during pregnancy. (2) Urinary phthalate metabolites and BPA concentrations were variable before and during pregnancy, but the magnitude of variability was biomarker specific. Variability was higher during pregnancy than before pregnancy for BPA and MBzP, but similar during and before pregnancy for MBP, MEP, and ΣDEHP. During pregnancy, MEP and MBP were less variable than BPA, MBzP (ICC = 0.25) and ΣDEHP metabolites. (3) Surrogate analyses suggested that a single spot urine sample may reasonably classify MEP and MBP concentrations during pregnancy, but more than one sample may be necessary for MBzP, DEHP, and BPA.

Analytical methodology was validated for measurements of phthalate metabolism, but is unclear for BPA. The study did monitor $^{13}C_{14}$- labeled 4-methyl-umbelliferone level (internal standard) as quality control for the measurements of phthalate metabolites. The LOD was reported for both phthalate metabolites and BPA. Only total BPA from urine was measured using appropriate mass spectrometric methods.

In conclusion, urinary phthalate metabolite and BPA concentrations were variable before and during pregnancy in this cohort. The primary strength of this study was the availability of multiple urine samples obtained both before and during pregnancy from the same woman. The urine samples were collected very early in pregnancy, allowing examination of exposures and exposure variability across the entire pregnancy. This study may provide benefit in evaluating studies examining gestational phthalate/BPA exposures and maternal/child health outcomes. The variability and surrogate category estimates can be used to assess the extent to which phthalate and BPA exposure misclassification may bias results in epidemiological studies. This study is more of an epidemiology study. However, the author also pointed out that this study may not be generalizable to other populations due to the source population, women from higher socioeconomic position.

Bisphenol A in human placental and fetal liver tissues collected from Greater Montreal area (Quebec) during 1998-2008 (Cao *et al.*, 2012)

The authors determined the concentration of BPA (free and BPA-glucuronide (BPA-glu)) in placental (n=128) and fetal liver (n=28) samples. The samples were obtained after elective pregnancy termination from 1998 to 2008. No information was available on the reasons for termination,

medical history, or lifestyle of the subjects. Average placental concentrations (ng/g) were 12.6 (BPA), 17.2 (BPA-glu) and 30.2 (total). Fetal age had an impact on BPA-glu, but not on BPA or BPA-total and the % of free BPA varied from 4.2 to 100% of total. Average liver concentrations (ng/g) were 9.02 (BPA), 19.1 (BPA-glu) and 25.8 (total). The % of free BPA varied from 12.4 to 99%.

A previously validated method was used for the sample extraction and derivatization and analysis of BPA. No mention was made of the method used for deconjugation. Two method blanks were analyzed with each batch and the samples were "corrected" for the background concentration. Free BPA was measured in all of the samples, but only a subset was analyzed from BPA-glu due to limited sample volume. GC-MS was used for the determination of BPA after derivatization. The method is commonly used and has been previously validated and undergone QC/QA evaluation. Deuterated BPA (d_{14}-BPA), which was added just before sample processing by the lab, was used in the analysis and quantification of the samples. Adequate QC was performed for laboratory work but not for sample collection. Samples were not collected with the goal of BPA analysis so collection controls were not available. Although there was no direct PK data, much was interpreted from the comparison of BPA and BPA-glu in the placental and liver samples. The general conclusion was that maternal liver and/or placental conjugation is highly variable based on the large variation (4.2 to 100) in the percentage of free BPA in the various samples. BPA-glu concentrations (placental) decreased with increasing fetal age, but similar trends were not noted for total or free BPA concentrations. This effect was also noted for the fetal liver samples. No explanation for such trends was suggested. Only 19 paired sets (placental:fetal) were available. Most showed placental:fetal ratios for total BPA close to 1, but there were a number of samples where this ratio did not hold.

The pooled samples and limited paired data limit the usefulness of the study. Additionally, the wide variability in percentage of free BPA create many questions about the methods used or integrity of the samples. Additionally the drop in BPA-glu with increased fetal age is not discussed at all. The study does not address any open questions about the placental transfer of different forms of BPA.

<u>The contribution of diet to total bisphenol A body burden in humans: Results of a 48 hour fasting study (Christensen *et al.*, 2011)</u>

This paper describes a controlled exposure study of BPA excretion in 5 fasted individuals over a 48 hour period to examine non-dietary sources of exposure. Possible sources of intake were evaluated through the use of diaries and total BPA was determined in quantitative collection of urine voids. Analytical methodology (LC/MS/MS) and validation procedures were well-described and the potential for contamination of samples by ubiquitous environmental BPA was carefully considered. Urine concentrations of total BPA generally decreased throughout the 48 hour fasting period and median values were comparable to those found in several large international cohorts that used untimed collection procedures (<1 ug/kg bw/d). The study authors concluded that approximately one third of total daily BPA exposure in these individuals came from non-dietary sources.

Analytical methodology was sufficiently validated and reported. Total BPA concentration in urine was the primary measurement used but the authors also spot checked aglycone levels to identify possible contamination (*i.e.*, >10% of total BPA as aglycone was considered as contamination). Appropriate mass spectrometric methods were used. Criteria for isotope dilution quantification and adequate demonstration of QC in sample preparation and analysis were met. Pharmacokinetic analysis was not performed in this biomonitoring study. These methodological data are not directly applicable for HI or RA; however, they do serve to increase confidence in estimates of aggregate daily BPA intake estimates from several large international urinary biomonitoring studies (<1 µg/kg bw/d).

Population variability of phthalate metabolites and bisphenol A concentrations in spot urine samples versus 24- or 48-h collections (Christensen *et al.*, 2012)

This study examined the statistical reliability of aggregate daily BPA excretion estimates based on analysis of concentrations of total BPA and diethylhexyl-phthalate metabolites in spot urine samples vs. those from urine voids collected quantitatively over a 24 hour period. Since biomonitoring studies typically rely on use of spot sampling methods, the statistical reliability of the assumption required testing. The authors report that concentration data from both spot and 24 hour sampling have generally similar central tendencies (*i.e.*, median, geometric mean) and variabilities for BPA, which suggests that spot sampling strategies are a valid way to estimate population distributions of daily intake with the exception of extreme values.

No defined dosing was done in this biomonitoring study. Pharmacokinetic analysis was not performed in this study. These biomonitoring and statistical data are not directly applicable for HI or RA; however, they do serve to increase confidence in estimates of aggregate daily BPA intake estimates from several large international urinary biomonitoring studies (<1 µg/kg bw/d).

Is lifestage-dependent internal dosimetry for bisphenol A consistent with an estrogenic mode of action in Sprague-Dawley rats when compared with a reference estrogen, ethinyl estradiol, and endogenous estradiol? (Churchwell *et al.*, 2014)

This paper analyzed samples from the NCTR GLP / NTP Technical Report E2176.01, which was developed to measure the potential toxicity of BPA exposure during fetal, neonatal, and adult life stages of Sprague Dawley (SD) rats. As part of this evaluation, the studies considered estrogenic mechanisms of action and therefore included concurrent reference estrogen treatment groups, and serum measurements of BPA and endogenous major sex hormones at neonatal (postnatal (PND) 4), weaning (PND21), and adult (PND80) stages. This paper primarily focused on describing the analytical strategies used to evaluate BPA internal dosimetry, its variation with age, and derived from this the relative estrogen receptor occupancy in dosed groups. This paper started with a pharmacokinetic study of a single oral dose of deuterated BPA (d6-BPA) given to SD rat pups on PND4 (and subsequent serum collection over 24 hours) to establish metabolic clearance rates and optimal post-dose serum collection timing. The main study then exposed pregnant Sprague Dawley rats to 9 different BPA doses (2.5 to 300,000 µg/kg/bw/day) starting on gestational day 6 until parturition. At PND1, direct dosing of pups was continued through the end of the study. This paper describes the BPA and sex hormone serum concentrations of these 9 dose groups, 2 reference estrogen dose groups, and two negative control groups from PND 4-80. Analytical

methods for the direct measurement (not calculation) of BPA-glucuronide (BPA-G), as well as BPA-aglycone (BPA, aglycone), testosterone, ethinyl-estradiol (EE2), and 17β-estradiol(E2) concentrations in rat serum used validated isotope dilution LC-MS/MS methods. In an attempt to eliminate any "background contamination", authors prescreened the environment and food, as well as the analytical equipment and reagents for the presence of exogeneous BPA. Even with this prescreening, low level (<2 nM) contamination of collected serum by BPA-aglycone was reported. Additionally, BPA-G concentrations in vehicle and naïve control rats were found to be equivalent (or similar) to the serum concentrations in the 2 lowest dosing groups of 2.5 and 8 µg/kg bw/d, indicating a low level of unintentional BPA exposure in the controls. Serum BPA, EE2, and E2 concentrations show that BPA or EE2 likely only began to occupy a majority of estrogen receptor α at the two highest doses. Additionally BPA to BPA-G serum ratios as a function of age demonstrated maximal BPA internal dose occurs in the youngest rats, showing a substantial increase in metabolic clearance after PND4.

Utility Statement:
The findings presented, in context with the NCTR 90-day study and the characterization of background BPA contamination, have extensive utility for HI and RA of BPA. The measurement of BPA-aglycone in serum collection from control groups after extensive method control efforts is useful as a benchmark of the low end of reliable BPA measurements in serum samples. Similarly, the measurement of unintentional BPA exposure in control groups (evidenced by BPA-G in serum of control groups equal to the 2.5 µg/kg bw/d dose) is useful to benchmark the lower limit of reliable low-dose controls in similar *in vivo* studies. While the unidentified source of BPA exposure to control groups could potentially limit the sensitivity of conclusions associated with the lower doses, the large number and ranges of doses and animals allows for robust HI and RA using this study. For instance, toxicological effects in estrogen-responsive tissues were only observed at the 2 highest doses, where serum concentrations were 3.5-4 orders of magnitude higher than controls. Additionally, use of several of the higher doses (80-2,700 µg/kg bw/d) of the low dose range demonstrates immature BPA metabolism only in neonatal rats (PND4) and is very useful for inter-species comparisons of internal doses. The 1-2% aglycone (of total BPA in serum) observed for rats with mature metabolism (PND 21 and 80) only started to deviate at the highest dosing concentrations (2,700 µg/kg bw/d and up) suggesting utility for extrapolation/interpretation of higher dose studies. The d6-BPA dosing study on PND 4 day old pups yielded pharmacokinetic parameters consistent with previous studies on 3 and 10 day old pups. These data showing age dependent metabolism allow for estimation of serum BPA-aglycone concentrations in native BPA low dose groups of young SD rats, doses where contamination will preclude reliable serum BPA measurements. The authors' identification of background contamination/inadvertent exposure, even in well controlled environments, underscores the need for researchers to adequately characterize materials and dosing concentrations, and to critically evaluate results for possible inconsistencies. For example, the authors noted that BPA-aglycone levels in PND4 rats were 17-58% of the total BPA, which exceeds values measured under nearly identical controlled (d6-BPA) conditions. Based on these data and previous literature, the authors discuss criteria for determining if BPA-aglycone concentrations from biomonitoring studies represent true exposures or unintended contamination.

Dermal penetration of bisphenol A in human skin contributes marginally to total exposure (Demierre *et al.*, 2012)

The authors obtained skin from two human cadavers (upper leg) and using a dermatome, sectioned 7200 µm thickness sections. Using a flow-through cell system with physiological saline, penetration studies with ^{14}C labeled BPA were performed. The integrity of the system was evaluated with tritiated water. Receptor solutions were sampled every hr up to 6 hr and every 2 hrs for 18 hr. At the end of the experiment, to capture the remaining radiolabeled BPA on the skin (stratum corneum), tape stripping and washing with ethanol and water was used. The flux was determined to be 0.022 µg/cm2/hr. 35% of the dose was recovered in the stratum corneum. The authors state that about 9-10 % of the applied dose was recovered in the saline receptor fluid at 24 hrs. No evaluation of dermal metabolism of BPA was undertaken, which is an important deficiency in this paper, since the skin has been shown to have some metabolic activity towards BPA. Determining the fraction of free BPA in the saline would be more useful than total radioactivity counts.

The methods used in this manuscript, using radioactivity and liquid scintillation counting, appear sound. Dosing was performed using carbon labeled BPA. Demonstration of QC in sample preparation and analysis appears adequate. This study is important, in that it provides a partial answer about dermal penetration of BPA in humans. BPA or its metabolites do cross the skin, thus dermal exposure to BPA contributes as a minor exposure pathway. Since the authors did not distinguish between metabolites and parent BPA, the utility of these data in a PBPK model is unknown at this time.

Potential sources of bisphenol A in the neonatal intensive care unit (Duty *et al.*, 2013)

The authors measured BPA concentrations in nutrition samples (breast milk and formula) and 2 urine samples (before and after feeding) of premature infants in the neonatal intensive care unit (NICU) to determine possible sources of BPA exposure. Along with nutrition samples and urine, the exposure of infants to medical procedures/devices was also recorded and used in the evaluation of exposure. The authors found that there was no difference between breast milk and formula and that a larger contributor to BPA exposure was medical interventions/devices. Infants with high (>3) device use had greater BPA exposure than infants with low (0-3) device use. Median BPA concentrations also differed with gestational age and gestational age was significantly associated with device use.

The methods used had been previously developed and validated. Blanks and positive control samples were analyzed during the study and precision and accuracy data is reported and acceptable. Although blank samples were collected, there was no presentation or discussion of the lab analysis results. Both conjugated and free BPA were analyzed in all of the reported samples. On-line solid phase extraction (SPE) and LC MS/MS were used for sample processing and analysis. Atmospheric Pressure Chemical Ionization (APCI) was used instead of electrospray ionization (ESI), most likely because the authors have more experience with the APCI method. While the methods referenced utilized labeled BPA, there is no mention that labeled BPA was used in the methods section of this study. Blanks and QC data were used.

The paper identifies possible sources of exposure but does not provide any PK or PK-associated data. There are a number of interesting findings, including equivalent BPA concentrations in breast milk and infant formula, the determination that infant formula contains BPA-glu, and the influence of medical device use on BPA exposure.

Fetal bisphenol A exposure: Concentration of conjugated and unconjugated bisphenol A in amniotic fluid in the second and third trimesters (Edlow *et al.*, 2012)
The authors report measurements of total and aglycone BPA in amniotic fluid collected during amniocentesis from women in the second (a contemporary collection) and third trimester (archived frozen samples) of pregnancy. Total and aglycone BPA was detected in a majority of second trimester samples but few third trimester samples, and in all cases, BPA concentrations were close to the reported limit of quantification (LOQ) (0.3 ng/ml).

Analytical methodology used was sufficiently validated, and both conjugated (total) and unconjugated BPA were measured. LC/MS with selected ion monitoring was used. The criteria of adequate demonstration of QC in sample preparation and analysis were met. Pharmacokinetic analysis was not performed in this clinical study.

These clinical data are not directly applicable for HI or RA; however, the use of sub-optimal analytical methodology makes the reliability of typical responses near the LOQ of questionable significance.

Distribution of bisphenol-A, triclosan and n-nonylphenol in human adipose tissue, liver, and brain (Geens *et al.*, 2012)
The authors developed analytical methods to measure BPA and two other chemicals in 11 stored human tissues (adipose, liver and brain) since 2002. The reported concentrations of free BPA in the various tissues are in disagreement with pharmacokinetic model predictions in humans. The authors attempt to account for sample contamination, and use literature results to explain why their findings suggest mg/day intake amounts versus the probable intake rate of < µg/day. There is no compelling explanation for their findings other than sample contamination.

Blank samples had BPA resulting in LOQs of 0.4 ng/g for fat and brain tissues and 0.3 ng/g for liver. Their method relied on Gas chromatography-electron capture negative ion mass spectrometry (GC-ECNI/MS). Total and free BPA measurements were taken in the liver. Native BPA measurements were made (no dosing). Internal standards included ^{13}C-BPA; ^{13}C-triclosan and D_8-4n-nonylphenol.

This paper has no utility. The expected BPA concentrations in tissues, if they could be measured, would be orders of magnitude lower than the reported values. The tissue samples appear to be contaminated with BPA and/or the BPA was hydrolyzed as the samples aged. The reported concentrations of BPA are in the nM range, when pM or lower values are expected.

Bisphenol A-glucuronide measurement in urine samples (Harthe *et al.*, 2012)
The authors developed and performed a single lab validation on a radioimmunoassay (RIA) for

the determination of BPA-glucuronide (BPA-G) in urine. The goal was to develop a simplified method for determining BPA-G in urine to address possible contamination issues with sample collection and handling when total BPA (free plus conjugated) is measured. In evaluating the method, fortification with BPA-G was used for recovery analysis, and 163 spot urine samples were collected. Twenty-four hour excretion profiles were also collected from 14 subjects and plasma samples were collected from 63 subjects in order to compare BPA plasma and BPA-G urine concentrations.

Method validation experiments and summary data were provided. In order to demonstrate the utility of the radioimmunoassay (RIA), both total and BPA-G were measured for a subset of the samples. GC/MS, with derivatization and after hydrolysis, was used to measure total BPA in 32 urine samples. Isotopically labeled BPA was used as an internal standard in the GC/MS analysis for total BPA concentrations in 32 samples. Stable isotopes were not used at any other point in the development or validation of the RIA. Only limited QC data were presented for reagent and "field" blanks, and some of the precision studies use the lowest acceptable number of samples to address precision and recovery. For a paper solely focused on a method, a more robust evaluation of the method would have been beneficial. No PK work was done for this paper; it is focused on the development of an RIA and not on PK.

This paper is solely focused on the development of a new RIA to assist in addressing contamination issues and does not address PK-related questions. While the RIA is validated (single lab) for measuring BPA-G, further studies using isotopically labeled standards would have been beneficial. The anti-BPA antibody used showed 95% cross-reactivity with BPA-G indicating that the method is not able to differentiate BPA in samples containing both aglycone (and probably BPA-sulfate as well, although this was not specifically evaluated) and BPA-G without additional processing. The usefulness of the method for addressing contamination issues is further limited since direct LC/MS/MS analyses for BPA aglycone, BPA-sulfate and BPA-G, as well as total BPA, have been published elsewhere. Any study that plans to measure total or free BPA would not benefit from the method.

A novel method for the quantitative determination of free and conjugated bisphenol A in human maternal and umbilical cord blood serum using a two-step solid phase extraction and gas chromatography/tandem mass spectrometry (Kosarac *et al.*, 2012)
A method for the quantification of aglycone BPA and BPA-glucuronide conjugate (BPA-G) in archived frozen human maternal and umbilical cord blood serum of 12 maternal–fetal pairs was developed. The aglycone BPA was extracted, derivatized, and analyzed by GC/electron impact(EI)-MS/MS. To determine the amount of conjugated BPA in serum samples, bisphenol A-d6 β-glucuronide was added to each sample prior to enzymatic deconjugation. This study probably is the first time that deuterium labeled bisphenol A-d6 β-glucuronide substrate was used to quantify and control the glucuronidase deconjugation and hydrolysis reaction. The method detection limit (MDL) and LOQ for BPA were 0.026 ng/mL and 0.087 ng/mL, respectively. The observed recoveries ranged between 65% and 88%.

The results demonstrated that BPA concentration in human maternal serum at mid-pregnancy and

at delivery ranged from <0.026 ng/mL to 10.4 ng/mL (≤46 nM; median 0.55 ng/mL or 2.4 nM, n = 12) and <0.026 ng/mL to 3.0 ng/mL (13 nM; median 1.5 ng/mL or 6.6 nM), respectively. Results for matching umbilical cord blood serum BPA concentration were in the range of <0.026–2.6 ng/mL (<11 nM; median 1.8 ng/mL or 8 nM). Only 2 mid-pregnancy serum samples out of 12 contained quantifiable amounts of BPA-G (0.3 and 0.55 nM), indicating that BPA–glucuronide is not abundant in either human maternal or umbilical cord blood serum. By contrast, 8 of the 12 samples contained aglycone BPA and the respective levels in individual samples were not reported. In general, the observation of aglycone BPA at a median value of 8 nM, but undetectable or >10-fold lower BPA-G values in serum samples suggests contamination by ubiquitous environmental aglycone BPA occurred during sample collection, storage, or analysis. Alternatively, deconjugation of conjugates in the archived samples cannot be excluded. Like some other attempts to quantify BPA in human blood cited by Kosarac *et al.*, these results are of no utility for HI or RA because of a high likelihood of contamination artifact.

Phthalates and bisphenol do not accumulate in human follicular fluid (Krotz *et al.*, 2012)
The authors analyzed follicular fluid for bisphenol A and phthalates after brief exposure to medical devices during an IVF cycle. The purpose was to determine if the accumulation of these analytes in the ovaries was occurring and if concentrations were sufficient to impair oocyte maturation. The sample size was 5 women, all undergoing IVF cycles. Hydrolysis was performed prior to analysis, suggesting that total BPA concentrations were being measured.

A contract laboratory was utilized for all of the BPA analysis. There are some method details presented in the paper, but reference to a previous publication for additional method validation information only addresses phthalates and not bisphenol A. Although not clearly stated, it appears that only total BPA measurements were made. LC-MS/MS was used for the analysis of the samples, but only limited information is given about the method and analytical parameters used. Stable isotopes were used in the analysis of BPA, but it is unclear which compounds were used and when they were added to the samples. Deuterated BPA (d_6-BPA) was used for recovery experiments to evaluate the accuracy of the method. The analysis of the samples, while not fully detailed, did use blanks and positive control samples to assess the method performance. Both "control" aliquots of media, as well as leachates were provided. However, there was no mention of field blanks or positive field controls being shipped with the samples. Only limited information is given about analytical method parameters and none of the QA/QC data are presented. There are no PK data in this paper and even the "occurrence" data for BPA are extremely limited (5 subjects). With no detectable BPA concentrations in any of the samples, there is a need for positive controls to establish that the collection and shipping did not interfere with BPA determination.

Determination of Free and Conjugated Forms of Bisphenol A in Human Urine and Serum by Liquid Chromatography−Tandem Mass Spectrometry (Liao *et al.*, 2012)
In this study, free, conjugated (BPA glucuronide or BPAG and BPA disulfate or BPADS), and substituted (chlorinated BPA; mono- [BPAMC], di-[BPADC], and trichloride [BPATrC]) forms of BPA were determined in human urine and serum samples, using SPE and liquid

chromatography− tandem mass spectrometry (LC/MS/MS) techniques. The instrumental calibration for each of the target compounds ranged from 0.01 to 100 ng/ mL and showed excellent linearity (r > 0.99). The limits of quantification (LOQs) were 0.01 ng/mL for free BPA and 0.05 ng/mL for the conjugated and substituted BPA. Respective recoveries of the six target compounds spiked into water blanks and sample matrices (urine and serum), and passed through the entire analytical procedure, were 96 ± 14% and 105 ± 18% (mean ± SD) for urine samples and 87 ± 8% and 80 ± 13% for serum samples. The optimal recoveries of BPAG and BPADS in the analytical procedure indicated that no deconjugation occurred during the SPE procedure.

The method was applied to measure six target chemicals in urine and serum samples collected from volunteers in Albany, New York. BPA and its derivatives were found in urine samples at concentrations ranging from < LOQ to a few tens of ng/mL. In serum, free and conjugated BPA were detected at sub-ng/mL concentrations, whereas BPA chlorides were not detected. The urine and serum samples were also analyzed by enzymatic deconjugation and liquid−liquid extraction (LLE) for the determination of total BPA, and the results were compared with those measured by the SPE method. To our knowledge, this is the first report on the occurrence of BPAG and BPADS in human serum.

Method validation parameters were provided. Free, conjugated (BPA glucuronide or BPAG and BPA disulfate or BPADS), and substituted (chlorinated BPA; mono- [BPAMC], di-[BPADC], and trichloride [BPATrC]) forms of BPA were measured. Native BPA and LC/MS/MS were employed with $^{13}C_{12}$−BPA as an internal standard. Extensive QC measurements were recorded. Individual urine and serum data were collected. This is the first study describing the occurrence of BPADS and BPA chlorides in human urine and serum samples. The newly developed analytical procedure is suitable for the analysis of different species of BPA.

<u>Evaluation of bisphenol A glucuronidation according to UGT1A1*28 polymorphism by a new LC-MS/MS assay (Lusin *et al.*, 2012)</u>

The authors measured glucuronidation of BPA by human microsomes from kidney, liver, lung and intestine. The analytical methods were deemed acceptable (LC-MS/MS). The *in vitro* studies were conducted in the µM range. The Km estimates for kidney, intestine, and liver were 119, 58, and 8.9 µM and the Vmax values, 4.8, 1.4, and 8.5 nmol/min/ mg protein. Using intestine microsomes may be problematic. The protein content of the intestine used by the authors was 3 mg/g for the intestine and 45 mg/g for the other tissues. Reports of 21 mg/g protein have been reported for the intestine (Cubitt *et al.*, 2011). Also, Groothuis and deGraff (2013) report that the role of the intestine in drug metabolism has been underestimated for years because of the lack of a good *in vitro* experimental method. These authors use precision-cut slices and compared the metabolic rate of enterocytes and hepatocytes. Nevertheless, for ingestion rates of low µg or pg quantities of BPA from food, gut metabolism is an important factor for lower oral bioavailability. Their reported maximal metabolic rate for gut microsomes of 1.4 nmol/min x 3 mg protein yields a rate of 4 nmol (1 µg) per min.

This study shows method development with details. Total and free BPA measurements were completed. Native BPA measurements were made as well as using d_{16}BPA. *In vitro* metabolic

constants for BPA metabolism were carried out in liver, kidney, and intestine. This paper has utility because extrahepatic metabolism was quantified in two organs relative to the liver. Unfortunately, the intestine metabolic constants may be in error because of the poor experimental methodology (microsomes vs precision cut slices). This study shows the importance of gut metabolism in understanding presystemic metabolism of BPA by both the gut and liver for the oral route of administration.

Analysis of polyfluoroalkyl substances and bisphenol A in dried blood spots by liquid chromatography tandem mass spectrometry (Ma *et al.*, 2013)

In this study, a liquid–liquid extraction and high-performance liquid chromatography/ tandem mass spectrometry method was used for the detection of perfluorooctanesulfonate (PFOS), perfluorooctanoate (PFOA), and bisphenol A (BPA) in dried blood spots (DBS). The method was validated for accuracy, precision, and sensitivity, by spiking of target chemicals at different levels on Whatman 903 filter cards, which is used in the collection of DBS by the newborn screening program (NSP). The field blanks were prepared from unspotted portions of DBS filter cards to determine the magnitude of background contamination. The method was applied for the measurement of PFOS, PFOA, and BPA in 192 DBS specimens provided by NSP of New York State. PFOS and PFOA were detected in 100% of the specimens analyzed. The concentrations of PFOS and PFOA measured in DBS were similar to those reported earlier in the whole blood samples of newborns. BPA was also found in 86 % of the specimens at concentrations ranging from 0.2 to 36 ng/mL (excluding two outliers). Only total BPA was measured. Further studies are needed to evaluate the sources of BPA exposures and health outcomes in newborns.

Samples were analyzed by LC/MS/MS, and method validation parameters were provided. $^{13}C_4$-PFOA, $^{13}C_4$-PFOS, $^{13}C_{12}$- BPA were used as internal standards. Extensive QC measurements were recorded. Individual spot data were collected. A HPLC-MS/MS method was developed for the analysis of PFOS, PFOA, and BPA in DBS collected from newborns, containing small amount of whole blood. This method was successfully applied for the analysis of target chemicals in 192 DBS specimens from the NYS NSP. The limit is the quantification of the exact volume of blood in each spot to determine the exposure level. This paper is not for PBPK, but methodology development. The outcome may be useful for epidemiology studies.

Bisphenol A is not detectable in media or selected contact materials used in IVF (Mahalingaiah *et al.*, 2012)

The authors test *in vitro* fertilization (IVF) media and some contact materials for the presence of bisphenol A. Contact materials were not tested directly, but “leachates” that had been in contact with the materials were tested for BPA. A contract laboratory was utilized for all of the BPA analysis. Some information on analytical methodology was noted, but more could be provided by reference or supplemental material. LC-MS/MS was used for the analysis of the samples, but only limited information is given about the method and analytical parameters used. Deuterated BPA (d_6-BPA), which was added just before sample processing by the lab, was used in the analysis and quantification of the samples. The analysis of the samples, while not fully detailed, did use blanks, and positive control samples to assess the method performance. Both “control” aliquots of media, as well as leachates were provided. However, there was no mention of field

blanks or positive field controls being shipped with the samples. Only limited information is given about analytical method parameters and none of the QA/QC data are presented. There is no PK data in this paper and even the "occurrence" data for BPA in the IVF materials is extremely limited and not well characterized. There is no information about the composition of contact materials or residual BPA concentrations. With no detectable BPA concentrations in any of the samples, there is a need for positive controls to establish that the collection and shipping did not interfere with BPA determination.

Human and Rat ABC Transporter Efflux of Bisphenol A and Bisphenol A Glucuronide: Interspecies Comparison and Implications for Pharmacokinetic Assessment (Mazur *et al.*, 2012)
In this study, the authors utilized an ATPase assay to evaluate BPA and BPA-G as potential substrates for the human and rat ATP-Binding Cassette transporters (ABC), P-glycoprotein (MDR1), multidrug resistance–associated proteins (MRPs), and breast cancer–resistant protein (BCRP). The results of this study indicated that BPA is a potential substrate for rat mrp2 and human MRP2, BCRP, and MRP3. The metabolite BPAG demonstrated the highest apparent substrate binding affinity for rat mrp2 and human MRP3 but appeared to be a non-substrate or potential inhibitor for human MRP2, MDR1, and BCRP and for rat mdr1a, mdr1b, and bcrp. These results suggest that in both rat and human, apical transporters efflux BPA into the bile and/or intestinal lumen. BPA-G would follow a similar pathway in rat; however, in human, due to the basolateral location of MRP3, BPA-G would likely enter systemic and portal blood supplies.

The study results suggest that the transport mechanism in rats shows a preference for BPA and BPA-G transport into intestinal lumen and hepatobiliary excretion, while in humans, shows a preference for BPA-G transport into the blood supply of the liver and small intestines. These findings are consistent with previous reports of dramatic difference in PK behavior of BPA between rodents and primates. These findings are also consistent with the PBPK model developed by Fisher *et al.*, 2011. The authors are careful to point out that additional interspecies testing and *in vitro* transport studies are necessary to fully assess systemic BPA clearance. The findings of this study may reduce the uncertainties in the assessment of PBPK model parameter values.

Bisphenol A concentrations in maternal breast milk and infant urine (Mendonca *et al.*, 2014)
The authors report total and aglycone measurements in breast milk from 27 mothers and urine from 31 of their infants. Total BPA was detected in 93% of the infants' urine samples and 75% of the breast milk samples; however, there was no significant relationship reported between BPA concentrations in breast milk and urine. Analytical methodology used was sufficiently validated, and both conjugated (total) and unconjugated BPA were measured. No controlled dosing with BPA was performed in this biomonitoring study. Criteria for mass spectrometric methods, isotope dilution quantification, and adequate demonstration of QC in sample preparation and analysis were met. Pharmacokinetic analysis was not performed in this biomonitoring study. These biomonitoring data are not directly applicable for HI or RA.

Urinary Free Bisphenol A and Bisphenol A-Glucuronide Concentrations in Newborns (Nachman

et al., 2013)

The study demonstrated BPA exposure in 11 neonates and 1 young infant (healthy newborns). The detection of BPA-glucuronide in all infants demonstrates universal exposure to BPA in the study population. BPA-glucuronide was the only detectable BPA compound in the urine of these newborns.

Analytical methodology was sufficiently validated; however, original data that met these criteria were in a separate publication (Fox *et al.*, 2011). Free BPA and BPA-glucuronide were measured. d6-BPA and d6-BPA-glucuronide were used as internal standards. Individual urine data were collected.

The detection of BPA-glucuronide in all infants demonstrates universal exposure to BPA in the study population. BPA-glucuronide was the only detectable BPA compound in the urine of these newborns. The absence of detectable concentrations of free BPA suggests that infants have 1 or more enzyme isoforms capable of complete metabolism of BPA or that enzyme activity in neonatal tissues is sufficient to quickly inactivate BPA. However, the study is limited to a small sample size.

Unexpected results in a randomized dietary trial to reduce phthalate and bisphenol A exposures. (Sathyanayana *et al.*, 2013)

This study describes a randomized trial of 10 families assigned to two diets designed to ascertain the effect of dietary replacement strategies that reduce exposure to BPA and DEHP (*i.e.*, without plastics). The dietary intervention strategies (total diet replacement vs. written recommendations) had minimal effects on baseline urinary levels of total BPA, which were similar to those reported for population-based survey of the U.S. population (*i.e.*, <1 µg/kg bw/d from NHANES). All relevant criteria were met for this study. Pharmacokinetic analysis was not performed in this biomonitoring study. These methodological data are not directly applicable for HI or RA; however, the urinary levels of total BPA reported were consistent with a number of larger human biomonitoring studies from the U.S. and other countries (*i.e.*, median aggregate daily intake <1 µg/kg bw).

Are Typical Human Serum BPA Concentrations Measureable and Sufficient to be Estrogenic in the General Population? (Teeguarden *et al.*, 2013)

The research reported a convergence of robust methods for measuring or calculating serum concentrations of BPA in humans from 93 published studies of more than 30,000 individuals in 19 countries. The study reports that typical serum BPA concentrations are orders of magnitude lower than levels measurable by many analytical methods and below concentrations required to occupy more than 0.0009% of Type II Estrogen Binding Sites, GPR30, ERα, or ERβ receptors. They report that occupancies would be higher, but still ≤ 0.04%, for the highest affinity receptor ERRγ. The study reports that the results show limited or no potential for estrogenicity in humans, and question use of certain methodology and reports of measurable BPA in human serum.

This study is a meta-analysis pooling data from multiple data sources. The paper utilized the human exposure and ADME (absorption, distribution, metabolism and elimination) data to

extend the assessment of human serum BPA exposure to the general population and test the hypothesis that human internal (serum) exposures to BPA are sufficient to induce biological responses through occupancy of estrogen receptors.

The paper is useful in HI and RA because of the thorough sourcing and review of epidemiological data and PK experimental and modeling studies. Additionally, in placing "mechanism of action" data in context with exposure data, the authors are able to identify a number of potential, but significant shortcomings in mechanistic and biomonitoring studies. Not the least of these is the potential for contamination during sample collection and analysis.

Racial disparity in maternal and fetal-cord bisphenol A concentrations (Unal *et al.*, 2012)
The authors measure BPA concentrations in both maternal serum and fetal cord serum in order to investigate whether any racial disparity exists in these values. Analytical method was reported, but very little data on method validation was given, and although blanks were discussed and it was stated that none of the blanks had detectable concentrations of BPA, it is unclear if blanks were carried through entire process or were simply water. An interesting observation about the method is that the authors chose to use APCI instead of ESI for BPA detection; this tends to produce higher LODs. Also, the column and method separation would have had limited ability to separate BPA from other possible interferences; thus, the column had insufficient theoretical plates, and the gradient was too quick. Only total BPA was measured after deconjugation. LC-MS/MS was used for the analysis of the samples, but only limited information is given about the method and analytical parameters used. Deuterated BPA (d_6-BPA), which was added just before sample processing by the lab, was used in the analysis and quantification of the samples. Water blanks were run, but there is no information on additional reagent blanks, positive control samples or variability (replicate analysis). There was no information on specific exposure habits. It is unclear if samples were taken after a fasting period and the maternal and fetal samples were taken on different days, with at least a few days and at most a few weeks separation. Given the lack of exposure data, this is an epidemiological study and cannot offer information about PK.

There are no PK data in this paper and even the epidemiological data available from the study are very limited. With only 27 patients across 3 ethnic groups, it is difficult to draw solid conclusions. Perhaps most detrimental is the time separation between maternal serum and fetal cord serum samples. With such large time differences and the transient nature of BPA it is difficult to draw any conclusions from the differences in BPA concentration.

Potential External Contamination with Bisphenol A and Other Ubiquitous Organic Environmental Chemicals during Biomonitoring Analysis: An Elusive Laboratory Challenge (Ye *et al.*, 2013)
The paper presented several case studies using the quantitative determination of BPA and other organic chemicals (*i.e.*, benzophenone-3, triclosan, parabens) in human urine, milk, and serum to identify potential contamination sources when the biomarkers measured are ubiquitous environmental contaminants. Contamination with target analytes during biomonitoring analysis could result from solvents and reagents, the experimental apparatus used, the laboratory environment, and/or even the analyst. For biomonitoring data to be valid - even when obtained

from high-quality analytical methods and good laboratory practices - the following practices must be followed to identify and track unintended contamination with the target analytes during analysis of the biological specimens: strict quality control measures including use of laboratory blanks; replicate analyses; engineering controls (*e.g.*, clean rooms, biosafety cabinets) as needed; and homogeneous matrix-based quality control materials within the expected concentration ranges of the study samples.

Analytical methodology must be sufficiently validated and for all case studies. LOD and standards are mentioned for some cases. On-line SPE-HPLC-MS/MS and isotope dilution–high performance liquid chromatography was used. Quality control blank (QCB) and quality control (QC) samples were mentioned for case studies.

The author provided case studies on unintended external contamination in the measurements of ubiquitous environmental chemicals. This is not a PBPK paper, but a summary of suggestions to avoid unintended external contamination during analysis. The study authors suggested:

- Evaluate the QCB (*i.e.*, reagent blank) by checking both the calculated concentration and the S/N ratio of the QCB peak to assess any systematic or non-systematic contamination.
- Prepare the QCBs using the same procedure and apparatus as the unknown study samples to avoid missing labware and apparatus potential contamination.
- Use guard columns to filter out potential interference contaminants from the SPE mobile phases or separate the chromatograhic peak of the target analyte in the study samples from its interference peak in the HPLC mobile phases.
- Conduct replicate analyses (*e.g.*, 5%) of study samples to identify random contamination from the laboratory environment and/or analyst, especially when the number of study samples is small. When limited sample volume precludes replicate analyses, include additional blanks randomly placed within the analytical batch.
- Prepare the samples for analysis in a controlled environment (*i.e.*, biological safety cabinet, clean room) if contamination from the analyst or the environment (*e.g.*, air, dust) is suspected.
- Use homogeneous matrix-matched QC materials at concentrations within the expected concentration ranges of the study samples.
- When possible, participate in external quality assessment programs or use standard reference materials to evaluate the accuracy of the measurement.

<u>Blood and urinary bisphenol A concentrations in children, adults, and pregnant women from China: Partitioning between blood and urine and maternal and fetal cord blood (Zhang *et al.*, 2013)</u>

The authors report blood- and urine-based biomonitoring of total BPA in paired maternal and cord blood samples as well as in reference adults from the Chinese population. Criteria on analytical methodology validation, mass spectrometric methods, isotope dilution quantification, and adequate demonstration of QC in sample preparation and analysis were met. Only “total” BPA was reported from this hospital-based human biomonitoring study. Pharmacokinetic analysis was not performed in this study.

These methodological data are not directly applicable for HI or RA. The impact of potential contamination of blood and urine samples was discussed by the authors but the data presented did not permit a critical evaluation since only total, and not aglycone, BPA was reported. Intravenous treatments of subjects prior to blood collection appeared to elevate blood levels of total BPA approximately 10-fold vs. those subjects not receiving IV interventions, which suggests iatrogenic exposures to BPA may occur. While the importance of reported differences in blood levels for total BPA between reference groups of adults and the maternal/cord samples is difficult to interpret, the urinary levels of total BPA were consistent with a number of larger human biomonitoring studies from other countries (*i.e.*, median aggregate daily intake < 1 µg/kg bw).

Other Mammalian Data

Bisphenol A disposition in the sheep maternal-placental-fetal unit: Mechanism determining fetal internal exposure (Corbel *et al.*, 2013)

The authors evaluated the pharmacokinetics of BPA and its glucuronide conjugate, BPAG, in pregnant sheep. A chronically catheterized experimental design was used (one catheter in the carotid artery, the jugular vein and amniotic cavity) in each ewe, which allowed for BPA and BPAG measurements in maternal and fetal circulation after dosing the dam or fetus with BPA or BPAG. BPA or BPAG was infused for 24 hrs through the maternal jugular vein. Kinetic studies were systematically carried out over different periods of pregnancy (over 108 to 117 days). The paper describes the procedures in detail. WinNonlin software was used to characterize the kinetics of BPA and BPAG and Systat software was used for statistical comparisons. The most important findings were that when BPAG was administered to the mother in late pregnancy (4 months of pregnancy), the BPAG was not detected in the fetus, nor was BPA detected in the maternal or fetal plasma. This suggests that once BPAG is formed from BPA by UGT enzymes, de-conjugation in either the fetus or adult is not an important consideration. In the late stage fetus, BPA is metabolized to BPAG, and the metabolite is unable to readily cross the placenta so it builds up in the fetal plasma and amniotic fluid. BPA crosses the placenta readily.

Compounds were administered intravenously into the jugular vein for 24 hours. The infusions of BPA and BPAG were at fairly high doses, in the 2-5 mg/kg/day range with LOQs of 1, 50, and 6.8 ng/ml for BPA, BPAG, and BPAS for plasma and amniotic fluid. Aglycone BPA, BPA-sulfate, a minor metabolite, and BPA-G, the principal metabolite, were directly analyzed using separate LC/MS/MS responses. The study reported intra-and interday coefficients of variation. Eleven ewes and their fetuses were used.

This study is important because it provides data on the behavior of BPA and its metabolite BPAG with regard to the placental-fetal unit and the stability of BPAG in the body. The animals were surgically manipulated over a long period of time to collect data during different phases of pregnancy. Pregnancy did not have an effect on the maternal kinetics of BPA (or BPAG) compared to non-pregnant ewes. BPA fetal kinetics follow maternal kinetics, while BPAG in the fetus will build up and not follow maternal kinetics. Measurements of BPA and BPA-G in amniotic fluid after maternal dosing were lower than those in maternal circulation, which is

consistent with observations in rat and monkey.

Pharmacokinetics of bisphenol A in serum and adipose tissue following intravenous administration to adult female CD-1 mice (Doerge *et al.*, 2012)
This study reports the pharmacokinetics of BPA in serum and adipose tissue of adult female CD-1 mice following intravenous administration (deuterated BPA 100 µg/kg body weight). Concentrations of un-conjugated (active) and conjugated (inactive) BPA in serum and adipose tissues were determined by LC/MS/MS. The half-life of total serum BPA was 6.6 hours. The half-life and clearance of un-conjugated BPA were 0.8 hours and 10 L/h per kg. The half-life of un-conjugated BPA in adipose tissues was 7.0 hours.

The use of d6-BPA is extremely beneficial, eliminating uncertainty about the influence of background BPA on the low concentrations being measured. This paper should offer information/guidance in making interspecies extrapolations. However, a good correlation between body weight and a parameter does not guarantee a good prediction of the parameter of interest. The PK of BPA in adult CD-1 mice is well characterized. The clearance rate of d6-BPA (aglycone) from the adipose tissue is consistent with the non-persistence of BPA. This manuscript highlights the allometric scaling for the projection of human clearance following IV and oral administration from animal data.

High Bioavailability of Bisphenol A from Sublingual Exposure (Gayrard *et al.*, 2013)
This manuscript reports comparative bioavailability of BPA following intravenous, orogastric, and sublingual administration. This was a crossover study. Six dogs received 0.05 mg/kg and 5 mg/kg BPA after intravenous and sublingual administration, respectively. A 20 mg/kg BPA dose was given to same dogs by orogastric gavage. Blood samples were collected up to 24 hours. BPA and BPAG (glucuronide BPA) in plasma samples were simultaneously quantified using an Acquity ultra performance liquid chromatograph coupled to a Xevo triple quadrapole mass spectrometer. The absolute bioavailability of BPA was 70 ± 31% and 0.72 ± 0.28% following sublingual and orogastric administration, respectively. The concentration ratio of BPAG to BPA in plasma was approximately 100-fold lower following sublingual administration than after orogastric dosing. This indicated that more parent BPA was available in the systemic circulation following sublingual than orogastric administration.

Analytical methodologies were sufficiently validated and reported. Aglycone and BPA-glucuronide (BPA-G) were measured directly. Native BPA was used for dosing but at high enough levels that contamination was not an issue. LC/MS/MS procedures were adequate. Labeled BPA and BPA-G were used as internal standards. Six dogs were used in the study.

This study is useful for characterizing the absorption kinetics of BPA by sublingual and oral routes of administration. In this case (sublingual), one should expect that the concentration of BPA in the systemic circulation will be much higher than exposure following oral administration if one assumes that the absorption of BPA only takes place in the gastrointestinal tract. A limitation of this study is the sampling of blood from the jugular vein, which drains the oral cavity prior to mixing, and does not give an accurate representation of systemic exposures.

However, and as the authors state, "the results of Teeguarden *et al.*, (2011) (a food-based clinical study) do not support sublingual absorption as a major contributor of dietary BPA to a much higher than expected human internal exposure." Therefore, this paper is limited in utility for HI of dietary exposures.

Differential accumulation of BPA in some tissues of offspring of Balb-C mice exposed to different BPA doses (Mita *et al.*, 2012)
The authors subcutaneously injected BPA (0.1 and 1 mg/kg bw/d) in mice throughout pregnancy and lactation (gestational day (GD) 1-PND 7). The dams were not dosed further and were sacrificed on PND 21. The offspring were untreated after weaning and sacrificed at PND 90. The authors report tissue levels of BPA in both dams and their pups with significant differences between dams and pups and between female and male pups.

The analytical methodology used was sufficiently validated. Only aglycone BPA measurements were reported. Mice were dosed with native BPA. LC with UV (220/280 nm) detection was used. Pharmacokinetic analysis was not performed in this study. These methodological data are not directly applicable for HI or RA. The data reported in this paper are inconsistent with a broad body of evidence from controlled pharmacokinetic studies in experimental animals, including mice and humans, showing that BPA is rapidly eliminated from the body within 24 hours, even from adipose tissue. Maternal tissues were collected 14 days after the last dosing and pup tissues after 83 days. This major pharmacokinetic inconsistency when coupled with the relatively non-specific analytical detection method used make these data of minimal reliability.

Fetal liver bisphenol A concentrations and biotransformation gene expression reveal variable exposure and altered capacity for metabolism in humans (Nahar *et al.*, 2012)
The authors measured BPA and conjugates in 50 first and second trimester healthy fetus livers. The livers were obtained from an NIH-funded birth defects laboratory tissue bank at the University of Washington. Information on storage of the livers was given. Healthy adult livers served as controls (n=2). Hepatic fetal liver gene expression data for four genes related to metabolism and enterohepatic recirculation were measured and compared to adult control livers. As expected the expression levels in the fetus were lower than in adult livers. Measured mean concentrations of BPA and its conjugates in the fetal livers were 2.99 (13.1 nM) and 0.63 ng/g. The data are suspect because of the high levels of free BPA, which could be due to either uncontrolled conjugate hydrolysis by β-glucuronidase in the liver homogenates or introduction of contamination by ubiquitous aglycone BPA during tissue collection, storage, and analysis.

This paper reported method development with details. Total and aglycone BPA measurements were taken in the liver. Native BPA measurements were made (no dosing). Criteria on mass spectrometric methods, isotope dilution quantification, and adequate demonstration of QC in sample preparation and analysis were met.

The expected BPA concentrations in tissues - from even upper bound aggregate exposures in the general population (<1 µg/kg bw/d) - would be orders of magnitude lower than the reported values and even below the detection limits reported. This paper has no utility for HI or RA. The

tissues samples appear to be compromised from contamination with BPA and possibly artifactual BPA hydrolysis in the tissue.

Concurrent determination of bisphenol A pharmacokinetics in maternal and fetal rhesus monkeys (Patterson *et al.*, 2013)
This study describes the PK of BPA in maternal and fetal rhesus monkeys. The dose was 100 µg/kg body weight of d6-BPA given intravenously. LC/MS/MS analytical method was used to determine the concentrations of aglycone (active) and conjugated (inactive) d6-BPA in maternal and fetal rhesus monkey serum, amniotic fluid, and placenta. The study indicated that the concentrations of aglycone in the serum of fetal monkey is only 45% of maternal monkey (based on AUC) but the conjugates are 4.4-fold higher in the serum of fetal monkey than that of maternal monkey. The higher concentrations of conjugated metabolite in fetal monkey appear to be due to slower elimination of the metabolite.

This study should be useful in hazard analysis, RA, and understanding and critically evaluating other biomonitoring studies in both humans and animals. The PK data are useful in understanding the PK of BPA and its conjugated metabolite in the fetal rhesus monkey and should assist in evaluating results from human monitoring data, especially when considering reported concentrations of BPA (aglycone) in tissue, urine, and blood. The study is especially important for understanding the formation and elimination pattern of BPA conjugated metabolite.

PBPK models

Commentary: Dermal penetration of bisphenol A - consequences for risk assessment (Gundert-Remy., 2013)
This commentary discusses procedures to be used in making route-to-route extrapolations for BPA from PBPK modeling of oral and dermal exposures. Unfortunately, the authors use uncertain assumptions about absolute bioavailability of BPA in humans from both dermal and oral routes and ignore the prominent role of Phase II metabolism of BPA in the gastrointestinal (GI) tract that occurs before systemic metabolism in the liver. This commentary is not applicable to HI or RA because of the significant flaws in the pharmacokinetic approach.

Functional UDP-glucuronyltransferase 2B15 polymorphism and Bisphenol A concentrations in blood: results from physiologically based kinetic modelling (Partosch *et al.*, 2013)
The authors used a previously developed PBPK model for BPA to simulate human exposures to BPA. The model assumed a distribution of metabolic constants for Km and Vmax (based on *in vitro* studies with polymorphic forms of UGT2B15). The goal was to better understand the wide range of reported BPA serum levels reported in the literature. The theoretical exercise has merit in understanding inter-individual metabolic variability based on liver microsome data from 15 human donors but falls short in several key areas. The *in vitro* enterocyte (gut) metabolism was incorrectly used in the model by not accounting for differences in mass of the GI tract and liver. Thus the role of first pass metabolism in the GI tract was underestimated. Several hepatic and extra-hepatic UGT isoforms are active towards BPA *in vitro*, thus the clearance of BPA by

metabolism was probably underestimated based on IVIVE by assuming the predominance of a single specific hepatic isoform of UGT. The authors stated that polymorphic forms of UGT2B15 cannot explain the wide range of reported serum concentrations of BPA.

No information was reported on analytical methods used to generate the data used in the PBPK model. The simulation exercise reported in this paper has key deficiencies resulting in inaccurate dosimetry predictions for the aglycone BPA when administered by the oral route. Therefore, the utility of the simulation exercise is limited to observations on the range of simulated serum aglycone BPA concentrations that could occur as a consequence of polymorphic forms of a key metabolic enzyme. This PBPK model needs to be recalibrated. The model prediction of peak serum levels ranging from near 1-72 pg/ml (0.4 nM to 29 nM aglycone BPA) assuming an oral intake of 1 μg/kg does not seem plausible.

Prediction and evaluation of route dependent dosimetry of BPA in rats at different life stages using a physiologically based pharmacokinetic model (Yang *et al.*, 2013)
This manuscript describes a PBPK model developed in neonatal and adult rats to extrapolate age-dependent pharmacokinetics of BPA and its conjugated metabolites. The PBPK model was calibrated in adult rats using studies on BPA metabolism and excretion in the liver and gastrointestinal tract and pharmacokinetic data with BPA in adult rats. For immature rats, the hepatic and gastrointestinal metabolism of BPA was inferred from studies on the maturation of phase II enzymes coupled with serum time course data in pups. The adult rats received 100 μg/kg of d6-BPA by intravenous administration and oral gavage. The pups received oral gavage on postnatal days 3, 10, and 21.

Both aglycone BPA and BPA conjugates were measured using d6-BPA. HPLC/MS/MS and an internal standard of $^{13}C_{12}$-BPA were used. QC measurements were recorded. Individual serum data were collected.

The paper's findings and discussion are useful for HI and RA. This BPA PBPK model predicted the kinetics of aglycone BPA and its metabolites in young rats. Despite the uncertainty in model parameter estimates for describing small intestinal BPA metabolism in rat pups and enterohepatic recirculation of BPA conjugates, the PBPK analyses provide some insights into the probable role of the GI tract in the kinetic behavior of BPA and its metabolites. Species comparisons of BPA pharmacokinetics between rats, non-human primates, and humans suggest that immature rat BPA dosimetry for oral administration does not appear to be representative of BPA dosimetry in neonatal non-human primates and humans. To extrapolate from toxicity studies in neonatal rats (PND10 and younger) to infant children, the dose would need to be adjustment to a much higher level in humans.

Neurotoxicology Studies

Summary

Thirty-six studies were reviewed. Species examined were mouse (20 studies, utilizing 10 strains), rat (12 studies, 3 strains), and rhesus monkey and Lacuane sheep (1 study each). There were two

human population studies. The studies were primarily exploratory academic studies that were not designed to yield NOAEL values. The majority (22) of the studies addressed the effect of BPA exposure on behavioral endpoints. Many of the behavioral studies also included cellular and molecular endpoints such as receptor expression. Reviewers identified 7 of the studies as useful for HI, and, of these, one was also found useful for RA. The 36 studies are summarized below and discussed individually in the following pages.

BPA exposure was via the oral route (gavage or dietary) in 28 studies, and via subcutaneous (sc) or intraperitoneal (ip) injection in 9 (one study utilized both oral and sc routes). A general limitation for most studies is the lack of information on dose certification and homogeneity of the dosed diet. This information is essential for dose confirmation in feeding studies. The degree of control for exogenous exposure to BPA through the environment (diet, cage, bedding, food and water containers) was mixed, and many times not reported. Control was deemed excellent in only 9 studies.

Investigators in this set of papers were largely focused on the effects of developmental BPA exposure. In 27 studies, exposure occurred over some portion of gestation; in many of these, dosing to the offspring was extended through weaning. In some, pups were directly exposed to BPA, while others maintained exposure to the dams and exposed the pups via lactation. Lactational exposure was frequently cited as a deficiency by reviewers because investigators did not generally use a dose that was high enough to ensure adequate transfer of BPA to pups via lactation.

BPA doses ranged widely from the ng/kg to mg/kg range. Only 11 studies utilized at least 3 doses. Only one third of the studies included an estrogenic challenge as a positive or reference control; ethinyl estradiol was the most common choice. Exposure validation was attempted by measuring BPA plasma levels in only 4 of the animal studies. Human exposures were estimated from single urine samples. Four studies examined short-term or acute responses to BPA, and only one of those tested behavioral responses at the predicted Tmax following BPA exposure.

The endpoints examined were diverse. Behavioral endpoints included various assays for learning and memory, as well as behaviors thought to be related to emotional states (e.g., anxiety). Neuroanatomical endpoints included measurements of dendritic spine density and volumetric measurements of hypothalamic nuclei. Molecular analyses of various receptors, mRNA detection, and gene expression targets were conducted by some investigators.

The most common reviewer-identified deficiency regarding statistical analyses had to do with inadequate accounting for litter effects. Other frequent criticisms had to do with over-interpretation of marginally significant results and inappropriate extrapolation of findings to humans. There remains confusion in the BPA literature as to what constitutes a relevant human exposure.

One paper was identified as having been conducted in such a way that results could be used for both HI and RA. This study found little effect of low dose exposure to BPA on a number of behavioral parameters in adult, ovariectomized female rats treated acutely.

Neese *et al.* (2013) examined the effect of BPA on working memory in middle-aged ovariectomized (OVX) rats. Two doses of BPA (5 and 50 µg/kg) were tested and 17β-estradiol was used as a positive control. Exogenous sources of BPA were well controlled in the environment. Exposure was by oral bolus delivered via pipette tip. Animals were dosed 10-30 minutes prior to behavioral testing, which occurred over a period of 8-10 weeks following OVX. Three behavioral tests were conducted measuring working memory. There were no positive findings in the BPA treated groups. The positive control impaired performance on the working memory tasks, consistent with previous work.

Six additional studies that did not meet criteria for RA were identified as useful for HI.

Ferguson *et al.*, (2012) examined the effect of pre-and post-natal BPA exposure on behavioral parameters in the juvenile rat. Two doses of BPA were used (2.5 and 25 µg/kg/d) and two doses of ethinyl estradiol (5 and 10 µg/kg/d) were used as positive controls. Positive controls resulted in expected effects with an estrogen exposure. Exogenous sources of BPA were well controlled in the environment. Exposure was by oral gavage starting at gestation day (GD) 6 through delivery, and pups were then directly orally treated at the same dose through postnatal day (PND) 21. Locomotor, learning, and exploratory behaviors were evaluated. The overall conclusion of this study was that there is little or no effect of developmental exposure of BPA on juvenile rat behavior at these doses.

Two papers were by Cao *et al.* (2012, 2013). These studies measured estrogen receptor expression in various regions of the rat brain after either oral or sc exposure to BPA. Gestational exposure and postnatal exposure were examined separately in separate studies. Positive controls were included and environmental exposure to BPA was well controlled. Effects of BPA on receptor expression were reported. The relevance or relation to any (patho)physiological effects of BPA, including behavior, have yet to be confirmed.

He *et al.* (2012) examined the effect pre- and postnatal exposure to low doses of BPA on the neuroanatomy of sexually dimorphic regions of the rat brain. Methodology was the same as in Ferguson *et al.* (2012). Two doses of BPA (2.5 and 25 µg/bw kg/d) and two doses of ethinyl estradiol (5 and 10 µg/bw kg/d) were employed. Exogenous sources of BPA were well controlled in the environment. Exposure was by oral gavage starting at gestation day (GD) 6 through delivery, and pups were then directly orally treated at the same dose through postnatal day (PND) 21. Pups were sacrificed at 21 days and brains fixed and stained. Volumetric evaluation of the sexually dimorphic nucleus of the preoptic area (SDN-POA) was carried out. SDN-POA volume was found to be enlarged in the positive control groups for both males and females. In the BPA-treated groups, SDN-POA volume was slightly larger in males but not females. The authors noted that the BPA effect in males had not been observed at higher BPA doses in other studies.

A study by Kundakovic *et al.* (2013) examined the effect of BPA (3 doses) on DNA methylation patterns and behavioral endpoints in mice. Exposure occurred orally during gestation. Environmental exposure to BPA was partially controlled; there was no positive control. Testing in juveniles reported dose-related changes in play, exploratory, and anxiety-related behaviors.

Changes in methylation patterns from specific brain areas were also observed.

A study by Viguie *et al.* (2013) examined the effect of gestational BPA exposure on thyroid function in sheep. Administration was via diet; there was no positive control. PK analysis was conducted on both dams and newborns to verify exposure. Total and free T4 were reduced in the newborns. The effect was reversed by 2 months of age.

Human studies:

Lastly, there were two observational studies conducted in humans that should be described. The first, by Hong *et al.* (2013), reported on a cohort of 1008 South Korean children ages 8-11. BPA exposure was estimated from single urine samples and was associated with behavioral changes assessed by two different widely used behavioral assessments, without significant gender differences. The second study, by Perera *et al.* (2012), reported on a cohort of 198 children in a long-term study of environmental health in a population of minority women in New York City. Behavior was evaluated between ages 3-5 yrs. BPA exposure was estimated from single urine samples from mothers taken in the latter half of pregnancy and in the children at 3-4 yrs of age. Some behavioral changes were noted, primarily in boys. The authors of these studies appropriately cautioned against over-extrapolation of these findings given the limited nature of the BPA sampling and the observational nature of the behavioral reports.

Summary and conclusions:

Measuring an effect of BPA exposure on neurodevelopment remains problematic, particularly at low doses. While there is an established literature on the effect of exogenous estrogen exposure on behavior and neurodevelopment in rodents, allowing for some consensus on the behavioral endpoints and brain regions on which to focus, results of low-dose BPA studies have not yet been consistent enough to be placed in context with this literature. The most carefully controlled studies have tended to support the null hypothesis that BPA has little to no effect on neurodevelopment at low-dose exposures. Most of the studies reviewed here, particularly those that examine changes at the level of gene expression, yield findings that are difficult to interpret. No study succeeded at developing unifying hypotheses that explain both reported molecular and behavioral findings.

Individual Study Reviews
Oral Exposure

Perinatal bisphenol A exposure promotes hyperactivity, lean body composition, and hormonal responses across the murine lifespan (Anderson *et al.*, 2013)
The goal of this study was to assess effects of pre- and post-natal BPA exposure on spontaneous activity, energy expenditure, body composition, and hormones/adipokines at three ages in male and female mice. Wild type female agouti mice (6 weeks of age) were placed on 1 of 4 phytoestrogen-free diets (AIN-93G): 1) control (n=11 litters); 2) 0.05 µg BPA/kg chow (n=14 litters); 3) 50 µg BPA/kg chow (n=9 litters); 4) 50,000 µg BPA/kg chow (n=13 litters). BPA was obtained from the National Toxicology Program. There was no positive control group. After 2

weeks, females were paired with 8 week old pseudoagouti males. Caging was polycarbonate-free (material not specified) and water reported to be "BPA-free" but bottle material was not specified. Diets continued until offspring weaning at postnatal day 22 when all offspring were placed on the control diet. Only wild type (*a/a*) offspring were evaluated. The authors state that "approximately" 1 pup/sex/litter was evaluated. However, there were 21 total offspring evaluated from the 50 µg BPA/kg chow group and only 9 litters. Thus, it appears that same-sex pups from the same litter were counted as separate subjects.

Postweaning, offspring were housed with siblings but the number/cage is not specified. At 3, 6, and 9 months of age, subjects were individually housed for 7 days for acclimation. They were then weighed and evaluated every 5 sec in 20-min intervals for 72 hrs for O_2, O_2 for lean body mass, CO_2 production, spontaneous activity, and food intake. Afterwards, body composition was measured via NMR imaging (unanesthetized). At 9 months of age, after the 72 hr period of testing, mice were fasted for 5 hrs and then received an oral glucose tolerance test (gavage of 2 g glucose/kg). Glucose and insulin levels were measured (via ELISA) at baseline, 15, 30, 60, and 120 min. At 10 months of age, mice were sacrificed and serum levels of adiponectin and leptin measured (via ELISA).

All marginally significant findings ($p>0.05$ and <0.10) were reported; however, the results reviewed here only describe findings at $p<0.05$. At weaning, 0.05 µg/kg diet offspring weighed less than controls. Data presented in all figures for the 50 µg/kg group represent more than 1/sex/litter. O_2 is altered in females of all BPA groups at least at one of the three ages and is, in general, increased relative to control females. At the 3 and 6 month assessments, the data indicate a dose-response trend. The same endpoint was increased at 9 months of age in BPA-exposed males. CO_2 production is altered in 6 month old females of the 50,000 µg/kg BPA group and in 9 month old females of the 0.05 µg/kg BPA group. This same endpoint was only affected at 3 months of age in the 50 and 50,000 µg/kg BPA-exposed males. The respiratory exchange ratio (CO_2 production/O_2 consumption) was not affected by BPA exposure. There was a significant sex effect for O_2 consumption corrected for lean body mass, but there is no detail of treatment-related differences. In general, BPA-exposed females were more active than same-sex controls at all ages, while males exposed to the 50,000 µg/kg diet were more active only at 3 months of age. Food intake was generally decreased in BPA-exposed females, but only significant in the 0.05 µg/kg BPA group at 6 months of age. There were no significant differences in BPA-exposed male offspring. Females of the 0.05 µg/kg group weighed less and had lower fat mass than same-sex controls at 6 months of age. Females of the 50,000 µg/kg group had significantly lower baseline glucose levels. Females of the 0.05 µg/kg group had significantly lower adiponectin levels; however, there was no indication of a dose-response.

The study design incorporated three different BPA dose groups and the BPA exposure duration is a typical one (throughout gestation and lactation). Oral dietary exposure is appropriate and both sexes were evaluated. However, there is no information on chow consumption and thus, no information on the actual amount of BPA ingested. Since consumption changes throughout gestation and lactation, the doses of BPA likely changed during this time as well. Nor is there any information about the dams' body weight throughout gestation and lactation. Given that an adult female C57Bl/6 mouse weighs ≈ 18 g and gains 10-16 g during pregnancy, this likely means an

average body weight of ≈ 31 g. A pregnant C57Bl/6 mouse consumes ≈5 g chow/day. For the low and high BPA diets here (0.05 µg/kg and 50,000 µg/kg), this translates into BPA daily doses of ≈ 0.0078 and 7.8 µg BPA/kg body weight, respectively. That dose is an extremely small dose. The authors cite two references supporting their claim that those BPA doses "capture human physiologically relevant exposure", but one of those references is an earlier mouse study from the same lab in which free and conjugated BPA were measured in postnatal day 22 mouse liver and compared with human fetal liver samples from first or second trimester pregnancies (Anderson *et al.*, 2012), an inappropriate comparison in terms of developmental age and organ choice. Given the limited BPA that was transferred via lactation (Doerge *et al.*, 2010), the effects seem limited to prenatal exposure and any amount that may have been direct to the pup by food consumption prior to weaning. This particular strain of mouse (agouti) is typically used for epigenetic studies. The glucose tolerance test was well-conducted. Without indicators of variance in most of the figures or in any of the text, it is difficult to determine the magnitude of the effects. It appears that the same subjects were examined at the 3 ages, but separating group-housed male mice for 7 days and then returning them to the home cage can cause aggression and severe fighting. The statistical analyses appear to have been conducted adequately initially – that is, the initial analyses seem to be separated by sex (although this is stated differently in the "Data analysis" section) as the first sentence for each effect describes a p value for each sex. However, when there is only a marginally significant overall effect, post-hoc comparisons to the control group are conducted. The authors also misstate some of the information in the references they cite. For example, reference #46 treated rats with 2.5 or 25 µg/kg BPA, not 2.5 or 5 µg/kg BPA. Finally, the finding of decreased body weight and decreased body fat seems contradictory to the majority of developmental BPA studies.

This study has no utility for HI and no utility for RA. The amount of BPA consumed cannot be determined unless chow intake is measured and reported; thus, the actual doses of BPA are not known. Further, not all dose groups contained a minimum of 10/sex/group.

<u>Prenatal Exposure to Low Doses of Bisphenol A Increases Pituitary Proliferation and Gonadotroph Number in Female Mice Offspring at Birth (Brannick *et al.*, 2012)</u>
The objective of this study was to determine whether prenatal exposure to low doses (below the oral reference dose - ORfD) of BPA affects the developing pituitary, specifically gonadotroph cell number and parameters of hormone synthesis. The authors base their hypothesis in part on publications by Takahashi and Oishi (2000) reporting placental transfer of BPA after a single dose of 1 g/kg to pregnant rats; and on a paper by Fernandez *et al.* (2009) reporting disrupted function of the HPG axis in rats following subcutaneous injection to neonatal female rats of 500 or 50 µg/d BPA (dose range 2.5 – 62.5 mg/kg body weight) for 10 days.

The study was non-GLP using mice with mixed FVB, C57BL/6 background. Pregnant mice were dosed orally with either 0.5 or 50 µg/kg/day of BPA or vehicle beginning on Embryonic Day 10.5 through Embryonic Day 18.5. BPA source and number of animals per dosing group were not provided. BPA was dissolved in ethanol and diluted in tocopherol-stripped corn oil; negative control was with tocopherol-stripped corn oil. There was no positive control. All animals were housed in a facility with a 12L:12D photoperiod and fed a standard mouse diet. There was no mention of controlling for dietary estrogens or BPA leachate from caging or food and water

containers. Pups were harvested on PND1; heads were fixed for later sectioning. Pups were collected from 5-7 different litters per treatment group. For each treatment group, 6-8 pituitaries were examined. Pituitaries were sectioned and immunostained for proliferating cells (mKi67), gonadotrophs (LHβ and FSHβ), pituitary progenitor cells (PIT1), stem cells (SOX2). No statement was made about whether slide readers were blinded to treatment group. Pituitaries were also isolated and mRNA extracted for quantitative RT-PCR. Samples were run in duplicate. Samples were normalized to GAPDH. Statistical methods utilized ANOVA, two-tailed t-test (P = 0.05 for significance).

The reported findings were as follows. BPA-treated females but not males showed modestly (~10-30%) increased numbers of mKi67 positive (proliferating) cells. The effect was weak, and non-dose dependent. BPA-treated females and males showed modestly increased (~20%) numbers of LHb and FSHb immunoreactive cells (gonadotrophs); this effect was not dose dependent and no positive control was included. LHβ and FSHβ immunoreactive cells were not mKi67 positive leading to the conclusion that the apparent increase in the number of LHβ and FSHβ labeled cells was not due to increased proliferation of those cells. The mKi67 (proliferating) cells could not be positively identified. They were neither PIT1 (pituitary progenitor) cells nor were they SOX2-positive (stem) cells. LHβ mRNA and FSHβ mRNA increased slightly but the effect was only in females and was not dose-dependent. Other markers for gonadotropin activity were assayed: Egr1 and Nr5a1 and GnRH receptor. mRNA expression levels showed no consistent pattern of change. mRNA levels for other major hormones (POMC, GH, and TSH) also showed no changes. Prolactin protein levels and mRNA expression were unchanged. However, in a control experiment, the authors showed that prolactin mRNA expression may not be detectable until day 10. This control experiment was used to determine the time course of expression of PRL using CD-1 mice which were not treated with BPA. Pups at P1, P5, P10, and 4–6 mo of age, with six biological replicates for each age were evaluated with PRL mRNA expression observed at P10 and in adult mice.

The authors describe a modest finding of a slightly increased number of proliferating cells in the pituitary of rat pups exposed prenatally to BPA. The finding was not dose-dependent and was found only in females and not males. The identity of the cells positively labeled for proliferation could not be ascertained. They were not gonadotrophs, and did not appear to be other types of pituitary precursor or stem cells. The functional relevance of the finding was unclear. There were inconsistent changes in LH and FSH mRNA levels in females but not males, and no changes in mRNA levels of other pituitary hormones (GH, TSH, POMC, or PRL). The relevance to humans is not clear. The authors overstate the significance of their findings and conclude that low dose BPA significantly affects the developing pituitary in female mice, without a plausible mechanism and without a hypothesis to explain discordant findings. There was no measurement or calculation of fetal exposure. There was no control for external sources of BPA exposure. **This study has no utility for HI and no utility for RA.**

<u>Prenatal bisphenol-A exposure alters sex-specific estrogen receptor expression in neonatal rat hypothalamus and amygdala (Cao *et al.*, 2013)</u>

The goal of this study was to quantify the effects of prenatal, low dose exposure to BPA on expression of estrogen receptors, ESR1 (ESRα) and/or ESR2 (ESRβ) in neonatal hypothalamus

and amygdala. Rats were obtained from the breeding center of the National Center for Toxicological Research. To limit environmental contamination with estrogenic substances, the rats were maintained in polysulfone cages equipped with glass water bottles, and fed low phytoestrogen chow. Other environmental conditions included a 12:12h dark/light cycle, humidity of 45-55%, and room temperature of 22±1°C. Food and water were provided ad libitum. After pairings with males, pregnant dams were isolated in separate cages. The aqueous vehicle for drug preparation was 0.3% carboxymethylcellulose sodium salt (CMC). Ethinyl estradiol (EE) served as a positive control. From GD6 through GD21, dams were left untreated (*i.e.*, naïve), or gavaged with either vehicle, 2.5 µg BPA/kg BW/day, 25 µg BPA/kg BW/day, 5 µg EE/kg BW/day, or 10 µg EE/kg BW/day. The timing of dosage was postulated to cover a "critical window" for sexually dimorphic effects. The precision of drug delivery was achieved by using a Hamilton precision pump in conjunction with an animal weight scale. Male and female pups (n = 5-8/sex/group, 1/sex/litter) were decapitated one day after parturition. Their brains were frozen, cryosectioned into 20 µM coronal slices, and prepared for *in situ* hybridization histochemistry. Select brain regions were sampled, including regions of interest (ROIs) from the amygdala, anterior hypothalamus, and posterior hypothalamus. Uneven hybridization conditions were avoided by processing all of cryosections for each gene at the same time (*i.e.*, two large batches). Radio-labeled sense and antisense RNA fragments for ESR 1 and ESR2 genes were used as probes to detect receptor mRNA. After exposing tissue slides to X-ray film for 7-27 days depending on the gene and ROI, radiographic 14C labeled microstrips were used to construct optical density curves, and convert results to nCi/g tissue. Receptor quantification and distribution was carried out with imaging software. A custom-made statistical program was devised for two-way ANOVA analyses of sex- and exposure-mediated differences in the intensity of staining for ESR1 and ESR2, with consideration given to specific, anatomically matched brain regions (*i.e.*, regions of interest/ROI). Autoradiographs were sorted by treatment, and assigned to Groups: Grp1, naïve; Grp 2, vehicle; Grp 3, EE 5; Grp 4, EE10; Grp 5, BPA 2.5; and Grp 6, BPA 25.

As expected, sex-related differences could be seen in naïve tissues from Grp 1 in ESR1 and ESR2 expression, although these differences did not achieve statistical significance in some of the brain regions sampled. In general, ESR1 and ESR2 expression was decreased by gavage treatment with vehicle (Grp 2) vs. Grp 1. Some ROI exposed to either EE or BPA were found to have significantly increased mRNA levels for ESR1 and ESR2 when compared to Grp 2 tissues. The EE and BPA effects in ROIs from the posterior hypothalamus and amygdala were more dramatic than ROIs of the anterior hypothalamus. These results were suggested to reflect possible interactions between stress (*i.e.*, from gavage) and prenatal BPA exposure. Authors hypothesize that changes in ESR expression could cause behavioral and activity alterations, but the functional significance could not be established.

The effects of EE treatment on ESR expression demonstrate the effective use of a positive control and selection of a critical developmental period for responsiveness to BPA exposure. The investigators utilized stringent experimental controls to demonstrate regional effects on ESR expression with low-level, prenatal BPA exposure. Some limitations to the study include (1) the use of only 2 doses of BPA, where a minimum of 3-4 doses is needed to establish the slope of a dose-response relationship if linear or non-monotonicity; and (2) some statistical analyses were

performed on tissues that were derived from as few as 4 radiographs/ROI/sex. Overall, this paper provides sound scientific methodology. The study is a mechanistic study with end points that do not address the the End Point Measure criterium. Findings from this study should be placed in the context of findings from other studies conducted with the same experimental design. **This study has utility for HI. Because no firm conclusions could be drawn on the (patho)physiological effects, including behavior (*e.g.*, Ryan *et al.*, 2010; Ferguson *et al.*, 2011, 2012), or BPA mediated changes in mRNA levels of ESR1 and ESR2, the results do not have utility for RA.**

Perturbation of male sexual behavior in mice (*Mus musculus*) within a discrete range of perinatal bisphenol-A doses in the context of a high- or low-phytoestrogen diet (deCatanzaro *et al.*, 2013)
The goal of this study was to assess effects (and interactions) of soy-based and soy-free diets and pre- and post-natal BPA exposure on anogenital distance, sexual behavior, reproductive organ weights, and urinary levels of estradiol, testosterone, and creatinine in male mice. In Experiment 1, two weeks prior to breeding, CF-1 female mice (n=170) were placed on a low phytoestrogen chow or maintained on a soy-based chow. From gestational day 10 through lactational day 9, pregnant females were given 1 g of peanut butter containing 0, 0.175, 1.75, or 17.5 µg BPA (Sigma-Aldrich). There was no positive control group. Cages were polypropylene and water bottles were glass. Offspring were weaned on postnatal day (PND) 27 and ano-genital distance measured. Males were housed 3-4/cage and remained on the same diet as their dam. At PND 60, 1/litter was sacrificed for wet and dry weight measurement of testes, vesicular-coagulating, and preputial glands. On PND 90, 1/litter was paired with a female and left undisturbed for 3 weeks although females were monitored for signs of pregnancy during this time. If there was no sign of pregnancy after 24 days, that female was removed and a new female placed into the cage. In Experiment 2, all females were maintained on the soy-based diet throughout, and BPA doses in peanut butter were 0, 17.5, 175 and 1750 µg/g peanut butter. BPA treatment times, housing and wean dates were the same as Experiment 1. At ≈ PND 79, urine was sampled from 1/litter for analysis of estradiol, testosterone, and creatinine and an additional 1/litter was sacrificed for wet and dry weight measurement of testes, epididymides, vesicular-coagulating and preputial glands. At ≈ PND 95, each male was paired with a hormonally primed female for 2 hr and latency and number of mounts, intromissions, and ejaculations were measured.

In Experiment 1, dam body weight, gestation length, litter size and weight, weaning pup body weight, ano-genital distance, and sex ratio were not altered by BPA treatment or type of diet. Wet and dry weights of the vesicular-coagulating glands were decreased in mice on the soy-based diet exposed to 17.5 µg BPA (testes and preputial glands were unaffected). In general, BPA treated males exhibited a longer time to insemination than controls, regardless of diet. In a somewhat unorthodox post-hoc comparison, males on the soy-based diet treated with 17.5 µg BPA had a longer latency to insemination than controls on the same diet. In Experiment 2, gestation length, litter size and weight, and sex ratio were not altered by BPA exposure, but there is no mention of dam body weight, weaning pup body weight or ano-genital distance. BPA treatment did not alter adult body weight, wet or dry weights of testes, epididymides, preputial glands, vesicular-coagulating glands or urinary levels of testosterone, estradiol, and creatinine. Number of and latency to mounts, intromission, and ejaculation were not altered by BPA treatment. However, males treated with 17.5 and 175, but not 1750, µg BPA exhibited fewer intromissions. Number

of ejaculations was reduced in males treated with 17.5 µg BPA.

The sample sizes are adequate and the litter is the unit of analysis. Several BPA doses were evaluated, and there was good control of exogenous exposure to environmental estrogens or BPA. The duration of BPA treatment is appropriate, given the concern about potential effects due to developmental exposure. In some of the statistical analyses of Experiment 1, there are significant main effects of BPA with no significant interaction with diet and yet, the individual BPA groups within each diet are compared across diets. As one example, analysis of latency to insemination detected a significant main effect of BPA but neither the main effect of diet nor the interaction was significant. Yet, the post-hoc comparisons compared males on the soy-based diet in the highest BPA group (17.5 µg) to control males on the soy-based diet as well as control and BPA treated males on the low-phytoestrogen diet. The number of subjects/group is sufficient ranging 12-20. There is no mention of blind assessment for some of the endpoints. In particular, the assessment of male sexual behavior could be troublesome if not conducted blind to treatment condition. Some of the endpoints appear to indicate dose-response trends (*e.g.*, latency to insemination in Expt. 1) while others do not (*e.g.*, most of the wet/dry weights of reproductive organs). The BPA effects on male sexual behavior seem unrelated to BPA-induced alterations in urinary gonadal hormone levels. While there is no detail of dam pregnancy and lactational body weights, the authors state that the lowest dose in Expt. 1 (0.175 µg/day) is approximately equivalent to 3.5 µg/kg bw/day, and their highest dose in Expt. 1 (17.5 µg/day) is approximately equivalent to 350-500 µg/kg bw/day. Given the limited amount of BPA transferred via lactation (Doerge *et al.*, 2010), the effects seem limited to prenatal exposure and any amount that may have been direct to the pup by food consumption prior to weaning. The BPA doses that produced statistically significant effects on the measured endpoints are more than an order of magnitude above the highest estimates for human intake. **This study has no utility for HI and no utility for RA.**

Developmental treatment with bisphenol A causes few alterations on measures of postweaning activity and learning (Ferguson *et al.*, 2012)

The goal of this study was to assess the effect of pre- and post-natal low dose BPA exposure on postweaning activity and learning in the juvenile rat. This first author has previously investigated the effect of BPA and demonstrated (1) no effect of BPA on two pre-weaning sensorimotor behaviors (righting reflex and slant board behavior) in the rat and (2) an increase in the size of the sexually dimorphic nucleus of the preoptic area (SDN-POA) following BPA (males) or ethinyl estradiol (EE) (males and females).

The study was non-GLP. BPA was dissolved in 0.3% carboxymethylcellulose (CMC) solution. Pregnant females (Sprague Dawley rats from the NCTR breeding colony) were gavaged beginning on GD6. On PND 1, the eight offspring in each litter (after culling to 4/sex/litter) were orally treated (with the same dose as their dam had received) daily through PND 21. On PND 21, two offspring/sex/litter were weaned. Post-weaning, only one offspring/sex/litter was behaviorally assessed and the other served as a cagemate. There were 6 treatment groups: Naïve control (restraint but no gavage to control for stress), 12/sex; vehicle control 0.3% CMC/kg bw/day, 12/sex; BPA, 2.5 µg/kg bw/day, 11/sex; BPA 25.0 µg/kg bw/day, 12/sex; EE2 5.0 µg/kg bw/day, 11/sex; EE2 10.0 µg/kg bw/day, 12/sex. Exposure to exogenous environmental estrogens

was extremely well controlled. Rats were housed in polysulfone cages with polysulfone microfilter tops. Water was dispensed from glass bottles and low phytoestrogen chow was stored in metal containers. This environment began for the F0 generation at PND 21 and continued throughout the remainder of the study. Behavioral endpoints were assayed sequentially between PND 26 and 79. None of the behaviors reported here were sexually dimorphic. Observers were blinded to treatment.

- Novelty preference (PND 26-29) (note: due to technical problems, some data were lost from up to 3 animals of each group except the low dose BPA group)
- Open field activity (PND 40-42)
- Motor coordination (rotarod) (PND 43-44)
- Spatial learning and memory (Barnes maze) (PND 47-50)
- Acoustic startle (PND 54)
- Spatial learning and memory (water maze) (PND 75-79)

The litter was the experimental unit in all analyses. Treatment groups were compared to the vehicle control. For datasets in which there was a repeated measure (*e.g.*, test days 1–5 for watermaze endpoints), within-group correlations were modeled using the heterogeneous AR1 correlation structure (Little *et al.*, 1996). When data were not normally distributed, a log transform or an ANOVA on ranks (*i.e.*, Kruskal–Wallis ANOVA) was conducted. Statistical significance was defined as $p<0.05$.

Overall, behavioral alterations due to BPA exposure were weak, inconsistent, or not present, generally failing to reject the null hypothesis. Findings in the estrogen treated group were somewhat more prominent, as expected for a positive control. In general, the authors found more effects on activity measures and not on learning and memory. The authors summarized the effects of BPA and EE2 on specific behavioral parameters as follows:

- Novelty preference: Relative to the VEH group, both EE2 groups exhibited increased activity in the novelty preference assessment, an effect somewhat more prominent in males.
- Open field activity: Effects noted in males only: both the BPA and EE groups were significantly more active. Study authors note that a hypoactive vehicle group may be responsible for significance.
- Rotarod: Significant differences for latency to fall measurements were noted between sexes but not to treatment.
- Barnes maze: Latency to locate the escape box was increased in both sexes of the low dose EE2 group
- Water maze: BPA or EE2 treatment had no significant effects.
- Acoustic startle: Neither BPA nor EE2 exerted significant treatment effects.

The authors report findings of weak or non-significant effects of developmental exposure to low level BPA on the behavioral tests under study. Appropriate control for environmental BPA and an appropriate positive control lend validity to the study. Because only low doses of BPA were used, a threshold for BPA to affect these behaviors was not determined. **This study has utility for HI but only limited in utility for RA.** Although BPA levels were not directly measured in this study, these animals are from the cohorts treated with Delclos *et al.* (2014). The potential for similar internal exposures in the controls and the lowest dose limit utility of this study for RA

(see Churchwell *et al.* (2014)).

Postnatal exposure to low-dose bisphenol A influences various emotional conditions (Fujimoto *et al.,* 2013)

The goal of this study was to determine the behavioral effects primarily related to "anxiety" and "depression" of an indirect developmental exposure between postnatal days (PNDs) 1 and 7. Male and female rats were indirectly exposed to BPA through lactation. Their dams drank water containing 1 ppm of BPA during this time frame, resulting in a calculated exposure of 24 µg/kg/day dose of BPA.

Ten female Wistar rats were purchased at gestational day (GD) 10 (Kyudo Corp., Japan). They were fed a soybean based rat chow (CE-2, CLEA Japan Inc.) and tap water was contained in glass bottles. Caging type is not specified. BPA exposure to offspring (5 litters) was indirect, by adding 0.1 ppm BPA (Sigma Aldrich Co.) to the water bottles on offspring PNDs 1-7. There were 5 control litters. At PND 1, litters were culled to 4/sex/litter. Weaned rats (PND 21) were group housed with same-sex siblings (number/cage not specified but likely 4/ cage). Three behavioral tests were conducted with the offspring: a single 10 minute open-field test (OFT, PND 42), a single 5 minute elevated plus maze session (EPM, PND 49), and a single 15 minute forced swim test (FST, PND 63). Methods state all tests were manually or automatically recorded with a computer based tracking system. It is not clear which endpoints were manually scored or if these were scored blind to treatment. A two-way (sex x exposure) ANOVA with a post-hoc Fisher's protected least squares difference (PLSD) test was used to determine significant effects. Only behaviors were evaluated.

Analysis of duration of OFT rearing showed significant main effects of sex and exposure but no significant interaction; however, the authors then performed inappropriate post-hoc tests in which they evaluate control males against BPA males. A main effect of exposure indicates that both sexes differed from both sexes of other treatment groups. The appropriate post-hoc comparison would be to compare the average of the BPA males and females to the average of the control males and females. Most endpoints in the EPM exhibited significant main effects of sex (but no significant effect of exposure and no significant interaction), indicating that males and females of all groups differed. However, the authors then evaluate sex differences within each exposure group (an inappropriate post-hoc comparison). There were main effects of exposure and sex in many FST endpoints; however, the authors evaluated sex differences within each exposure group – which is only appropriate if there was a significant interaction. The main effects of exposure seemed to indicate higher durations of immobility, a shorter latency to immobility, and lower levels of limb movement in the BPA exposure group. The authors concluded that BPA's action on androgen receptors may be responsible for the demasculinization/feminization of male behavior.

The design of experiment is suboptimal for several reasons. BPA exposure to rodent offspring indirectly through lactation from dams exposed to 24 µg/kg bw/day BPA has minimal value. Levels of BPA in milk would be ≈ 0.003 % of dam serum (Doerge *et al.*, 2013). Thus, the exposure to the offspring would be ≈ 0.1 µg/kg bw/day. In a rat, this would result in blood levels in the femtomolar range, which is at least an order of magnitude below almost all physiological

receptor binding Kd known. Thus, there is no plausible mechanism of action. Although the number of subjects/group is adequate (n=16-20/sex/group), the litter is not the unit of analysis. There were 5 control litters and 5 BPA-treated litters, a major limitation of experimental design. There are multiple reports describing the drawbacks of ignoring litter effects (Haseman *et al.*, 2001; Holson *et al.*, 2008), and it is quite clear that the litter must be the unit of analysis and that same-sex siblings cannot be considered as individual subjects. Thus, when analyzed correctly, this study has n=5/sex/group. It is not clear why two of the three behavioral tests were conducted during adolescence/puberty (PND 42 and PND 49) and the FST was conducted at adulthood. Further, the authors did not explain why there was an object inside the open field or why only one FST test was conducted. A typical FST consists of two days and behavior is only measured on the second session (when presumably, the animal learns there is no escape).

This study is of no utility for HI and no utility for RA. The study assessed only one BPA dose (24 µg/kg bw/day). The litter was not selected as the unit of analysis. The caging material was not specified and the diet was soy-based. The exposure level to the offspring would be so low that their peak BPA blood levels would be predicted to be in the femtomolar range. Thus, the BPA levels would be below the Kd of almost all known receptor ligands and affinities.

<u>The effects of bisphenol A on emotional behavior depend upon the timing of exposure, age and gender in mice (Gioiosa *et al.*, 2013)</u>

The purpose of this study was to determine the behavioral effects, primarily related to anxiety and possibly depression, of developmental exposure to 10 µg/kg/day of BPA with respect to the timing of the exposure, maternal environment, sex and age at testing. A comparison of prenatal exposure from PND11 until birth versus postnatal exposure (birth to weaning) was made.

Female CD-1 mice were fed a soy-based diet ad lib throughout the study. This diet was specifically chosen to avoid the "side-effects" of soy-free diets. Caging and water bottles were polycarbonate, but were stated to be new. After mating and beginning on gestational day (GD) 11, the pregnant dams (mating procedures and age not specified) consumed 0.1 ml corn oil/50 g bw/day without (n=27) or with (n=15) 10 µg/kg BPA (source not specified) included until offspring postnatal day (PND) 8. The concentration of the BPA in the corn oil was not given, and it appears that one exposure/day to BPA in corn oil occurred. Within 12 hr after birth, litters (culled to 4–6/sex/litter) were cross fostered. The 15 litters exposed to BPA during gestation were cross-fostered to control dams (*i.e.*, corn oil only during pregnancy) and vice-versa. This resulted in 15 litters exposed to BPA only prenatally and fostered to control dams and 15 control litters fostered to dams which were exposed to BPA during gestation and continued to receive BPA throughout lactation. The remained 12 control litters were cross-fostered within this treatment group to control for any effects of the fostering procedure. Thus, all litters were cross-fostered. BPA or corn oil exposure ended at offspring weaning (PND 25) at which time the offspring were group-housed with same-sex siblings. Behavior of the offspring was tested at PND 28-30 and PND 70 with different subjects assessed at each age. A 20 minute novel environment test was conducted at PND 30, and at PND 70, a 5 minute free-exploration open field session and a 5 minute elevated plus maze (both adult tests were conducted on the same day). Analysis of videorecording of the behavioral data was performed by an observer blind to treatment. Data were analyzed via two-way ANOVAs with sex and treatment as factors. Post-hoc

comparisons were done using Tukey's HSD test, which the authors state does not require an overall statistical significant ANOVA result.

The control group exhibited a significant sex difference in the time spent in the novel environment; however, neither BPA group (prenatal exposure only or lactational exposure only) exhibited a significant sex difference via Tukey's HSD test. This effect appeared to result from decreased time in the novel environment by BPA-exposed females. Lactationally-exposed male and female offspring entered the novel environment significantly more quickly than controls. Both BPA groups exhibited a significantly shorter duration of RA behavior. Lactationally-exposed females had a higher percentage of time engaged in self-grooming than control females. Latency to enter the open field was not affected; however, lactationally-exposed mice had a significantly shorter duration of time in the open field than controls. Lactationally-exposed females had a significantly shorter duration of time in the center of the open field and fewer returns to the home cage, but this is likely due to their overall shorter duration in the entire open field. Prenatally- and lactationally-exposed females had a significantly shorter duration of RA behavior than control females. In the analysis of elevated plus maze activity, prenatally- and lactationally-exposed females had a significantly longer duration in the closed arms and significantly fewer open arm entries than control females. Lactationally-exposed males had a significantly longer duration while lactationally-exposed females had a significantly shorter duration in the central area than same-sex controls.

The postnatal exposure data have minimal utility because the experimental design is suboptimal. BPA levels in milk would be ≈ 0.003% of dam serum (Doerge et al., 2010). Thus, offspring lactational exposure from a dam exposed to 10 µg/kg bw/day would be ≈ 0.03 µg/kg bw/day. This would result in peak blood levels in the femtomolar range in a mouse, at least an order of magnitude below almost all physiological receptor binding Kd. Thus, no plausible mechanism of action can be proffered. In the prenatal exposure group, although the dams' dose is relatively high, the calculated peak levels of free BPA present in fetal serum from oral BPA doses of 10 µg/kg/day to a mouse dam would be less than 100 pM in rodent and, because of rapid elimination, levels would be in the less than 1 pM within approximately four hours.

Other potential shortcomings include the authors purposely choosing to use a soy-based diet and polycarbonate caging and water bottle material. Only one BPA dose was evaluated. There was no control group that was not cross-fostered. Cross-fostering can induce "stress"-like effects in offspring. The authors appear to have selected the post-hoc comparisons without regard to the overall ANOVA results. That there were virtually no BPA levels present in the neonates from lactational exposure and the authors reported that lactational BPA exposure produced the same or greater effects as prenatal exposure casts doubt upon the validity of their results. A soy-based diet was used, which could confound any estrogen-related effects produced by BPA and there was no control for potential environmental estrogen exposure via caging and water bottle material. Only one dose of BPA was tested. **This study has no utility for HI and no utility for RA.**

<u>Low oral doses of bisphenol A increase volume of the sexually dimorphic nucleus of the preoptic area in male, but not female, rats at postnatal day 21 (He *et al.*, 2012)</u>
The objective of this study was to determine whether prenatal exposure to low doses of BPA

affects the neuroanatomy of sexually dimorphic regions of the rat brain. The size (volume) of the sexually dimorphic nucleus of the preoptic (SDN-POA) area of the rat brain varies with gender (3-8 times larger in males) and is known to be developmentally affected by steroid hormones (testosterone, estradiol).

The study was non-GLP. Sprague-Dawley rats were dosed by oral gavage 2.5 and 25 µg/kg bw/d BPA and 5 and 10 µg/kg/d ethinyl estradiol (EE2). Vehicle controls: 0.3% carboxymethylcellulose (CMC); naïve controls were removed from the cage and restrained but not dosed. Exposure to exogenous environmental estrogens was extremely well controlled. Rats were housed in polysulfone cages with polysulfone microfilter tops. Water was dispensed from glass bottles and low phytoestrogen chow was stored in metal containers. Adult SD rats were mated and females were dosed starting at GD6; following parturition pups were dosed orally from PND1-21. At weaning on PND 21, pups underwent perfusion with PBS, followed by perfusion-fixation with 4% PBS-paraformaldehyde, and brains were removed. Endpoints assayed and methodology used: After 48 h of postmortem fixation followed by 20% sucrose-PBS treatment for 48 h, each brain was sectioned coronally into 30 µm thick slices. Brain slices were freefloating and preserved in 2.5×10^{-4} sodium azide-PBS solution for 4–15 months. A total of 155 rat brains were evaluated (n=10–15/sex/group). One offspring/sex/litter was used in volumetric analyses. The SDN-POA was defined by regions co-stained with calbindin-D28K (CB28) and DAPI and imaged using NIH Image J software. The surrounding structures served as negative controls for CB28 labeling. To minimize potential bias in immunofluorescence processing, brain slices obtained from both sexes and several treatment groups were prepared in batches. Given that the SDN-POA structures can be bilaterally asymmetrical, the left and right volumes for each subject were averaged. All volumetric evaluations were conducted blind to treatment. The left and right volumes were averaged for each subject and this average volume was used in statistical analyses. Average SDN-POA volumes were analyzed using a two-way ANOVA with factors of treatment, sex, and the interaction (SigmaPlot V. 11, Systat Software Inc., Chicago, IL). Where appropriate, comparisons to the vehicle control group were conducted using the Holm–Sidak method.

SDN-POA volumes were found to be enlarged in the positive control EE2 groups for both males and females. The SDN-POA volumes of females treated with 5.0 or 10.0 µg/kg/day EE2 were 142% and 181% larger than those of same-sex vehicle controls, respectively. For males, SDN-POA volume was significantly larger (22%) at the higher EE2 dose only. The average SDN-POA volumes were unaffected by BPA in females, but were found to be significantly larger in males when compared to those of vehicle control males. The SDN-POA volumes for males in the 2.5 and 25.0 µg/kg/day BPA groups were 32% and 21% larger than those of vehicle control males, respectively. The finding in males was somewhat unexpected and has not been confirmed in other studies; specifically, substantially higher BPA doses have been reported to have no volumetric effect on the SDN-POA in either sex. The authors thoroughly examined other explanations for the finding, including methodological issues surrounding measurements of the SDN-POA, as well as potential non-estrogenic pathways for BPA action.

This study is a companion to a separate study that evaluated other physical and behavioral parameters. Findings from this study should be placed in the context of findings from other

studies conducted with the same experimental design as the endpoints evaluated in this study are mechanistic and do not address the End Point Measure criterium. **The study has utility for HI.** Because no firm conclusions could be drawn on the (patho)physiological effects (including behavior, *e.g.*, Ferguson *et al.*, 2011, 2012) of BPA mediated changes in the SDN-POA, the results do not have utility for RA. Although internal dosimetry for BPA and EE2 were not evaluated in this study, Churchwell *et al.* (2014) provide relevant pharmacokinetic commentary for this dosing paradigm.

Sex and dose-dependent effects of developmental exposure to bisphenol A on anxiety and spatial learning in deer mice (*Peromyscus maniculatus bairdii*) offspring (Jasarevic *et al.*, 2013)
The goal of this study was to assess the effects of dietary BPA or ethinyl estradiol exposure on serum BPA levels, spatial learning, and anxiety behavior in male and female deer mice offspring. Two weeks before breeding, 25 female virgin deer mice were placed on 1 of 5 diets: 1) low phytoestrogen diet, 2) 50 µg/kg diet BPA (source not specified), 3) 5,000 µg/kg diet BPA, 4) 50,000 diet µg/kg BPA, or 5) 0.1 µg/kg diet ethinyl estradiol (EE2, positive control). Caging material was polypropylene and water bottles were glass. It is not clear how long females remained with males (n=25) for breeding, but it is clear that experimental diets were available during breeding so that males were also exposed. Some dams remained on the experimental diets for ≈ 12 months and serum sampled from controls, those on the 50,000 µg/kg BPA diet, and those on the EE2 diet for measurement of conjugated and unconjugated BPA. Only litters with 2 or more offspring were used. Some females were bred more than once to obtain sufficient numbers of subjects. At weaning, offspring were placed on the control diet and housed with 1-2 same-sex siblings until ≈ postnatal day 60 when they were individually housed. Spatial learning was measured 1 week later for 2 trials/day for 7 consecutive days in a Barnes maze. If subjects did not enter the escape hole within 30 sec, a barn owl recording was played for motivation, but it is not clear if other subjects were nearby during this time. Latency, path length, errors, and search strategy (*i.e.*, random, serial, or direct) were averaged for the 2 trials/day. Latency, path length, and errors are numerical values, but it is not clear how search strategy averages were determined. Anxiety behavior was measured 1 week later via an elevated plus maze (EPM) session.

Reported serum levels of conjugated and unconjugated BPA averaged ≈ 58-59 and 5.5 ng/ml, respectively, in dams on the 50,000 µg/kg BPA diet. The presence of unconjugated BPA in the blood of control and EE2-treated mice at levels up to 0.8 ng/ml and an unusually high aglycone fraction in blood of high dose-treated mice (5.5/59 = 9% when compared to gavage administration studies where the aglycone fraction in mice was 0.5% of the total BPA at C_{max}, Doerge *et al.*, 2011), suggest that sample contamination could have produced spurious results. BPA effects on spatial learning were complex, in part, due to the complicated statistical analyses. For this review, only analyses which took the litter of origin into account will be detailed. There were significant sex effects on the probability of direct search strategy (DSS) use on test days 5 and 7 (days 1-4 and 6 could not be analyzed): control males used the DSS more than control females on days 5 and 7; males of the 50 µg/kg BPA diet group used the DSS more than same-treatment females on day 7 only; females of the 0.1 µg/kg EE2 diet group used the DSS more than same-treatment males on day 7 only. Significant effects of BPA or EE2 exposure included

(relative to same-sex controls): 50,000 µg/kg BPA males used the DSS less on days 5 and 7; 5,000 µg/kg BPA males used the DSS less on day 7; 5,000 µg/kg BPA females used the DSS more on day 7; 0.1 µg/kg EE2 males used the DSS less on day 7; 0.1 µg/kg EE2 females used the DSS more on day 7. Analysis of latency indicated: control males had shorter latencies than control females; 0.1 µg/kg EE2 males had longer latencies than same-treatment females; relative to control males, 5,000 µg/kg BPA males, 50,000 µg/kg BPA males, and 0.1 µg/kg EE2 males had longer overall latencies. BPA or EE2 exposed females did not differ from control females. Control males committed fewer errors than control females; relative to control males, 50,000 µg/kg BPA and 0.1 µg/kg EE2 males made more errors. Control males had a longer EPM open arm duration than control females; relative to control males, 50,000 µg/kg BPA, 5,000 µg/kg BPA, or 0.1 µg/kg EE2 males had shorter open arm durations. Similarly, control males exhibited a shorter closed arm duration than control females. 50,000 µg/kg BPA, 5,000 µg/kg BPA, and 0.1 µg/kg EE2 males had longer closed arm durations than control males.

Both sexes were evaluated and a positive control group included. The statistical analyses are complicated, and they were done twice because some dams were bred twice: once using the individual as the unit of analysis and again, using the litter as the unit of analysis. This is problematic since it appears (but not stated) that dams remained on BPA or EE2 diets until re-breeding. Gestation duration for deer mice is 22-26 days and offspring are typically weaned at 18-24 days of age so subjects from the first breeding resulted from dams on the treated diets for ≈ 54-64 days while subjects from the second breeding resulted from dams on the treated diets for 94-114 days. Longer durations treated diet consumption could be a potential confound. It is not clear if males had access to the different diets during breeding. If so, they should have remained in that group for subsequent breedings (*i.e.*, a male bred with a BPA female in the 1st breeding should never have been bred with a control or EE female in the 2nd breeding). Further, since dams were bred twice, some of the subjects resulted from the same dam, and it is not known if the same male was used for the 2nd breeding. Suppl. Table 1 indicates there were 3 males tested from litter 5 of the 5,000 µg/kg BPA diet. It is not clear how this was handled in the analyses and this table does not indicate which litters resulted from the 1st and 2nd breedings. Search strategy categorization was done manually but whether this was blind to treatment status is not stated. There is no description of food intake during gestation/lactation so actual BPA and EE doses are not known. Pregnant deer mice typically weigh ≈ 23.0 g and consume ≈ 0.17 g/day/g body weight (Carlsen, 2012); thus, the lowest BPA dose here (50 µg/kg) would result in ≈ 8.5 µg BPA/day/kg body weight. Using the litter as the unit of analysis, there was only one statistically significant effect at the 50 µg/kg dose. It should be noted that there could have been some direct pup exposure as deer mice began to eat solid food at ≈ 15 days of age (Hammond and Kristan, 2000). Given the limited BPA transferred via lactation (Doerge *et al.*, 2010), the effects seem limited to prenatal exposure and any amount that may have been direct to the pup by food consumption prior to weaning. **This study has no utility for HI and no utility for RA.** Number of subjects/group is less than 10 and some subjects were same-sex siblings. Dams were bred twice and thus, the subjects resulted from dams having differing durations of BPA exposure.

<u>Perinatal BPA exposure demasculinizes males in measures of affect but has no effect on water maze learning in adulthood (Jones *et al.*, 2012)</u>

The goal of this study was to assess effects of oral BPA treatment during gestation and lactation on Long-Evans male and female offspring performance on measures of depression, anxiety, and spatial learning/memory. Adult female Long-Evans rats trained to consume corn oil from a syringe as preparation for BPA treatment were paired with a male and sperm plug positive females were individually housed in polysulfone cages with BPA-free water sacks. Diet was a standard chow (presumably soy-based, but not specified). BPA treatment began on gestational day 7 and continued through lactational day 14. Each treatment group consisted of 3 plug positive females, except as noted: 1) corn oil vehicle, 2) 5 µg/kg bw/d BPA, 3) 50 µg/kg bw/d BPA, 4) 500 µg/kg bw/d BPA (n=2 for this group) and, 5) 5,000 µg/kg BPA. There was no positive control group and the BPA source is not specified. Litters were culled to 4/sex, except for the 500 µg/kg bw/d BPA group in which litters were culled to 6/sex and group housed. All offspring in a litter were used and considered as individual subjects. Behavioral testing began at postnatal day 90. Standard procedures were used for water maze, forced swim, and elevated plus maze (EPM) testing; however, it is not clear that data from the forced swim test were collected blind to treatment status. Water maze endpoints were analyzed via repeated measures ANOVA but it is not clear how the 4 daily trials were handled. EPM endpoints were analyzed in two separate analyses: the first analysis separated the two sexes (one ANOVA/sex) and the second analyses used t-tests to assess sex differences within each treatment group. Analysis of day 2 forced swim test endpoints are described as using a repeated measures ANOVA; but it is not clear what the repeated measure is if only day 2 data were analyzed. Although not described in the methods text, the results indicate that forced swim test endpoints were analyzed similarly to the EPM data; that is, two separate sets of analyses.

There were no significant effects of BPA treatment on any water maze endpoint. Females swam significantly faster than males. In the sex-separated analyses of EPM endpoints, 500 µg/kg females produced significantly more fecal boli than control females. Females of the 5 µg/kg group exhibited a significantly higher percentage of open arm entries than control females. In the analyses of males only, 5 µg/kg bw/d males had a significantly higher distance traveled than control males. In the second set of elevated plus maze analyses, the expected sex effects were apparent in the control group. Those sex effects in the control group are listed as significant in the text and in Figures 1 and 2; however, Table 1 lists a different (and not significant) p value for distance traveled and mobility duration. Similar t tests for the 5 µg/kg bw/d group indicated a significant sex effect only for number of fecal boli. With the exception of number of center entries, all of the significant sex effects reported for the control group were apparent in the 50 µg/kg group. Significant sex effects were noted in the 500 µg/kg bw/d group for distance traveled, mobility duration, and center entries. In the 5,000 µg/kg bw/d group, significant sex effects were noted for mobility duration and number of fecal boli. When forced swim test endpoints were analyzed separately by sex, there were no significant treatment effects in either analysis. When the control group was analyzed for sex differences using t tests, females had a significantly shorter duration of immobility, increased number of rotations, and a longer latency to immobility. In the 5 µg/kg group, females also had a significantly shorter duration of immobility, and decreased numbers of immobile and mobile episodes. In the 50 and 500 µg/kg bw/d groups, females had a significantly shorter duration of immobility and increased number of rotations. In the 5,000 µg/kg bw/d group, all collected endpoints exhibited significant sex

differences which were in the same direction as the control group (*i.e.*, immobility duration, number of rotations, latency to immobility, and number of immobile and mobile episodes).

Both sexes were evaluated; however, there was no positive control group. Although the number of subjects/group is adequate (n=12/sex/group), the litter is not the unit of analyses. The 12 control males were produced from 3 litters and the twelve 500 µg/kg bw/d males were produced from only 2 litters. There have been multiple reports describing the significant drawbacks of ignoring litter effects, and it is quite clear that the litter must be the unit of analysis and same-sex siblings cannot be considered as individual subjects. Thus, when analyzed correctly, this study has n=2-3/sex/group. Further, treatment groups were culled to litters of different sizes. Although this may not have had major effects, culling to different sizes can affect later development (Chahoud and Paumgartten, 2009; O'Dowd *et al.*, 2008). It is not clear if the error bars on the figures are standard error of the mean or standard deviation. Given the limited BPA transferred via lactation (Doerge *et al.*, 2010), the effects seem limited to prenatal exposure and any amount that may have been direct to the pup by food consumption prior to weaning. The litter was not the unit of analysis in this study, and it is not clear if the diet was a low phytoestrogen chow. **This study has no utility for HI and no utility for RA.**

<u>Maternal bisphenol A oral dosing relates to the acceleration of neurogenesis in the developing neocortex of mouse fetuses (Komada *et al.*, 2012)</u>

The purpose of the study was to evaluate the mechanisms that promote abnormal stem cell proliferation, differentiation, and migration of intermediate progenitor cells (IPCs) and radial glial cells (RGCs) within the ventricular (VZ) and sub-ventricular (SVC) zones during neocortical development of mouse fetuses exposed *in utero* to BPA. C576BL/6 mice were purchased from Japan SPC, Inc., housed in polypropylene cages with stainless steel lids, and maintained in a 12:12 h light-dark cycle at a temperature of 24 ±1°C and a relative humidity of 55 ±5%. The Guidelines of an Animal Research Committee were followed. Food (a soy-based diet) and water were provided *ad libitum*. Exposure to non-dietary estrogenic contaminants was avoided or controlled by using glass water bottles with Teflon seals, and using a supplier that provided a certification analysis for each lot of food. BPA (Sigma Aldrich, purity unknown) was prepared weekly in a corn oil suspension, and administered via oral gavage to pregnant dams at embryonic day (E) 8.5 to E13.5. Fetuses were collected at E14.5. A preliminary bracketing experiment (N=3 litters) reported that BPA dosing at 20 µg/kg bw/day did not produce hyperplasia in the fetal cortical plate, but BPA at 200 µg/kg bw/day did, so that higher dose was used for the remainder of the study. The calculated intake level for the high BPA dose was 3 µg BPA/day; however, the embryonic serum levels were not determined. Negative control dams received an equivalent volume of corn oil; a positive control was not employed.

Head size and body weight measurements were obtained from male and female subjects from BPA (N=6) and control (N=6) litters, and no BPA differences were found. Another set of female only fetuses from high BPA dosing (N=3 litters) and control (N=3 litters) were labeled for S-phase kinetics by administering sequential intraperitoneal injections of 2 thymidine analogs, 5-Chloro-2′-deoxyuridine (CldU) followed by 5-Iodo-2'deoxyuridine (IdU). Fetal brains were preserved for tissue sectioning and staining with a general morphology dye (hematoxylin and eosin) or immunostaining. Cell cycle kinetics were determined by counting the number of CldU

and IdU positive cells in the cortical layer in anatomically matched sections. Antibody detection of CldU, IdU, neural class III β-tubulin, Ki67 (nuclear protein marker for proliferative cells), Tbr2 (T-brain gene-2, transcriptional marker for differentiation of IPCs), Pax-6 (paired box protein, marker for differentiation of RGCs), nestin (marker of radial fibers of RGCs), and DAPI (4',6-diamidino-2-phenylindole, tags DNA) was conducted.

The study reports findings of hyperplasia (increased thickness) of the cortical plate (CP) of the dorsal telencephalon in BPA fetuses, suggestive of accelerated neurogenesis. This was accompanied by increased exiting of cortical cells from the cell cycle; altered nuclear migration along radial fibers; decreased proliferation of neural stem cells; and reduced/delayed differentiation of RGCs to IPCs. The authors concluded that *in utero* BPA exposure inhibited the proliferation and promoted the differentiation of radial glial cells and intermediate progenitor cells and shortened the radial fibers of radial glial cells in the SVZ/VZ. Those effects were suggested to be caused by accelerated neurogenesis of neural stem/progenitor cells in the dorsal telencephalon.

The litter was not the unit of statistical analysis used in this study. The 9 female fetuses/group were drawn from three control and three BPA litters. Only one sex was evaluated, although the endpoints could have been examined in both sexes. The diet was high in phytoestrogens. The authors did not state whether the cell counting was done blind to treatment. Only one BPA dose was studied. **This study has no utility for HI and no utility for RA.**

<u>Effect of fetal exposure to bisphenol A on brain mediated by X-chromosome inactivation. (Kumamoto *et al.*, 2013)</u>

The objective of this study was to test the effect of prenatal BPA exposure at a critical period of brain development in mRNA levels of XCI and other X-linked genes related to brain development. Twenty pregnant ICR mice received either BPA in corn oil at 0.02 (n=7) or 50 (n=5) mg/kg bw/day or corn oil (n=8) on day GD 6 and 15 by "compulsory oral administration." Filtered tap water and normal diet (CE-2, CLEA Japan) was provided *ad libitum*. Weight and anogenital distance (AGD) of female pups was measured on PND2, 4, and weeks 3 (PND21) and 7 (PND28). Cerebrum mRNA analysis was conducted using real time PCR. Estradiol (E2) levels in serum were measured. Statistical analysis used Mann-Whitney U test for differences between means; $p<0.05$ was considered significant.

Increased body weight at all the time points in the high dose group and on PND21 in the low dose group were reported. Cerebrum weight was increased on PND2 and 4 in the high dose group but the cerebrum:body weight ratio was decreased for the high dose female pups on PND4 and 21 and for the low dose on PND21 only. XCI related genes had changed mRNA expression compared to vehicle control: Xist mRNA was downregulated in the high dose on PND21 and 28 and in the low dose on PND28. *Tsix* mRNA was upregulated in only the high dose animals on PND2, 21 and 28. Other genes that showed decreased mRNA expression in the high dose or both high dose and low dose female pups were Fmr1 (PND21 and 28, high dose only), Gdi1 (PND21 and 28 high dose and PND28 low dose), Nlgn3 (PND21 and 28 high dose and PND28 low dose), and Pak3 (PND21 and 28 high dose and low dose), AR (PND28 high and low dose). Increases were seen in mRNA levels of ERα in the low dose group on PND28. AGD was reported as

shortened on PND 4, 21, 28 in high dose and PND21 in low dose only. Serum E2 levels measured lower on PND21 in the low and high dose groups but not on PND28.

This study has several limitations. Animal number is low (n=5-8/group). There is no assurance that the intended exposure to BPA was achieved. The normal diet that was provided did not control for phytoestrogen exposure. It is not clear if the housing material was BPA free. Litter effect has not been controlled adequately. The data analysis is not adequate. A two-way ANOVA must be run first and then a post hoc test to determine between groups differences because there are both dose and developmental time point factors have to be considered. The rationale for using Mann-Whitney U tests rather than parametric tests was not clear based on the graphical data presented. There was a large number of measurements for each statistical unit (the litter) – 15 measurements (mRNA from 10 genes, body weight, cerebrum weight, cerebrum relative weight, serum E2, and AGD) at 4 time points. Type 1 errors are highly likely in this type of scenario. Mann-Whitney test does not adequately control for repeated measures in each group. For many genes that were measured, the expression levels are very low. As such, large errors are likely. None of the genes, except *Tsix*, had changes varying more than 35% of control levels. The only changes in expression that look to be possibly significant are the increased expressions of *Tsix*. However, since expressions are increased rather than decreased and *Tsix* suppresses over expression of X chromosome genes, it is not clear whether this is a detrimental effect. The claim that there are big differences in the control of the expression of genes related to X chromosome gene expression is not supported by the data. It is noted that large differences are not seen in any of the various genes evaluated in the control or treatment groups except *Tsix.* Authors report BPA affecting anogenital distance, estradiol levels, and estrogen receptor levels. These have not been seen by other investigators as well as in the NCTR BPA studies using the same doses used in the authors' study. **Given the limitations of this study, this study has no utility for HI and no utility for RA.**

<u>Sex-specific epigenetic disruption and behavioral changes following low-dose *in utero* bisphenol A exposure (Kundakovic *et al.*, 2013)</u>

The objective of the study was to examine the consequences of maternal exposure to BPA during pregnancy by evaluating epigenetic and behavior changes in juvenile and adult male and female offspring. Pregnant BALB/c (n = 12-17/group), housed individually in BPA free (polysulfone) cages received 2, 20, and 200 μg/kg bw BPA or vehicle (tocopherol stripped corn oil) daily via oral administration from GD 0–19. The identity of the diet or the type of water bottles used is not identified. At weaning on postnatal day (PND) 28, 6 male and 6 female offspring from 7-8 litters/group were sacrificed, whole brains were dissected to obtain prefrontal cortex, hippocampus, hypothalamus sections and RNA and DNA were extracted. Quantitative real-time PCR was used to assess gene expression for 1) estrogen receptor (ER) genes: *Esr1*, *Esr2*, *Esrrg* and 2) DNA methylation genes (*Dnmt1* and *Dnmt3A*). Subsequently, *Esr1* methylation was assessed by PCR and pyro-sequencing. Remaining animals were housed n=3 animal/cage (same sex, same dose group) underwent behavioral testing: anxiety-like (observations of home-cage social behavior and open-field testing), and social (social approach and aggression toward a stimulus mouse) from PND 30 to PND 70 [male and female offspring from vehicle (n = 8–10), 2 μg/kg bw/day (n = 10–12), 20 μg/kg bw/day (n = 10), and 200 μg/kg bw/day (n = 12) BPA

litters]. Main statistical analysis was performed using a multilevel regression aimed at fitting the log[BPA] *versus* DNA/RNA expression levels plot for males and females respectively; $p<0.05$ for the fitting was considered significant. Complimentary analysis was done by two-way ANOVA with sex and treatment as factors and Tukey post hoc analyses of dose effects, and, in some cases, t tests to determine group differences.

Multilevel regression analysis showed the most pronounced non-linear effects in the hypothalamus where statistically significant, sex-specific quadratic changes (U-shaped in females and inverted U shaped in males) were seen in *Esr1, Esr2, Esrrg*, and DNA methylation gene *Dnmt1*expression. In the prefrontal cortex a sex-specific linear change (female decrease at a faster rate than males) was seen in *Esr2* and *Dnmt1* expression, and a sex-specific quadratic change was seen in *Esrrg* and *Dnmt3a* expression. In the hippocampus, a sex-specific linear change (female decrease and male increase) was seen in *Esr2*, and a linear change in both sexes (decrease) was seen in *Esrrg*. The changes were such that the low doses of BPA (2 μg and 20 μg/kg bw/day) resulted in a reversal of the normal sex-specific ER related gene expression, which was confirmed in vehicle group: BPA-treated males had similar or elevated mRNA levels of *Esr1* (hypothalamus), *Esr2* (all three brain regions), and Esrrg (cortex, hypothalamus) compared with females. Similar results were also reported in two-way ANOVA analysis of the same data, with the male expression levels for ER genes first increasing (2 and 20 μg/kg bw/day) then decreasing, and female levels generally decreasing or remaining unchanged. Not all the changes were identified as significant in both analyses for example, *Esr2* in prefrontal cortex in males and females.

DNA methylation in 17 CpG sites in untranslated exons A and C of *Esr1* gene from prefrontal cortex and hypothalamus of males and females exposed to 20 μg/kg bw/day prenatal BPA (two regions that show significant changes in *Esr1* mRNA levels) was examined. Prefrontal cortex DNA had increased DNA methylation at CpG sites 1, 2, 3, and 8 of exon A in males but not females. The hypothalamus had decreased DNA methylation at CpG sites 1, 2, 3, 4, 6, 7, 10, and 11 of exon A of females but not males.

Home cage social behaviors analyzed at PND 30 and PND 40 reported changes that were consistent with a disrupted sexual dimorphism in play behaviors. For example, chasing, usually greater in males than in females, did not show a sexual difference and fighting, typically a male-specific behavior was only observed in a female cage at the 200 μg/kg bw/day dose. Exploratory and anxiety-like behavior assessed at PND 60 and social approach assessed at PND 70 reported changes with BPA treatment reversing the sex differences in these behaviors. There was an increase in aggression at the highest dose on PND 70 for both sexes. BPA treatment increased maternal behaviors (maternal care) at the highest dose. Controlling for the increased maternal care did not change the outcome for the vast majority of the measures in this study.

Limitations of the study include: (1) The number of animals per each group in the gene expression and methylation study and how the analysis deals with the litter effect. The study utilized n=6 pups/sex with sometimes multiple (1-2) pups per sex from each litter in each group. The results would be more convincing if one pup/sex/litter had been used. (2) Although the cages appropriately are BPA free, there is no mention of controlling for phytoestrogens in the diet or

BPA exposure from water bottles. (3) The epigenetic changes are only evident in exon A, a region that displays the smallest homology (63%) with the human gene. It is unclear if similar effects could also occur in humans. (4) If ER methylation mediates the altered ER expression reported here, there is no description of how BPA could affect *Dnmt1* and *Dnmt3a* expression. There is a disconnect between the methylation in the *Esr1* gene DNA CpG islands in the frontal cortex and hypothalamus (shown in Figure 4) and the *Esr1* gene expression/ mRNA levels (shown in Figure 1). In the hypothalamus *Esr1* methylation is decreased (Figure 4E) in females but their *Esr1* expression is decreased (Figure 1D) not increased as it would be expected. Conversely, there were no effects on *Esr1* methylation in males (Figure 4D) and yet there were changes in male gene expression (Figure 1D). The same disconnect is true in the frontal cortex where males had a significant increase in DNA methylation in *Esr1* (Figure 4B) but no real effect on the gene expression (Figure 1A). (5) There is no description of how the study controlled for analyst bias in behavioral measurements, or if there was any automation of the data collection or analysis. The study suggests prenatal BPA exposure as a possible hazard that may affect neuronal epigenetic pathways and behavioral outcomes. The results would need to be confirmed by other laboratories. **Thus, the data have limited utility for HI and no utility for RA.**

Organizational effects of perinatal exposure to bisphenol-A and diethylstilbestrol on arcuate nucleus circuitry controlling food intake and energy expenditure in male and female CD-1 mice. (Mackay *et al.*, 2013)

The purpose of this study was to determine if perinatal BPA exposure could affect adult metabolism and hypothalamic energy balance circuitry. The arcuate nucleus (ARC) of the hypothalamus is involved in regulating feeding behaviors. Immunopositive peptidergic proopiomelanocortin (POMC) neurons primarily exert anorexogenic effects, whereas Agouti Gene Related Protein (AGRP)/NYP positive neurons are associated with orexogenic effects (hyperphagia). The development of hyperleptinemia, and/or decreases in leptin mediated cell signaling cascades in some brain regions, are suggested as phenotypes of obesity. The study authors' hypothesis is that exposure to BPA or DES perinatally alters (masculinizes) the development of the melanocortin system.

Virgin female CD-1 mice (Charles River) were housed in polysulfone cages equipped with glass water bottles and fed low phytoestrogen chow (AIN93G, Research Diets). Weight matched females were divided into 4 groups and mated with males. Sperm plug positive dams were single housed and maintained from GD0 throughout pregnancy and nursing on AIN93G diets containing either no estrogenic additives (negative control), 1µg/kg diet BPA (low dose BPA, average weekly maternal dose of 0.36 µg/kg bw/day), 20 µg/kg diet BPA (high dose BPA, average weekly maternal dose of 7.20 µg/kg bw/day), or 4 µg/kg diet DES (positive control, average weekly maternal dose of 1.41 µg/kg bw/day). BPA and DES (Sigma Aldrich, >99% purity) were first prepared by dissolving the powders in small volumes of methanol. Then, the "appropriate amount" of drug concentrate was stirred in 1.5 kg oil. The treated oils were forwarded to Research Diets where they were incorporated into the mouse chow. Litters were adjusted on PND2 to 10-12 pups/litter, which were equally matched for sex where possible. To induce obesity, pups from cohort 1 (2 males and 2 females per litter) were placed on a high fat diet (HFD, Research Diets) starting at 3 months. Mice were assessed at 4 months for glucose

tolerance (tail vein samples collected at selected intervals before and after glucose challenge of 2g/kg body weight); and at 3 and 5 months for (a) locomotion and energy expenditure [kcal/h (3.941 X VO2 X 1.106 X VCO2)/1000] and (b) glucose tolerance (as above)). After sacrifice at 5.5 months of age, HFD mice from Cohort 1were assessed for (a) plasma concentrations of the adipokines IL-6, insulin, leptin, and resistin, and (b) body fat as determined by dissected weights of perigonadal, retroperitoneal, sc fat pads, and interscapular brown adipose tissue (BAT). Gene expression was performed using quantitative RT-PCR (qRT-PCR, 1 animal/sex/litter and postnatal diet), and levels were reported relative to housekeeping genes. Cohort 2 was sacrificed at 3 months of age. Data from 2 brains per litter per sex were pooled (The Methods did not specify whether pooling was done prior to analysis). Their brains were dissected and processed for (a) immunodetection of vesicular glutamate transporter 1 (vGlut) and glutamic acid decarboxylase 67 (GAD67) in POMC, and (b) immunodetection of ARC sections and tissue punches for density of POMC and AgRP fibers, NPY and ERα receptors. Littermate effects were controlled statistically by pooling littermates of the same sex and postnatal diet. Pups that exhibited abnormal, stereotypic behavior were excluded from data analyses.

As expected, the HFD increased body weight and fat pad weights compared to subjects on the AIN93G diet. Overall, maternal weights and fat pad weights of pregnant dams were not affected by either BPA or DES exposure. The most significant reported finding was that male and female mice perinatally exposed to the high dose BPA were hyperleptinemic. There was no significant effect of treatment on body weight or food intake in male pups. For female pups on the HFD diet, there was significantly greater food intake relative to control, DES, or low BPA cohorts.

In terms of locomotion, energy expenditure, and glucose tolerance, only the high BPA males demonstrated statistically significant (higher) levels of energy expenditure (kCal/hr, mirrored by high VO2 and VCO2), and impaired glucose intolerance. With the exception of the reduced energy expenditure levels observed in high BPA females during light period locomotion, no meaningful differences in energy, locomotion, or glucose tolerance was found in the female pup cohorts. No clear pattern could be discerned from exposure to DES or BPA in HFD pups on circulating adipokines. For example, female HFD mice exposed to high- but not low- BPA had elevated leptin levels, but the DES counterparts had reduced levels relative to HFD controls.

Histological sections of brain tissue suggested increased co- staining for ERα and POMC fibers in females exposed to BPA (both doses), but not males. High BPA males on the HFD, but not females, had significantly higher levels of the orexigenic peptides, AgRP and NPY. In the arcuate nucleus (ARC), there was no apparent difference in the expression of the vGlut (incorporates excitatory glutamate into synaptic vesicles) or GAD67 (an enzyme critical to synthesis of inhibitory neurotransmitter GABA), suggesting that the local balance between excitatory and inhibitory synaptic transmission was not significantly altered. No treatment-related effects on ObRb (leptin receptor) levels were observed.

The authors conclude that high perinatal BPA exposure exerted sex differences. In BPA males, but not females, there was a decrease in POMC fiber staining in the PVN and an increase in the expression of orexigenic peptides AgRP and neuropeptide Y. Unlike high BPA males, the female counterparts did not have increased glucose tolerance or decreased POMC fiber staining. The

protective effects of estrogen signaling in females were postulated for this difference (*e.g.*, increased POMC mRNA expression).

As reported, the data would suggest complex trends in sexually dimorphic phenotypes resulting from early BPA exposure, but a strong, dose-dependent link or clear pattern between BPA exposure and obesity was not demonstrated. In addition, the positive control group showed few positive findings for exposure to DES. The authors did not provide a unifying hypothesis for their findings and overextended their findings to humans on the basis of inconsistent data. **This study has no utility for HI and no utility for RA.** Although it appears that adequate controls were used to limit environmental exposure to estrogenic contaminants, the methods were not clear on pooling data from littermates. There was no measurement or estimation of blood levels of BPA in pups, which were exposed only through nursing postnatally.

Sex specific impact of perinatal bisphenol A (BPA) exposure over a range of orally administered doses on rat hypothalamic sexual differentiation (McCaffrey *et al.*, 2013)
The objective of this study was to determine whether prenatal exposure to low doses of BPA affects the sexually dimorphic nucleus of the preoptic area (SDN-POA) and/or the anteroventral periventricular (AVPV) nucleus.

The SDN-POA and AVPV play a critical role in male sexual behaviors. The AVPV has a role in generating the preovulatory surge in females. Over the course of early brain development, SDN-POA becomes comparatively larger in males. In contrast, females develop a larger AVPV, which has more dopaminergic neurons. The investigators postulate that the perinatal, sexually dimorphic effects of physiologic estrogen and dopamine (as measured by the regional levels of the rate-limiting enzyme in dopamine biosynthesis, tyrosine hydroxylase, TH) may be mimicked and/or altered by perinatal exposure to estrogenic substances such as BPA.

The study adhered to the Society for Neuroscience guidelines and University of Rochester animal care and utilization committees (UCAR), but was non-GLP. Timed pregnant Long Evans rats (source, Charles River) arrived at GD4 and were housed in polysulfone cages equipped with glass water bottles, bedded with wood chips. The rooms were maintained in a 12:12 h light-dark cycle at a temperature of 74°C, and a relative humidity of 30-70%. Food (Purina 5001 rodent chow) and water provided ad libitum. From GD12 through PND 10, dams were orally dosed with either corn oil vehicle (negative control, n=11), 17 β estradiol/corn oil (n=2, positive control) or BPA/corn oil. BPA dosing levels were: 10 (n=12), 100 (n=12), 1000 (n=10), or 10,000 (n=11) µg/kg bw/day. The source and purity of BPA was not specified. To avoid the stress of oral gavage, dietary administration of BPA was achieved by pipetting the test solution onto a piece of Nilla Wafer® and dropping the cookie on cage bedding. Note that the precision of drug delivery was not discussed (see Ferguson and Boctor, 2009). After weaning at PND21, pups were randomly assigned to 1 of 4 experimental groups. To generate the data, four males and four females/litter/group were used. Between PND 65-68, the offspring were sacrificed and sexed. After transcardial perfusion with paraformaldehyde, the brains were removed and fixed (sucrose/paraformaldehyde), and shipped at -80°C to NCSU for coronal sectioning. The boundaries and density of the SDN-POA was estimated by immunolabeling for calbindin-D28K

(CB28). AVPV sections were immunostained for TH. Avidin-biotin was used for signal amplification. One offspring/sex/litter was used in volumetric analyses. The volumes of the SDN-POA and AVPV were determined via stereology using an optical fractionator with systematic random sampling. The paper does not state the number of readers or if they were blinded to sex and dose. Stereology provides 3D interpretations of planar cross-sections, using geometric principles. Cell density was quantified from color images by using MCID Core Imaging software for a regional point counting area of $1mm^2$. Sensitivity of the animal model was assessed by comparing vehicle and estradiol exposed female tissues using a Student's t-test (pooled variance). The left and right volumes were averaged for each subject and this average volume was used in statistical analyses. Average SDN-POA volumes were analyzed using a two-way ANOVA with factors of exposure and sex, followed by one-way ANOVA for within sex differences. Two sample t-tests were used to evaluate sex differences within each exposure group. Fisher's least significant difference was performed post-hoc to determine significant effects.

There was a reported reduction in males, but not significantly in their female counterparts, in the volume of SDN-POA relative to control counterparts. No dose dependency of this effect could be discerned. However, for each dose group, the larger SDN-POA volume in males was maintained. This finding was matched by a decrease in CALB-immunoreactive (CALB-ir) cells in males, although curiously, the high BPA dose groups (1000 and 10,000 µg/kg bw/day) did not show this effect. The authors suggest that the reduction in SDN-POA volume and cell number in males could exert a demasculinizing effect on male sexual behaviors. The main BPA effects on AVPV-ir were observed in tissues from both sexes with no clear dose-dependent pattern. The authors conclude that the data demonstrate perinatal BPA exposure alters the structure and composition of SDN-POA and AVPV.

The primary strengths of this study were (a) the animal environment (*e.g.*, caging materials) but not diet was well controlled for contamination with estrogenic substances; (b) multiple doses of BPA were employed, and; (c) the food wafer method of BPA delivery avoided confounding effects of more stressful drug dosing measures. There were several limitations to the methodology, and only some will be listed here: (a) *in vivo* exposures were not verified through analysis of blood or urine for levels of BPA; (b) it is generally accepted that one pup per litter should be included per assay to remove litter bias. In this paper, the authors noted that "Four males and four females per litter were used to generate the data," so it is not at all clear that the litter is the unit of analyses; (c) in terms of tissue preparation, it is not clear if brain slices were processed in batches containing samples from both sexes and several treatment groups, in order to control for bias in tissue processing and immunolabeling; (d) although it was previously reported that SDN-POA structures can be bilaterally asymmetrical (He *et al.*, 2012), the investigators did not discuss how differences in asymmetry were addressed; (e) the source and purity of BPA was not specified. **This study has no utility for HI and no utility for RA.**

<u>Working memory in bisphenol-A treated middle-aged ovariectomized rats (Neese *et al.*, 2013)</u>
The purpose of this study was to determine if chronic oral exposure to BPA would alter working memory in middle-aged ovariectomized rats. These authors have previously characterized the

effect of chronic exposure to estrogens, including 17β-estradiol, ERβ selective SERMs, and soy phytoestrogens on performance of middle-aged female rats on an operant working memory task, showing reduced performance. The authors' hypothesis is that low dose BPA, orally administered, will reduce performance on a working memory task.

The study was non-GLP. Long-Evans rats were ovariectomized at 10-12 mo, and allowed to recover for 2 weeks. Doses included 5 and 50 µg/kg BPA dissolved in a 2% ethanol solution prior to mixing in tocopherol stripped corn oil (vehicle). Doses were delivered via an oral bolus once per day with a polysulfone pipette tip approximately 10–30 min prior to operant training or testing each day, so that testing would coincide with peak serum concentrations of BPA. As a positive control, 17β-estradiol was delivered by implant at a stable physiological dose of 20–30 pg/ml. Rats treated with 17β-estradiol (positive control) were also given a single oral dose of the 0 µg/kg treatment (vehicle control) prior to training or testing each day. Environmental conditions were well controlled. Dosing of BPA was via a polysulfone pipette tip. 17β-estradiol was via a silastic capsule. Cages were polysulfone, diet was low soy, and water was BPA free from glass water bottles.

Daily BPA treatments lasted for 8–10 weeks. This study was conducted in three separate cohorts of rats; therefore cohort was included as a factor in all statistical analyses. The final sample sizes for each treatment dose were as follows:

50 µg/kg bw/day BPA (BPA50), cohort 1, n=12; cohort 2, n=14; cohort 3, n=16 (total N=42)
5 µg/kg bw/day BPA (BPA5), cohort 1, n=12; cohort 2, n=13; cohort 3, n=15 (total N=40)
0 µg/kg bw/day BPA (oil), cohort 1, n=11; cohort 2, n=12; cohort 3, n=16 (total N=39)
10% 17β-estradiol (estradiol), cohort 1, n=9; cohort 2, n=11; cohort 3, n=16 (total N=36)

Cohorts were tested 4 months apart. Three behavioral tests that evaluate working memory were performed: CA – cued alternation, NCA – noncued alternation, DSA – delayed spatial alternation. The reader is referred to the manuscript for details of the training, equipment, and rationale. Statistical methods utilized (author's text): "The behavioral data were analyzed using the SPSS for Windows. Treatment group and cohort were included in the analyses as between subject factors and significance was set at $p< 0.05$. When appropriate, Tukey post hoc tests were run for pair-wise comparisons. For CA, cumulative errors across all sessions served as the main measure of learning, and were analyzed using ANOVA for the treatment group and the cohort. For NCA, the overall proportion correct across the ten sessions served as the primary measure of learning, and was analyzed using a 4 (treatment group)×3 (cohort)×10 (session) mixed ANOVA where session was a repeated measures factor. For DSA, the proportion correct across the 25 test sessions was first averaged across blocks of 5 test sessions to produce five 5-session test blocks. Proportion correct at each intertrial delay across the 25 test sessions was then analyzed using a mixed 4 (treatment group)×3 (cohort)×5 (block)×5 (delay) repeated measures ANOVA with block (1–5) and delay (0, 3, 6, 9, 18 s) serving as repeated measures factors. Error pattern analyses were also conducted to determine if rats were more likely to respond incorrectly following a correct or an incorrect lever press. A "win-stay" error was defined as an incorrect response on the same lever that had been correct on the previous trial, whereby the rat responded correctly on the n-1 trial, but incorrectly on the nth trial. A "lose-stay" error was defined as an incorrect lever press on the same lever that had been incorrect on the previous trial, whereby the rat responded incorrectly on the n-1 trial as well as on the nth trial. Win-stay and lose-stay errors

were analyzed separately using a mixed 4 (treatment group)×3 (cohort)×5 (block) repeated measures ANOVA with block (1–5) serving as a repeated measures factor. The latency to lever press following a correct and an incorrect response was also analyzed separately using a mixed 4 (treatment group)×3 (cohort)×5 (block) repeated measures ANOVA with block (1–5) serving as a repeated measures factor."

There were no treatment-related findings for chronic treatment with either dose of BPA. The positive control 17β-estradiol impaired DSA performance in middle-aged rats to the same degree as previously reported. This is an exceptionally well-done study. The authors pointed out in the discussion that these results are specific to the memory tasks assessed, and that effects of BPA on social or emotionally based behaviors (anxiety, etc.) cannot be ruled out. **This study is useful for HI and RA.**

Social behavior is perturbed in mice after exposure to bisphenol A: a novel assessment employing an IntelliCage (Ogi *et al.*, 2013)

The purpose of this study was to assess the effects of gestational and lactational exposure to BPA on adult offspring drinking behavior in group-housed mice. From gestational day 0 through lactational day 21 (*i.e.*, postnatal day (PND) 21 for offspring), sperm plug positive C57Bl/6J mice were gavaged with 0.01% ethanol (vehicle) or 500 µg/kg BPA (from Wako, Osako, Japan). There was no positive control group. There is no information on caging or water bottle material and the diet is not specified. Offspring were weaned at PND 21 and group-housed (2-5 same-sex mice/cage), but it is not clear if treatments were housed separately. There were two control cohorts and more than one subject/sex/litter was selected for a total of 16/sex in the control group. BPA subjects came from 6 litters: 8 females were obtained from 4 of those 6 litters and 8 males were obtained from 5 of those 6 litters for a total of 8/sex. Thus, in the BPA group as in the control group, more than one subject/sex/litter was obtained. At PND 77 for females or PND 91 for males, groups of 8 (same-treatment, same-sex) mice were placed into the IntelliCage to measure drinking behavior. It is not stated if those groups of 8 were familiar cagemates or strangers. The IntelliCage system fits inside the four corners of a large rodent home cage. In each corner, there is a small chamber allowing only one mouse to enter at any time. The interior of each chamber allows access to a water bottle via nosepokes. Although not explicitly stated, it appears that water was only available via accessing those chambers. For the first 3 days in the apparatus, mice were allowed free access to all four water sources (that is, the gates covering the end of the water bottles were open). Beginning on the fourth day, a nosepoke (after entering any corner chamber) was required to gain 7 seconds access to water. This schedule remained in effect for the next 12.5 days. A normal 12:12 hour light/dark schedule was in effect. The endpoints were averaged over the diurnal periods and over the nocturnal periods. Data were analyzed separately by sex via Tukey's HSD tests and those appear to be followed by Wilcoxon rank sum tests to determine sex differences within each group. Extreme values were not included, but it is not specified how outliers were determined. Several endpoints were analyzed: total number and total duration of nocturnal corner visits, number and duration of nocturnal visits without drinking, number and duration of nocturnal visits with drinking, and the same endpoints for diurnal corner visits. Total number of nosepokes, average number of nosepokes/visit, and ratio of number of nosepokes with drinking to total number of nosepokes were analyzed. A "corner preference bias" and a "corner preference variance" were calculated as well as a "random

interval" and "different-animal visit interval rate".

There is no information on gestational/lactational body weight or food/water intake. The Discussion states that there were "no significant differences in the maternal behavior" due to treatment, but there are no methods or data results presented for this. There is no information on litter endpoints (*e.g.*, birth weight, litter size, sex ratio). Number of corner visits during the nocturnal period was significantly increased in both groups relative to the diurnal period. Number of visits without drinking during the nocturnal and diurnal periods was significantly more than visits with drinking, except for BPA-treated males during the diurnal period. Relative to same-sex controls, BPA-treated females exhibited significantly fewer number of visits and fewer visits without drinking during the diurnal period only. Total duration of visits and duration of visits without drinking during the diurnal and nocturnal periods were increased in BPA-treated males relative to same-sex controls as was visit duration with drinking during the diurnal period. Females performed significantly more total nose pokes and more nose pokes/visit than males, but there were no significant BPA effects on those measures. BPA-treated males had a significantly higher preference bias than control males, indicating a preference for one of the four corner chambers. BPA-treated males also exhibited significantly lower total interval rate and lower interval rate with drinking than control males, indicating they were slower to visit the same corner chamber that the preceding animal visited.

Most studies using the IntelliCage incorporate a spatial learning task; that is, subjects have free access to all chambers but each is assigned a specific chamber in which it can access to water (since mice are identified via transponders, the gate covering the water bottle end will not open unless the correct mouse has performed the nosepoke). Normal mice quickly learn to suppress their entries into chambers without access and increase entries into the chamber with access. Here, the authors instead calculated new endpoints such as Corner Preference Bias, Corner Preference Variance, and Random Interval Rate. A quick literature scan did not show other IntelliCage studies which used those endpoints; thus, it is difficult to interpret these results. The authors discuss the effects in terms of altered social behavior and differences in impulsivity and/or exploration. Given that only visit number and duration and nose poke number were measured, how those endpoints directly translate into BPA-induced differences in social behavior, impulsivity, and exploration is unknown. There are many more direct measures of social behavior, impulsivity, and exploration. In addition, all subjects in the IntelliCage were of the same treatment group; thus, any alterations in social behavior were between same-treatment mice. Both sexes were evaluated; however, there was no positive control group and only one dose of BPA was evaluated. Although the number of subjects in the control group is adequate (n=16/sex), the BPA group consisted of 8/sex. Further, the litter is not the unit of analysis as there were 11 control litters and 6 BPA treated litters. There are multiple reports describing the severity of ignoring litter effects; it is quite clear that the litter must be the unit of analysis and that same-sex siblings cannot be considered as individual subjects. Finally, it is not specified that exposure to exogenous estrogens or BPA were controlled in caging material, water bottle material, or diet.

This study has no utility for HI and no utility for RA. This recommendation is based on the

commingling of littermates in statistical analyses; the lack of adequate controls for environmental conditions that would minimize inadvertent exposure to estrogenic substances; use of only one dose level of BPA; fewer than 10 subjects/group; lack of a positive control group, and; use of behavioral protocols with the Intellicage that have not been validated.

Anxiogenic Effects of Developmental Bisphenol A Exposure Are Associated with Gene Expression Changes in the Juvenile Rat Amygdala and Mitigated by Soy (Patisaul *et al.*, 2012)
The objective of this study was to determine whether continuous oral exposure to BPA during gestation through puberty would affect anxiogenic behavior in juvenile and adult rats and whether BPA effects can be affected by the amount of soy in the diet.

The study was non-GLP. Wistar rats were bred in house and reared on a phytoestrogen-free diet over several generations. Phytoestrogen levels in the soy diet "approximated levels in a traditional Asian diet or consumed by infants reared on soy formula." BPA (1 mg/L) or ethinyl estradiol (EE, 50 µg/L) was administered in drinking water. No statement was made about composition of the water bottles. Water consumption was measured biweekly and daily intake was used to estimate amount ingested. For the pups, daily intake was estimated by dividing consumption by the number of pups in the cage. There were 5 exposure groups: Soy diet; Soy-free diet; BPA + soy diet with BPA intake estimated to be ~22 (F) or 18 (M) µg/d; BPA (soy-free) with BPA intake estimated to be ~23 (F) or 25 (M) µg/d; EE positive control (soy-free diet plus water containing EE) with intake estimated to be ~0.9 (M/F) µg/day. Doses were chosen to produce free BPA serum levels by the authors' estimation in the ng/mL range. Serum BPA levels were reported as < 2 ng/mL for all dosed groups of dams. Levels in the pups were lower than in the dams. Maternal genistein levels ranged from 21-41 ng/mL for aglycone and 0.8-46 ng/mL for glucuronated genistein. Much smaller genistein (0.6-1 ng/mL) and no gluc-genistein were present in the PND12 animals. Authors stated that they were 'in the range of vegetarian populations that consume soy-rich food but below those seen in soy-formula fed infants". However, the presence of aglycone BPA in an untreated group within a factor of 2 from the treated groups suggests sample contamination produced spurious results. Methodology for measuring BPA and genistein and its glucuronide was from a previous article (Coughlin *et al.*, 2011) but much of the serum concentration data for both BPA and genistein were uninterpretable, even by the authors. Dams were segregated in different rooms according to diet. There was no mention of whether food containers were BPA free.

Dams were placed on their respective diet at least a week prior to mating and remained on their assigned diet for the duration of the experiment. Animals were dosed with BPA or EE from GD6 to PND40. Pups were weaned on PND21 into same sex and exposure groups (3-5 pups/cage). At 3 months they were separated into same-sex pairs. Juvenile behavior was assessed after weaning but prior to puberty (PND 24-28). Adult behavior was assessed from PND 60-70. Behavioral endpoints were assessed using the elevated plus maze (EPM) (juveniles and adults) and light/dark (L/D) box (juveniles only). All females were tested in estrus. Observers were blind to treatment groups. A subset of animals tested for juvenile behavior was sacrificed on PND 34, brains were removed, flash frozen, and sectioned to expose the amygdala, which was then removed by micropunch. Samples of the amygdala were analyzed for gene expression

normalized to *Gapdh*, and expressed as fold change. Only genes showing greater than 20% change in expression were considered positive. For behavioral and gene expression endpoints, statistical analysis included a 3-way ANOVA with gender, BPA exposure, and diet as factors. For gene expression, proven outliers were excluded from analysis, and nonparametric testing was used when necessary.

For the juvenile exploratory behavior using L/D box test, there was no effect of gender so data were collapsed across gender for all groups. The primary finding was that BPA–treated animals showed more anxiety-related behavior, but only in the soy-free diet group. EE was positive in this test. BPA had an apparent opposite effect in the soy-fed group, which the authors interpreted as a mitigating effect of diet. In the elevated plus maze test (juveniles), performance was broken into four parameters: (1) % animals that entered the open arm, (2) latency to enter the open arm, (3) mean number of open arm entries, and (4) total time spend on open arms. For parameters 2, 3, and 4, there was no effect of gender so data were collapsed across gender for all groups. For parameter 1, the only significant difference observed was that a slightly smaller % of soy-free BPA-fed males entered the open arms. EE had no effect. For parameter 2, there were no significant differences between groups. For parameter 3, both soy-fed groups showed significantly more open-arm entries than the soy-free groups. Significance was also achieved between the BPA-treated soy-free animals and the soy-free controls. EE had no effect. For parameter 4, authors claim a significant effect of soy diet on time spent in the open arms in the text, but did not mark the data as significantly different in the graph. EE had no effect.

For the elevated plus maze test (adults), data were reported by gender. EE exposed animals were not included in this test. The rationale the authors presented was that no changes had been observed in the juveniles and that EE treated animals would be sacrificed for gene analysis. For parameter 1, there were no significant differences. For parameter 2, the most significant finding was that males fed the soy-free diet took 4X longer to enter the open arm. For parameter 3, females across all groups showed more open arm entries than males, but there were not large differences within gender across the groups. For parameter 4, females across all groups showed spent more time in the open arms than males, but there were not large differences within gender across the groups. The authors noted that BPA-treated males in the soy-free group were more similar to females than in the other groups.

Of 48 genes examined in the amygdala from juveniles, a significant interaction with BPA was found for 8. In the soy-fed animals, no significant gene expression changes were noted for BPA. In the soy-free fed animals, 6 genes decreased 1.5-2-fold with exposure to BPA. Two genes that showed differential exposure in males vs females in controls were similarly expressed in BPA-treated groups. EE treated groups did not shows significant differences relative to other groups. The authors stated that ‘none of the BPA-induced changes were recapitulated by EE.’

The study had a number of limitations. There was no mention of control for BPA exposure in the food containers. The authors agree that some BPA exposure through food may have occurred. The dose received from each pup is uncertain given that pups were not singly housed. There is no description of accounting for litter in the statistics. Although not clearly stated, there are ~30-50

juveniles per dose group in the behavioral analysis (Figure 2A). As such, there likely were only 4-5 litters/group; *i.e.* the effective n in the study is 4-5 animals/group. Given this, the statistical differences between the groups may be overstated. The estrogenic control (EE) had no effect on either gene expression or behavioral endpoints in juveniles and was not evaluated for behavioral effects in adults. There were no consistent findings across the two behavioral tests for BPA treatment. Opposing results of BPA depending on diet are difficult to understand, especially when diet alone cannot be demonstrated to have an effect. It is not stated how many animals, biological repeats, or technical repeats were used to perform gene analysis. Absent these data, no conclusions can be drawn on the significance of the findings. It is extremely difficult to correlate gene expression changes with behavioral observations. The authors overinterpreted their gene expression findings and extrapolated their findings well beyond what the data can support. The relevance to humans is speculative. **This study has no utility for HI and no utility for RA.**

Single exposure to bisphenol A alters the levels of important neuroproteins in adult male and female mice. (Viberg and Lee, 2012)

The objective of this study was to determine whether postnatal exposure to BPA during a critical period of synaptogenesis alters proteins involved in synaptic transmission. The study used NMRI mice (source, Scanbur, Sollentuna) and was conducted in accordance with the Uppsala University and Agricultural Research Council, but was non-GLP. BPA (purity >99%) was sonicated with lecithin and peanut oil to emulate mouse milk for final concentrations that would correspond to doses of 0.32, 3.2 or 4.8 mg/kg bw. These high dose levels were justified by the authors because, unlike repeated dosing studies, the intent of the study was to investigate the effects of BPA at a critical (PND10) period of brain development.

Upon receipt, pregnant mice were housed in “plastic” cages maintained in a 12:12 h light-dark cycle at a temperature of 22°C. Food (Lactamin, Stockholm, Sweden) and tap water were provided ad libitum. The materials used in bedding and water bottles were not specified. Within 48 hours of birth, the litters were culled to 10-14 pups. There is no description of how many pups and from how many litters were randomized for each dosing group of the study. Males and females were separated at week four and housed in separate rooms. At PND10, male pups were orally administered via a metal gastric tube a single dose of 0.32, 3.2, or 4.8 mg/kg body weight. All female pups received a dose of 4.8 mg/kg body weight. Control subjects received vehicle. Male mice were sacrificed 24 h or 5 months after BPA dosing; all females were sacrificed 5 months after dosing (animal sacrifice method was unspecified). At sacrifice, the brains were removed, and the cerebral cortex (CC) and hippocampi (HC) were dissected out and frozen at -80°C. The CC and HC were homogenized in RIPA lysis buffer; the homogenates were centrifuged and the supernatants were collected and maintained at -80°C. Protein content of the homogenates was determined using the Bicinchoninic acid (BCA) colorimetric method. Samples of equivalent protein content were added to nitrocellulose membranes, fixed, blocked, and incubated overnight with the selective primary anitibodies for CAMKII, synaptophysin, GAP43, and tau; the expression was detected with secondary HRP conjugated antibodies using conventional slot-blot methods. Note that the methods do not discuss controlling for artifacts (*e.g.*, incubation in the absence of the primary antibody, or using positive controls). The intensity of the immunoreactive bands was detected using enhanced chemiluminescence techniques and

quantified by scanning/imaging software. Average protein band intensity from male subjects was analyzed using a one-way ANOVA approach, followed by Newman–Keul's post hoc test. Bands from female pups were analyzed using Student's t-test. 6 M and 8-9 F/groups were analyzed.

No difference was observed in protein band intensities among the samples obtained from male neonatal mice (24h post-BPA dose cohort). HC samples from adult male mice that received a single exposure to BPA at PND10 also did not demonstrate BPA mediated effects. However, the mean synaptophysin levels (*i.e.*, band density) were significantly elevated in BPA males (n=6) when normalized to control levels at exposures of 3.2 and 4.8 mg BPA/kg bw. CAMKII, GAP43 and tau levels were not significantly altered. Synaptophysin levels were also significantly increased in high BPA dosed females. CAMKII was decreased in BPA female samples (both CC and HC samples). The authors try to link these findings with BPA exposure during brain development and effects on proteins involved in memory (long term potentiation)

There were numerous methodological errors in the investigation, including but not limited to: (a) the animal environment (*e.g.*, use of tap water and "plastic" cages) suggests that the investigators did not carefully consider controlling for contamination with estrogenic substances; (b) a naïve control group was not included to address the potentially confounding variable of using a stressful (gavage) BPA dosing method; (c) a positive control was not included to test method sensitivity; (d) it is generally accepted that either one pup per litter should be included per assay, or the litter be used as the statistical unit, in order to remove litter bias. However, it appears that the tissue homogenates were obtained from littermates and protein expression results from each were considered independent measurements in statistical analysis; (e) the slot blot methodology did not control for membrane binding artifacts (*e.g.*, incubation in the absence of the primary antibody; (f) the slot blot assay is a relatively crude and low resolution assay which does not take into account regional differences, and the data were not augmented with other experimental approaches (*e.g.*, histology). **This study has no utility for HI and no utility for RA.**

Effects of developmental bisphenol A exposure on reproductive-related behaviors in California mice (*Peromyscus californicus*): A monogamous animal model (Williams *et al.*, 2013)
The purpose of this study was to assess the effects of dietary BPA or ethinyl estradiol (EE2) exposure on spatial learning, anxiety behavior, and territorial marking behavior in male and female California mice offspring. Adult California male and female mice (Genetic Stock Center, University of South Carolina, Columbia, SC) were housed in polypropylene cages with "BPA-free" water in glass bottles. The authors state that adult mice were purchased and then housed for 8 weeks in quarantine; however, the age at breeding was 8-12 weeks. Type of diet during this period was not identified. Two weeks prior to breeding, the females were placed on one of three diets: a low phytoestrogen diet; the same diet with 50 mg/kg BPA, or the same diet with 0.1 µg/kg EE2 as a positive control. BPA source is not specified. The BPA group was estimated to receive ≈ 150 µg BPA/day.

California mice are a monogamous species; thus, the male and female pair remained pair-housed for the study duration. To obtain sufficient numbers of offspring, some pairs were allowed to produce up to 3 litters. This implies that sires were exposed to the test diets, and litters from later

pairings may have differed from the first litter in which the sire was not exposed and dams had different exposure times. Final group sizes were 17 control litters (n=18-19 offspring/sex for testing), 18 BPA litters (n=12-15 offspring/sex for testing), and 9 EE2 litters (n=9-10 offspring/sex for testing). After weaning at PND 35, all offspring were placed on the low phytoestrogen control diet and individually housed.

Spatial learning was measured via Barnes maze performance at approximately PND 90 for two trials/day over 7 days. If a subject did not seem motivated to locate the escape hole, a barn owl recording was played. It is not clear if other subjects were in the test room at this time or how it was determined when or for what duration to play the recording. Latency, path length, errors, and search strategy were determined by averaging the two daily trials. It is not clear if the tester categorizing the search strategy was blind to treatment. While latency, path length, and errors are numerical values and can be easily averaged, it is not clear how the average of the search strategy for the two trials was determined. One day after completion of the Barnes maze assessment, subjects were assessed for anxiety behavior using an elevated plus maze during which data were automatically collected. Although not directly stated, the territorial marking behavior test seems to have followed the elevated plus maze assessment.

Only 5 males/treatment group were tested; all were non-littermates. For each pair, a control and a BPA-treated male were matched to be no more than 5 g different in body weight. Baseline urinary marking behavior was measured by placing each mouse individually into a filter paper lined cage for 1 hour. The next day, subjects in each pair were placed on opposite sides of a cage lined with filter paper but separated from each other by wire mesh. The duration of this session is not clear. Subsequently, each pair was housed together for one hour/day for 5 consecutive days, and on the next day, they were again placed on opposite sides of the two-chambered filter paper lined cage with wire mesh barrier. Marking patterns on the baseline day and days 1 and 7 in the mesh divided cage were scored by testers blind to treatment. Latency and error rate in the Barnes maze were analyzed as a split plot using factors of diet, sex, and day. Search strategy data were analyzed. Elevated plus maze data were analyzed using a split plot design with factors of sex and diet. Territorial marking behavior data were analyzed using a split plot.

Results: Average body weight of female offspring exposed to EE2, but not those exposed to BPA, was significantly lower than control females at PND 90. BPA exposure had no significant effects on body weight. The error rates and latencies to reach the Barnes maze escape hole were not affected by BPA or EE2 exposure, and there are no results presented for path length. There were no significant effects of BPA or EE2 exposure on search strategy use (direct, serial or random). BPA-exposed females had a significantly shorter duration in the open arms of the elevated plus maze than control females. No other elevated plus maze endpoint measured indicated significant BPA or EE2 effects. Although there were no differences in baseline territorial marking behavior, BPA-exposed males did not increase their marking behavior on days 1 and 7 in the wire mesh barrier cage as did control males and this difference in behavior was significant on day 7.

Both sexes were evaluated and a positive control group was included. The number of

subjects/group was adequate. The statistical analyses are complicated. However, only one BPA dose was evaluated. As noted above, there are also study design concerns with regard to variable test chow exposure levels among the sires and offspring, which could have resulted in paternally-induced effects of BPA and/or EE exposure. The gestation duration for California mice is 30-33 days and offspring were weaned at 35 days of age; thus, subjects from the first litters resulted from dams that were on the treated diets for approximately 79-82 days (and no sire exposure) while subjects from the second and third litters resulted from dams and sires that were on the treated diets for 109-150 days (for sires) and up to 218 days (for dams). Potential litter effects represent another, potentially confounding variable. Since the dam and sire were housed together throughout the study, some offspring resulted from the same parents, but different litters. The authors state that statistical analyses were used to determine litter effects for some test parameters; the methods that were employed are unclear. Categorization of search strategy type was done manually, but whether this person was blind to treatment status is not stated. There is no description of food intake during gestation or lactation so the actual doses of BPA and EE2 are not known; however, the authors state the BPA group received ≈ 150 µg BPA/day. Body weight of pregnant California mice was not stated; however, adult males typically weigh ≈ 38 g. If pregnant females weigh 25-30 g, the BPA dose here (150 µg BPA/day) translates to 5000-6000 µg BPA/kg body weight/day, doses higher than the criteria for this report. Further, there may have been direct pup exposure if the offspring began to eat solid food prior to weaning as most rodent species do.

This study has no utility for HI and no utility for RA. Some of the subjects were same-sex siblings. Some of the dam/sire pairs were bred several times, resulting in differing BPA and EE2 exposure amounts as well as paternal exposure for the second and third litters. Only one BPA dose was evaluated. It is not clear how results from this mouse species (a monogamous species) translate to other laboratory rodents or humans.

Gestational Exposure to Bisphenol A Produces Transgenerational Changes in Behaviors and Gene Expression (Wolstenholme *et al.*, 2012)

In this study, n=5/group female C57BL/6J (B6) mice received phytoestrogen-free chow with or without 5 mg BPA/kg diet 7–10 days before mating *ad libitum*, in what was estimated to be ~ 20 µg BPA/day or approximately 800 µg/kg bw/d. No mentioning if the caging and water bottles were BPA free. Embryos from pregnant females were collected 18 days after finding the plug, blood collected for BPA analysis, embryos extracted and brains frozen on dry ice from F1 (n= 3/group) and from F4 (n=4/group) generation. There was no positive (estrogen) control. For behavioral studies, to rule out effects from BPA induced maternal behavioral changes or lactation exposure, BPA group litters (n=4) were transferred to n=10 control dams (which now were made to foster mixed litters). On PND21 all litters were group housed by litter and sex, fed standard rodent chow containing phytoestrogens, and underwent behavioral tests. F1 brother-sister pairs (BPA n=9, control n=8) were used to produce the transgenerational lines.

Outcome measures included: microarray analysis of whole brain tissue of GD18 embryos, three biological replicates using Illumina MouseWG-6 version 2.0 Expression Bead Chips; rtPCR using brain RNA from GD18 embryos from F1 (n = 3 per group) and F4 generation (n =5–6 per group) with an iCycler iQ System (Bio-Rad) for TaqMan- and SYBR Green-based detection.

genes measured were *Esr1, Esr2, Gper* (previously called *Gpr30*), *Esrrg*, also *Oxt* and *Avp*, and their receptors *Oxtr, Avpr1a, Casp9*, *Dnmt1*, *Dnmt3a*, *Dnmt3b*, and *Slc1a1*; *B2M* and *Ppib* were used as control genes. BPA analysis using serum was conducted from n=3 control and n=6 BPA animals (this number does not agree with the reported n=5 in each cohort). Only unconjugated BPA was measured with LC/MS with a limit of quantitation of 0.5 ng/ml. Three pooled samples (two dams in each pool) showed unconjugated (free) BPA levels of 4.6, 3.9, and 2.0 ng/ml whereas dams from control phytoestrogen-free diet had BPA levels below the level of quantification. Juvenile social interactions were recorded on PND21 using a same age, sex, and prenatally treated mouse from another litter for 10 min using Noldus Observer software. Interactions were categorized into four groups (social, nonsocial, investigative, and play soliciting) and quantified. Anxiety was tested on PND22 on an elevated plus maze. Social preference tests on a three chambered social preference task device were performed on PND24 using same or opposite sex mouse. For gene expression data, normalized gene expression was calculated relative to the control male group. *P* values were corrected for multiple comparisons using the q value program in Bioconductor. Two-way ANOVA with sex and diet as factors was used. Significant results were assessed by Fisher's exact *post hoc* tests that adjust significance levels to take multiple comparisons into account.

The composite scores of social, nonsocial, and investigative behaviors were not significantly altered by BPA. The individual behaviors that made up this composite score showed some differences: social behavior ("sitting next to each other") and "play-soliciting behavior" were higher in BPA group, but "investigative anogenital sniffing" was lower. Social preference for an adult male was decreased in F1 BPA males. Also BPA males had less social interaction and BPA females more social interaction than controls. In general, BPA exposure increased social behavior and decreased nonsocial behavior in juvenile F2 and F4. In F1, qPCR changes were measured in *Esr1* (decrease), *Gper* (or *GPR30*) (increase), and *Esrrg* (increase), *Avp* (decrease), *Slc1a1* (decrease) (versus a previously measured increase seen by this group). *Oxt* displays a decrease and sex dependence trend (p=0.07). In F4, *Avp* and *Oxt* decreased.

The study is significantly limited due to a small n. Although the description lacks clarity on the details of breeding, it is stated that study starts with n=5/group; n=4 litters from the BPA group are cross-fostered for behavioral analysis. So it can be deduced that only one litter was available for genetic analysis in F1 and n=3 biological repeats refer to littermates. As such, the effective n in the genetic analysis arm of the study equals 1 and thus is severely underpowered. This may not be the case for F4, where larger numbers were available (either n=4 or n=5-6/group, inconsistent description). Interestingly, in F4 there were no statistically significant differences in any of estrogen receptor related genes measured. The results from behavior assessments also are not convincing. The composite score, labeled as "social interactions" does not change in F1, whereas its components either increase ("sitting next to each other" or "play soliciting behavior") or decrease ("investigative anogenital sniffing"), thus it is not clear what the adverse effect in humans would be. In F4, the main effect of BPA was to increase social interaction, an outcome that does not seem adverse. This notwithstanding, F4 has been derived from brother-sister breeding of descendants from just n=4 (at least 2 pairs/litter) BPA treated animals, and the measured changes can be due to genetic differences, independent of BPA exposure. An

intriguing finding was the decrease in *Avp* and *Oxt* gene expression in F4. However, due to the low effective n (n=4), these differences may be due to the exact genetic makeup of the littermate breeding pairs and not reflect changes in gene methylation as authors posit. **Given the small n, this study has no utility for HI and no utility for RA.**

Gestational and lactational exposure to bisphenol A affects anxiety- and depression-like behaviors in mice (Xu *et al.*, 2012)

The purpose of this study was to determine the behavioral effects, primarily related to "anxiety" and "depression," of developmental exposure to doses of either 400 or 4000 µg/kg bw/day BPA with respect to the timing of the exposure, maternal environment, sex, and age at testing. A comparison of prenatal BPA exposure from GD7 to GD20 to postnatal BPA exposure (PND1 to PND14) was made. Female ICR mice dams (age not given, wt was 25 to 30 g) and males were bred to produce offspring. Gestational exposure groups' dams were orally given sesame oil alone or with BPA present. Dams were fed a soy-free diet ad lib. There were two control sesame oil only groups (n = 10 litters for each) with one for gestational and one for lactational exposure to compare to the BPA exposure groups. There was no positive (estrogen) control. Both gestational (GD 7 to 20) and lactational (PND 1 to 14) BPA exposures at 400 or 4000 µg/kg bw/day levels were tested (n = 10 litters for each of the 4 groups). Concentrations of the BPA in the sesame oil were not given but apparently the animals were only orally dosed once a day so that 400 or 4000 µg/kg bw/day exposures occurred. At PND 1 litters were culled to range within 8 to 10 pups per dam with "relatively" equal numbers of each sex per litter. At PND 49 all the females were ovariectomized. At 56 days, behavioral testing was started in both sexes (one female and one male tested per litter) with the remaining animals in the litters used for organ weight determination or glutamate receptor protein levels in the hippocampus and amygdala. Four behavioral tests were performed each on successive days in the order of 1) open field activity, 2) dark/ light transition task, 3) mirrored maze, and 4) elevated plus maze. These were used to assess anxiety levels and depressive-like behavior. Analysis of the behavioral data was performed through an automated computer base tracking system (VideoMot2BWM; TSE System GmbH, Germany). Behavioral and body weight differences were determined using mixed repeated ANOVA measures. Two-way ANOVA with unidentified post hoc test was used to evaluate Western blot protein levels and reproductive organ/ body weight rations.

Gestational BPA was reported to decrease (≈ 10%) female body wt. but not affect male wt. while lactational BPA increased male and female body wt. but only at the 400 µg/kg/day. However, there was no effect of gestational or lactational BPA on reproductive wt/body wt. Open field activity was not affected by BPA in males and only affected female grooming behavior at 4000 µg/kg/day. There were modest and marginally significant decreases in parameters measured in the dark/light transition task of males exposed to BPA that may relate to increased anxiety. The females had slightly more pronounced decreases. In the mirrored maze there were very few effects on either sex, and all the changes reported as significant differed by less than 20% of control levels. Both exposure levels of BPA at both exposure windows (gestational and lactational) had the most pronounced effects on the elevated plus maze performance in females, decreasing entries and time spent in open arms of the maze and unprotected head dips by more than 50%. Males were also affected in a similar manner but to a somewhat lesser extent. Also,

both exposure levels of BPA at both exposure windows increased duration of immobility in both sexes in the forced swim test again indicating BPA may have had an increase in anxiety- and depression-like effect. The biochemical glutamate receptor data showed modest effects. Western blot showed that BPA may have some modest effects on two types of glutamate receptor subunits. There were very minimal effects on the NMDA receptor NR1 in both the hippocampus and amygdala (less than 20%). The biggest effect in the Glur1 receptors occurred in female hippocampus where levels dropped 25 to 30% from either gestational or lactational exposure. All other reported significant changes were 20% or less from control levels.

The major negative consideration in the study design is that the lactational exposure to BPA would be expected to be significantly lower than the gestational exposure. This obviously confounds comparisons of gestational versus lactational exposure. Other study shortcomings are noted. Although neutering females may make behavioral testing easier, it also makes the relevance of BPA exposure on females more difficult to interpret. A lot of changes in the behavioral parameters monitored were 25% to 15% greater or lesser than control. As well, body weight changes that differed from control by 10% were reported as significant. A better interpretation of the study would be facilitated if they are reproduced by independent investigators.

This study has limited utility for HI and no utility for RA. The study may have limited use for HI because; 1) the doses tested were very high, 2) all data generated on females was derived from mice ovariectomized during puberty and just prior to behavioral testing. Non-ovariectomized (normal human condition) mice were not tested. The study did report relatively robust effects on the elevated plus maze indicating BPA exposures may have increased overall anxiety and depressive-like effects. However, the effects of BPA on the remainder of the behavioral result were less substantial and not as compelling evidence that BPA affected anxiety. The biochemical glutamate receptor data showed modest effects at best.

Sex-specific effects of bisphenol-A on memory and synaptic modification in hippocampus of adult mice (Xu *et al.*, 2013a)

The purpose of this study was to determine the behavioral and biochemical effects, primarily related to learning/memory and synaptic plasticity/remodeling, of adult male and female mice exposure to doses of 400, 4000, or 40,000 µg/kg bw/day BPA for 12 weeks.

Ten week old female and male ICR mice were used in the experiments and maintained on a phytoestrogen-free water and diet ad lib. The BPA groups were orally given (fed) 400, 4000 or 40,000 µg/kg bw/day in 40 µl of arachis oil, while controls were given oil only. There was no positive (estrogen) control. It was stated initially in the methods that there were only 4 male and 4 female rats per treatment group. However, later in the methods it was stated that there were 22 mice (apparently they meant 22 male and 22 female per treatment group) in each group with 12 for behavioral testing, 6 for morphometric measurements, and 4 for Western blot protein analysis. Three behavioral tests were performed each to determine 1) open field activity (OPF, at 3 days post BPA), 2) Morris water maze (MWM, 5 days post BPA) and 3) step-down passive avoidance behavior (SPA, 11 days post BPA). These were used to determine learning and

memory, motor activity, and “anxiety”. Estrous cycles were checked in females during behavioral performance, and only data from diestrous mice were used for data analysis (1 to 2 animals /group of 12 or maybe 6). Although the analysis of the behavioral data was performed in part through an automated computer base tracking system (VideoMot2BWM; TSE System GmbH, Germany), it is not clear what the observer’s role was in evaluating the behavioral endpoint. It appears just one observer was involved in conducting the behavioral tests. It is not clear how much human evaluation of behavioral performance occurred and whether there were multiple evaluators for such data collection. Morphometric measurements for synaptic density were determined in glutaraldehyde fixed brains using TEM electron microscope methods at 3 days after BPA. Three days after the end of BPA, groups of mice were sacrificed for Western blot analysis of the synaptosomal and dendrosomal fraction of dissected hippocampus to evaluate synaptic “status” (levels of synapsin 1, PSD-95, NR1, and Glur1). Two- and three-way ANOVA was used to analyze the behavioral data while a one-way ANOVA was used to analyze the Western blot data.

In the OPF, there appears to be a dose-dependent increase in the time spent grooming by males (400% control) and females (200%). Time in the central area was increased in males (40,000 µg BPA) but unaffected in females. Frequency of rearing was increased in males and decreased in females at the low dose of BPA but not the high dose. BPA had no effect on female performance in the MWM and only a weak dose response effect of inhibiting male performance. The SPA was unaffected by BPA in females, while in the males only the highest dose decreased latency in the SDA. There was no effect of BPA on female synaptic density or other measures of synaptic morphology. BPA decreased the synaptic density in males at the low and high dose but not the intermediated dose. Also, it reportedly increased the width of the synaptic cleft to about 120% of control. BPA had no effect on any of the glutamate- or synaptogenic proteins measured in this preparation. BPA affected all these proteins in males but there was no dose response.

Limitations were minimal in the study. However, it would have been more desirable to perfuse the mice with the glutaraldehyde fixative upon sacrifice and not just post-fix the brain in this solution after removal of the brain from the skull. The results indicate that exposure to very high levels of BPA does not affect female mice. On the other hand, the data from these investigators indicates that these very high doses of BPA affected male behavior and may affect their hippocampal function. However, it is difficult to determine how this may be occurring. The 400 µg/kg bw/day dose had as much effect on the morphometric and synaptic/dendritic proteins as the higher doses. It also had as much effect at decreasing MWM performance which is a measure of learning and memory. **This study has limited utility for HI and no utility for RA.** The study indicates that BPA exposure has no effect in females even at a dose thousands-fold above human exposure levels. The data indicate that males may be susceptible to memory and learning deficits when exposed to high doses of BPA but the mechanism(s) by which this might occur are not obvious. There was no consistent exposure-response effect any of BPA on the behavioral data, and no dose response effects were seen in the morphometric and biochemical parameters with the 3 exposure levels tested, despite being spaced only 10-fold apart.

Perinatal exposure to bisphenol A inhibits synaptogenesis and affects synaptic morphological

development in offspring male mice (Xu *et al.*, 2013b)

The purpose of this study was to determine the effects of indirect perinatal exposure to BPA (through dams given 40, 400, or 4000 µg/kg bw/day) on hippocampal synapse morphology and genes related to synaptic plasticity and function in male mice. Dams were dosed with BPA during the GD7 to PND 21 perinatal period. Changes in hippocampal synapse morphology and genes related to synaptic plasticity and function in male offspring were determined during development (PND 14, PND 21) and late puberty/ very early adulthood (PND 56).

Female ICR mice (dams, wt was 25 to 30 g) and males were bred to produce offspring. Dams were fed a soy-free diet and water (container type not stated) ad lib. During the gestational and postnatal time frame, GD 7 to PND 21, dams were administered 40, 400, or 4000 µg/kg bw/day of BPA. The doses of BPA were dissolved in sesame oil, and volumes of 0.1 ml/ 30g of oil containing the BPA were "orally injected" (not clear how this was done) into the dams. Controls received the oil only. Blood levels of free or conjugated BPA were not determined in serum or urine of dams or offspring. It was stated that litters were culled to 10 pups/ litter with males and females being equal "when possible," but it looks like there were 6 males kept in each litter. The male mice were same sex housed at weaning but their numbers/cage not given. The males were sacrificed at PND 14, 21, and 56 for either electron microscope morphometric (EMM) analysis or synaptosomal (non-nuclear tissue fraction) protein analysis of the hippocampal CA1 region. For EMM analysis, it appears that there was probably an n = 3 (but maybe n = 6) for all 12 groups (4 treatments x 3 developmental time points) because there were 6 litters with apparently 6 males per litter. Tissues for EMM were prepared for a transmission EM "visualization" and Gundersen's (1988) methods were used to numerically estimate synaptic density for EMM analysis. Synaptic width, length curvature, and PSD thickness were also determined. For the synaptosomal protein analysis an n = 4 was apparently used. Traditional western blot techniques were used to quantify the levels of synapsin, PSD-25, Glur1, NR1, and β-actin. For the EMM data, a two-way repeated measures method was used to determine significance while a one-way ANOVA was used for synaptic proteins. A post hoc Tukey's test was used to determine significant between group differences.

There was a decrease in the numeric synaptic density (from 80 to 90% control), relative to control, produced by the high dose of BPA at PND 14, 21, and 56 while the intermediate dose did not change this parameter relative to control at these time points. The low dose decreased this parameter at PND 14 and 56 but not at PND 21. The high dose of BPA affected the synapse width (increased), active zone length (decreased), curvature (increased), and postsynaptic density (PSD) thickness (decreased) at almost all developmental time points. The intermediate BPA exposure had minimal effects on three of these four parameters with PSD thickness (decreased). BPA affected all the synaptosomal proteins (synapsin, PSD-25, Glur1, NR1) to the greatest extent at PND 56. However, there was no dose-response effect at this time point or at PND 14 and 21.

Design shortcomings included no maternal or offspring BPA levels in serum were determined. Mice were not perfused at sacrifice but brains were post-fixed making the fixation process less consistent and potentially interfering with the EMM morphometric measurements. The

hippocampal deficits are seen only in male mice and not females, which would be expected if an endocrine disrupter effect was the cause of the deficits. The authors cite work (Hajszan and Leranth, 2010) that this has been seen before in males, but Hajszan and Leranth (2010) saw effects in females as well as males. **This study has limited utility for HI and no utility for RA.**

Persistent overexpression of DNA methyltransferase 1 attenuating GABAergic inhibition in basolateral amygdala accounts for anxiety in rat offspring exposed perinatally to low-dose bisphenol A (Zhou *et al.*, 2013)

The objective of this study was to determine whether low-dose, perinatal exposure to BPA affects neural circuitry in a brain region associated with anxiety behaviors. The basolateral amygdala (BLA) and the central nuclei of the amygdala (CeA) have central roles in fear conditioning and anxiety behaviors. Electrical stimulation of this structure elicits fear responses in animals, whereas lesioning exerts anxiolytic effects. The BLA receives sensory inputs, and CeA provides the primary, inhibitory output.

The study was conducted in accordance with the Animal Care and Use Committee of Nanjing Medical University, but was non-GLP. Female Sprague-Dawley rats (source, Oriental Bio Service Inc.) were bred (methods unspecified) and administered daily oral doses of BPA throughout the entire period of pregnancy and lactation (time points and methods of verification unspecified). Pups were weaned at PND 21-22. The offspring were group-housed by litter and sex. 8-12 female offspring/group were evaluated for this study. BPA (source, Sigma-Aldrich) was dissolved in ethanol, further diluted in "water" and pipetted onto one cornflake, and fed to the rats to achieve an oral dose of 40 µg/kg/day. Vehicle treated dams served as negative controls. The experimental design did not include a positive control (*e.g.*, ethinyl estradiol). Rats were maintained in a 12:12 h light-dark cycle at a temperature of 25°C. Food (source unspecified) and tap water provided ad libitum. The materials used in caging, bedding and water bottles were not specified. Bilateral stainless steel cannulas were implanted near the BLA for drug delivery. After a 7 day recovery period, 0.5 µL drug per side were delivered bilaterally through an infusion catheter for 7 consecutive days. The drugs were: 5-aza-deoxycytidine (5-aza, DNA methylation inhibitor), L-methionine (L-met, methyl donor), midazolam (MDZ), picrotoxin (PTX, a non-selective, noncompetitive GABA receptor antagonist) or vehicle (saline). Mean group data were compared using Student's T test. An ANOVA approach was used for comparing >2 treatment groups, followed by Fisher's protected least significant difference.

For RT-PCR, PND 45 female offspring were anesthetized with 10% chloral hydrate. Their brains were dissected out and preserved in storage at -80°C. Blocks of tissue (50 µm thick sections) were cut with a cryostat along coronal planes. The BLA was micro-dissected on dry ice from the frozen sections. Total mRNA was extracted (TRIzol, Invitrogen). Gene-specific primer sequences for glutamic acid decarboxylase (GAD67, enzyme that catalyzes the decarboxylation of glutamate to GABA), DNA (cytosine-5)-methyltransferase 1 (DNMT1, 1 of enzyme subtypes that regulate cytosine residue methylation), glyceraldehyde 3-phosphate dehydrogenase (GAPDH, enzyme involved in glycolysis) were probed. Reverse transcription reactions were

carried out with an Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems) and High Capacity Reverse Transcription Kit (Applied Biosystems) per manufacturer's instructions.

For the open field test, rats (PND 38-45) were individually placed in plexiglass arena and their locomotion patterns were video-recorded. As referenced by a previous publication, locomotion was measured over a 30 min. period. The amount of time spent in the center and the number of rearings were quantified. The dependent variables were not specified (*e.g.*, movement time, distance traveled). The authors were also unclear on whether the investigators were invisible to the rats during testing, or whether the investigators were blinded to treatment at the time of behavioral assessment.

For the electrophysiology, rats were decapitated at PND45. The brains were maintained in carbogenated, chilled artificial saline solution. Coronal slices containing the BLA and external capsule (EC) were sectioned with a vibrotome to 400 µM thickness. Population spikes were obtained by stimulating the EC with a stainless steel field electrode, and recording field potentials with 2 M NaCl filled glass, extracellular electrodes. Square wave stimulus pulses of varied amplitudes were used to determine stimulus thresholds, maximum responses, input/output curves, and the amplitude of population spike (PS) amplitudes. Synaptic plasticity was assessed using paired pulses and high frequency stimulation (100 Hz for 1 s) paradigms to assess paired pulse inhibition (PPI) and long term potentiation.

RT-PCR results suggest a role for BPA in overexpression of DNMT1 and (subsequent) decreased expression of GAD67: (a) In BPA treated subjects, mRNA expression of DNMT1 was significantly elevated and GAD67 mRNA was suppressed. This GAD67 effect was mimicked in controls by treatment with the methyl donor MET; (b) decreases GAD67 BPA could be counteracted in BPA rats by treatment with the antagonist of DNMT1 (5-aza-Cdr), but not in control rats. Behaviorally, BPA rats exhibited significantly more anxiety-like behaviors, as evidenced by less exploration time in the center arena and increased number of rearing events, when compared to controls. The BPA effect was mimicked by treatment with 5-aza-CdR or picrotoxin, and counteracted by treatment with MET. Control rats were similarly affected by treatment with MET. These results suggest that the anxiety behaviors are associated with increased DNA methylation and decreased GABA-reception. The electrophysiology suggests that BPA affected synaptic plasticity. BPA rats exhibited a reduction in the magnitude of paired pulse inhibition. Instead, paired pulse facilitation was observed. The number of post synaptic spikes was also increased in BPA rats, an effect that was reduced towards control levels by treatment with 5-aza-CdR. Blocking GABAergic neurotransmission with PTX increased PSs in both control and BPA subjects. The number of PSs was also higher in BPA rats, mimicked by PTX, and reversed by 5-aza-CdR.

There were some methodological limitations in study design and reporting, including but not limited to: (a) some aspects of the animal environment suggests a possible lack of control contamination with estrogenic substances; (b) a positive control was not included to test method sensitivity; (c) the selection of a single dose of 40 µg/kg bw/day BPA; (d) lack of clarity about how many animals were bred and randomized for the study. On the other hand, the multi-

dimensional approach and statistical significance of results could suggest a possible link between BPA effects on gene expression, GABA mediated neurotransmission, and anxiety-like behaviors. The research could provide a scientific context for effects underlying anxiety by examining genetics and neurophysiology. **This study has no utility for HI and no utility for RA.**

Human behavioral studies

Bisphenol A in relation to behavior and learning of school-age children (Hong *et al.*, 2013)
The objective of this study was to investigate the relationship between environmental exposure to BPA and childhood neurobehavioral measures of cognition and behavior. The population studied was children age 8-11 (average, 9.05 ± 0.07 years old) recruited from rural and urban areas in South Korea. Data from 1008 children were included in the study. Emotional and behavioral problems of the children were assessed by their parents using the Korean version of the Child Behavior Checklist (CBCL). The CBCL is an age- and sex-standardized questionnaire composed of 108 items rated on a three-step response scale. The Learning Disability Evaluation Scale (LDES) is parent rated and consists of 88 items describing the observed characteristics of students with a learning disability. IQ was assessed using the Korean Educational Development Institute's Wechsler Intelligence Scales for Children (KEDI-WISC). IQ was also used as a covariate in analyzing the data. Urine samples were collected between 9:00 and 11:00 a.m. and analyzed for total (conjugated and free) BPA using the methods described by Matsumoto *et al.* (2003) with minor modifications. 1C12-BPA in acetonitrile was used as an internal standard. The limit of detection (LOD) was 0.15 µg/l. Urinary BPA concentration (µg/l) was standardized with creatinine (Cr) to control for individual differences in urine dilution. Blood levels of lead as well as urinary levels of phthalate metabolites (mono-n-butyl phthalate, MnBP; mono-2-ethyl-5-oxohexyl phthalate, MEOHP; mono-2-ethylhexyl phthalate, MEHP) and cotinine were also measured.

Statistical analyses were described as "Differences between children were estimated using Student's t-tests for continuous variables and chi-square tests for categorical variables. BPA concentrations (µg/g Cr) followed a log-normal distribution and were, therefore, log10-transformed for the statistical analysis. We performed multiple linear regression analyses (ordinary least squares) using the log10-transformed, Cr-standardized, urinary BPA concentration as the primary independent variable, and adjusting for numerous potential confounders including demographic and obstetric variables, psychiatric family histories, and biological levels of environmental toxicants other than BPA. The regression equation was also estimated including an interaction term between urinary BPA concentration and gender, or a quadratic term for urinary BPA concentration. All statistical analyses were performed using STATA 11.0 (STATA Corp., College Station, TX). All results were considered to be statistically significant when p-value was less than 0.05."

The authors reported that the median urinary BPA level was 1.28 µg/g Cr after Cr-standardization, and the nonstandardized value was 1.23 µg/l. These values were lower than the levels from the 2005–2006 NHANES data for 6- to 11-year olds (2.7 ng/ml) or 12- to 19-year olds (2.4 ng/ml), and the levels from the German Environmental Survey on Children. Using linear regression analysis, urinary BPA concentration was positively associated with the CBCL

total problems score and negatively associated with the LDES score. The effects were not modified by gender (*i.e.*, there was no sexually dimorphic association when analyzed for urinary BPA concentration x gender). Findings were consistent across subscores of the respective indices. Using nonlinear regression analysis, BPA concentration was negatively associated with several of the CBCL subscores and positively associated with some of the LDES subscores. Nonlinear associations with the CBCL scores for delinquent and aggressive behavior and externalizing problems were significant for males. Data were examined for covariables, and results were found to be similar when adjusting for demographic and obstetric variables, psychiatric family histories, and biological levels of environmental toxicants other than BPA. The only environmental toxicant found to have a positive association with BPA was MnBP.

The findings associating BPA with specific behavioral perturbations in children are inconsistent at best, both within this study, and across other population studies, which the authors thoroughly discussed. The methods used were observational, and thus limited to detecting associations. In addition, BPA measurements were taken at only a single time point. Although the sample size was large, no consistent conclusions about the association of BPA with behavioral alterations can be made. If anything, the data argue for a null effect of BPA. **This study has no utility for HI and no utility for RA.**

Prenatal Bisphenol A Exposure and Child Behavior in an Inner-City Cohort (Perera *et al.*, 2012)
The objective of this study was to assess BPA exposure and behavioral parameters in a long-term study in children. The population studied was African-American and Dominican women and their children in the Columbia Center for Children's Environmental Health (CCCEH) New York City cohort. Women and children were followed from pregnancy to child's age 5 years. Child behavior was assessed between 3 and 5 years of age using the Child Behavior Checklist (CBCL). Generalized linear models were used to test the association between BPA exposure and child behavior, adjusting for potential confounders. The analysis was conducted on 198 children (87 boys and 111 girls).

Prenatal BPA exposure was estimated from single spot urine samples taken between 24 and 40 wks gestation (mean 37.1 weeks). Childhood exposure was estimated from single spot urine samples taken between the ages of 3-4 years. The limit of detection (LOD) was 0.4 μg/L; concentrations below the LOD were given a value of LOD/2 for the statistical analyses. Urinary dilution was calculated from specific gravity (SG) measurements obtained using a handheld refractometer. All BPA concentrations were corrected for dilution by SG adjustment. Other environmental toxicants were also assessed. Phthalate exposure was estimated by urinary concentrations of phthalate metabolites; exposure to polycyclic aromatic hydrocarbon (PAH) was assessed by personal monitoring (methods referred to previous publication); environmental tobacco smoke (ETS) was monitored during pregnancy by questionnaire. BPA concentrations were dichotomized into high (upper quartile) vs low (lower three quartiles). Scores for the behavioral scales were normally distributed, and so were analyzed as continuous variables using linear regression.

The ranges of total SG-adjusted BPA urinary concentrations in maternal and child urine samples

were 0.24–38.53 μg/L and 0.42–73.50 μg/L, respectively, a more than 100-fold range. Geometric means for mothers and children were 1.96 and 3.94 μg/L, respectively. Urinary BPA is assumed to be in its conjugated form. The authors reported no significant differences between boys and girls in median and mean prenatal or postnatal BPA concentrations, whether SG-adjusted or not. Among boys, high prenatal BPA exposure (highest quartile vs. the lowest three quartiles) was associated with significantly higher CBCL scores (more problems) on Emotionally Reactive [1.62 times greater; 95% confidence interval (CI): 1.13, 2.32] and Aggressive Behavior syndromes (1.29 times greater; 95% CI: 1.09, 1.53). Among girls, higher exposure was associated with lower scores on all syndromes, reaching statistical significance for Anxious/Depressed (0.75 times as high; 95% CI: 0.57, 0.99) and Aggressive Behavior (0.82 times as high; 95% CI: 0.70, 0.97).

The study has several limitations, including the timing of urinary BPA measurement measured only once during pregnancy. In addition, there was a large variation on the time during gestation when BPA levels were measured. The conclusions of the study are limited by the qualitative nature of the single BPA urinary concentration measurements. Behavioral measurements are limited by the subjectivity of the parent reporting. Interestingly, the study's conclusions are not in agreement with other studies of larger cohorts (Braun *et al.*, 2011). **This study has no utility for HI and no utility for RA.**

Subcutaneous and IP exposure

Neonatal Bisphenol A exposure alters sexually dimorphic gene expression in the postnatal rat hypothalamus (Cao, *et al.*, 2012)

The objective of this study was to examine if BPA affects ERα, ERβ, and kisspeptin gene expression in the hypothalamus. Pups were born to timed pregnant Long Evans rats (n = 13) housed in thoroughly washed polysulfone caging with woodchip bedding, and fed a semi-purified, phytoestrogen- free diet *ad libitum* to minimize exposure to exogenous BPA, phytoestrogens, and other endocrine disrupting compounds. There was no mention if the water bottles were also BPA free. Beginning on the day of birth (defined as postnatal day zero (PND0)), n = 6–9 pups per sex per group from 3-4 litters received 0.05 mL SC injections of vehicle (oil:ethanol=9:1), 10 μg estradiol benzoate (EB), BPA 50 μg/kg bw, or BPA 50 mg/kg bw, once/day for 3 days (PND 0-2). It appears that sufficient precautions were taken to minimize cross-contamination between the litters and test articles, although Churchwell *et al.* (2014) provides commentary on the difficulty in unintentional exposure prevention. Pups were sacrificed on PNDs 4 and 10, crania rapidly frozen then cryosectioned into three serial sets of 18 mm coronal sections, and analyzed using in-situ hybridization histochemistry (ISHH) to determine expression of estrogen receptor genes *Esr1* (ERα), *Esr2* (ERβ), and *Kiss1* (*kisspeptin 1*). Areas of interest were: anterior hypothalamus (AVPV and MPOA), mediobasal hypothalamus (VMNvl and ARC, both rostral and caudal). Sense and anti-sense ^{32}S RNA probes for ERα, ERβ, and Kiss1 were added to brain sections fixed on slides, exposed to x-ray film for 14–40 days, and then quantified using a digital densitometry application. For Kiss1, to increase the signal, the hybridized Kiss1 slides were emulsion dipped developed, counterstained, and the quantified.

Two bilateral sections/animal were used, with the exception of ARC Kiss1, where 4 bilateral sections/animal were used. The values for each region of interest (ROI) were averaged. Two-way ANOVA with sex and exposure group as factors were used; then, one way ANOVA to analyze the effect of exposure within each sex. Significant effects were followed up with the Dunnett's Multiple Comparison post hoc test to compare with vehicle and t-tests to identify sex differences. All analyses were two-tailed and results were considered significant when $p \leq 0.05$.

EB administration eliminated the sexually dimorphic expression of ER1 and 2 and Kiss1 (F>M), mostly by reducing expression in females. In BPA-treated animals, there were reported changes that varied with dose, age, and ROI. In anterior hypothalamus, ERα expression was increased on PND 4 in both sexes receiving 50 mg/kg BPA; decreased on PND 10 in both sexes receiving 50 μg/kg in AVPV but not MPOA; decreased on PND 10 in both ROIs in females only receiving 50 mg/kg. ERβ expression was decreased on PND10 for both dose groups for both ROIs, resulting in reversal of sexual differences for MPOA and undetectable expression in AVPV. Kiss1 expression on PND10, when detected, was sexually dimorphic; both doses of BPA reversed the characteristic sexual difference (F>M) seen in vehicle controls. The finding has no statistical significance due to a reduction in animals (both males and especially females) with measurable expression levels. In mediobasal hypothalamus, ERβ expression was increased in males in cVMNvl on PND 10, and Kiss1 expression was increased in ARC in males on PNDs 4 but did not eliminate the sex difference in expression levels.

The study is a mechanistic study, not designed to be used for RA. The end points evaluated do not address the End Point Measure criterium. The following are some important points for consideration. Subcutaneous route of administration and dose results in an exposure much higher than oral administration. Although the BPA is administered at a sensitive window in rat brain development roughly corresponding to human fetal brain in the second/third trimester, the fetal BPA serum concentration is ~50% of maternal level. As such the exposure under these experimental conditions can be many orders of magnitude beyond expected human dietary exposure. Littermates were used in analysis (2-3 littermates/sex/group). As such, the effective n could be as small as n=3/sex/group. A limited number of doses was given (2 doses with 1,000 fold difference). Method sensitivity, especially for *Kiss1* expression is low and precludes from drawing firm conclusions. Additionally, no firm conclusions could be drawn on the (patho)physiological effects (including behavior, *e.g.*, Ferguson *et al.*, 2011, 2012) of BPA mediated changes in ERα, ERβ, and kisspeptin genes. **This study has no utility for HI and no utility for RA**.

<u>Adolescent exposure to Bisphenol-A increases anxiety and sucrose preference but impairs spatial memory in rats independent of sex (Diaz Weinstein *et al.*, 2013)</u>
The goal of this study was to assess the effects of BPA treatment during adolescence on measures of anxiety, locomotor activity, spatial memory, and sucrose preference in male and female Sprague-Dawley rats. At postnatal day (PND) 42, Sprague-Dawley rats (n=9/sex/group) (Charles River Laboratories) were group-housed in same-sex, same-treatment cages. Caging type was not specified, although glass water bottles were used. Diet was not specified and noted only as "rat chow". There was no positive control group. From PND 49 onward, each received 12

subcutaneous injections of saline or 40 µg/kg/day BPA (Sigma-Aldrich) (*i.e.*, on PNDs 49-60). On PND 55, three assessments occurred. Anxiety behavior was measured using the elevated plus maze apparatus (5 minute trial). Locomotor activity was measured in an open field (6 minute trial). Spatial memory was measured using the object placement test with a 1 minute inter-trial interval (3 minute trial). On PND 56, spatial memory was again assessed with a 10 minute inter-trial interval, and again on PND 57, with a 2 hr inter-trial interval (3 minute trials). On PND 58, subjects were food and water deprived and on PND 59-60, two bottles were placed on each cage, one containing regular water and the other containing a 10% sucrose solution. Table 1 in the paper seems to indicate that the BPA injections on PND 55 occurred after the anxiety and locomotor assessments, but before the spatial memory assessment. On PNDs 57-60, that same table seems to indicate that BPA injections occurred prior to any behavioral assessment. Body weights were measured during treatment.

BPA injections had no effect on body weight during the 12 day treatment period. BPA-treated rats (males and females) exhibited longer durations in the closed arms of the elevated plus maze, indicating increased anxiety. There were no other statistically significant effects on this assessment. BPA-treated rats (males and females) entered the inner sectors of the open field less, which may also indicate increased anxiety. Females were more active overall than males, an expected sex effect in this assessment. Spatial memory was not altered by BPA treatment with inter-trial intervals of 1 and 10 minutes. However, with the 2 hr inter-trial interval, BPA-treated rats explored the object in the novel place less. This effect seemed especially prominent in BPA-treated males. BPA-treated rats also exhibited an increased sucrose preference relative to controls; however, there were no sex effects.

Both sexes were evaluated and the expected sex effects on locomotor activity were detected. However, the expected sex effects on sucrose preference were not apparent. In part, this may be because the subjects were food and water deprived prior to this assessment. Food/water deprivation is not typically conducted prior to sucrose preference tests. The statistical analyses are appropriate. The behavioral assessments are those that are commonly conducted to evaluate anxiety, locomotor activity, and spatial memory. However, it is not clear whether the BPA injections on PNDs 55-60 occurred before or after the behavioral assessments on those days. If BPA treatment occurred prior to the assessments, the effects on spatial memory and sucrose preference may be due an acute effect of BPA treatment. In any case, all behavioral assessments occurred during the period of BPA treatment (PNDs 49-60) so it is not clear if any of the reported BPA effects would persist after treatment ended. It is not clear why the percentage of time with the new object in the object placement test ranges 46-56% using inter-trial intervals of 1 and 10 minutes, but increases to 60-70% with a 24 hour inter-trial interval. It is not clear if the data shown for exploration time (Fig. 3A) and T1 data in Table 3 are the total exploration time for both of the objects or the exploration time for the object that is to be located in the novel location. While the object placement test has been used to test spatial memory, the novel object recognition test is more widely used. The number of subjects/group is less than 10, only one dose of BPA was used, and it is likely that the diet was a soy-based diet. **This study has no utility for HI and no utility for RA.**

Prenatal exposure to Bisphenol-A impacts midbrain dopamine neurons and hippocampal spine

synapses in non-human primates (Elsworth *et al.*, 2013)

The goal of this study was to examine potential effects of BPA exposure on dopaminergic and glutamatergic neurons in young non-human primates. The study had two arms, a fetal stage and a juvenile stage, using two different non-human primate species. In the fetal stage study, adult female rhesus macaques (Macaca mulatta) were caged individually in stainless steel cages and fed Purina Monkey Diet #5045, a high protein diet containing soybeans; thus, it has the potential of containing high level of phytoestrogens. The diet was supplemented with seasonal fruits, seeds, and cereals. Although the identity of seeds used to supplement the diet is not specified, it could have included flaxseeds, another food source with very high phytoestrogen content. Water was provided *ad libitum*, using a BPA free set-up. Pregnant monkeys carrying male fetuses were excluded from the study. There were two cohorts in this study.

1. Oral BPA cohort: n=5/group pregnant monkeys received test article (deuterated BPA, d-BPA) or vehicle (unspecified) via food by lacing small pieces of fruit with 400-µg/kg body weight starting on GD 100 to cover the entire gestation till vaginal delivery ~GD165 and beyond. Neonates were euthanized 1 to 3 days after birth, brains fixed and the number of DA neurons in each ventral mesencephalon was quantified using tyrosine hydroxylase (TH) immunohistochemistry. Maternal blood samples for PK were only collected "within two weeks of birth".
2. Subcutaneous (SC) BPA cohort: pregnant animals received a total of 2.5 ml d-BPA 50 mg/ml (n=6) or vehicle (tocopherol-stripped corn oil, n=3) via SC silastic tubing implants (three sites) from GD 100 to 155. Fetuses were removed by hysterotomy at GD155, brains fixed and total number of spine synapses in the stratum radiatum of the CA1 subfield of the hippocampus and layers II/III of the dorsolateral prefrontal cortex (DLPFC) was determined by electron microscopy. Maternal blood samples were obtained at GD155 to determine serum levels of d-BPA using a solvent extraction step with an internal standard and LC-MS analysis.
3. In the juvenile stage study, pre-pubertal African green monkeys (Chlorocebus aethiops sabaeus; 14–18 months old; 2.5–3 kg) were initially housed in social group enclosures, then 2 per cage for the duration of the study. They received Harlan primate diet (#8773) supplemented with seasonal local fruits; water was dispensed from BPA free pipes. D-BPA at a dose of 75 mg or vehicle (n=4/sex/group) was administered via subcutaneous silastic tubing implants (two sites) for 30 days at which point the animals were euthanized. Brain samples were collected and the following analyses were performed using LC-MS analysis without a solvent extraction step and without an internal standard.

DA, homovanillic acid (HVA), 5-hydroxytrytamine (serotonin, 5HT), and 5-hydroxyindoleacetic acid (5HIAA) were separated by reverse-phase isocratic HPLC, detected by gel electrophoresis (data not shown), and quantified with respect to internal and external standards. DA neurons were evaluated by staining for TH using primary and secondary antibodies, following by stereologic counting using StereoInvestigator 7 software. The total number of spine synapses in the CA1 stratum radiatum of the hippocampus (critical in memory formation) and layers II/III of DLPFC (which is related to working memory and has a dense DA neuron innervation) were evaluated. For this, a systematic randomized protocol and predetermined rules were used to select the slides from the serial coronal sections and count the synapses using electron microscopy. Plasma d-BPA levels were determined before implant, at 4, 20, and 30 days after

implantation. The method used included only a precipitation step but no internal standard followed by LC-MS analysis. Working memory performance was evaluated by a 2-well spatial delayed response task in a custom-designed Wisconsin General Test apparatus. Statistical analyses included t-test for the prenatal study, oral cohort, or 2-way ANOVA with region and treatment as two effects for the SC cohort and juvenile study arm.

The results of the prenatal treatment included oral d-BPA dosing resulting in mean maternal plasma concentration of d-BPA 0.68±0.28 ng/ml. TH immunoreactivity in the midbrain of neonatal offspring decreased at birth. SC d-BPA implantation resulted in mean maternal serum level on GD 155 of 0.91±0.13 ng/ml unconjugated d-BPA. This was associated with a significant loss of spine synapses in the CA1 but not in DLPFC. In the juveniles, exposure resulted in d-BPA equal to 14.6±0.9 ng/ml at 4 days after implantation, 16.8±1.0 at 10 days after implantation and 13.1±1.4 ng/ml at 30 days after implantation. No change in any of the outcome measures was seen.

The studies are performed in non-human primates and include an appropriate number of animals. The study has several limitations. There is no certainty that either oral or SC dosing delivered continuous exposure to d-BPA, since the blood levels of BPA were measured only one time and only after the pregnancies were terminated. The differences in TH immuno-histochemistry seen at birth are not fully explored. It is not described whether there were any differences in the length of gestation between the vehicle and treatment groups. These animals were not at the same gestational age at birth and some differences could be expected due to different maturity levels. For the subcutaneous cohort, both groups were analyzed on GD155, but the control group only included n=3 subjects, thus limiting the strength of evidence of the finding. The authors hypothesize that changes seen in TH-immunoreactivity could have consequences in subsequent brain development whereas loss of spine synapses in hippocampus could impact memory processes.

The study has limited utility for HI and no utility for RA. The authors have not been able to provide a unifying hypothesis for discordant effects of BPA on spine density in fetal and juvenile primates, nor have they been able to explain an apparent anti-estrogenic effect of BPA in adult primates (Leranth *et al.*, 2008).

Estrogen-induced memory enhancements are blocked by acute bisphenol A in adult female rats: role of dendritic spines (Inagaki *et al.*, 2012)

The objective of this study was "to examine effects of acute BPA exposure, alone and in combination with estrogens on E2-induced memory enhancement and synaptic plasticity in ovariectomized (OVX) and gonadally intact, cycling female rats." A total of 83 OVX and 18 intact female Sprague-Dawley rats, 3 months of age, were pair-housed with *ad libitum* access to a low phytoestrogen diet. Caging and water bottle material are not stated. The authors' focus was on the acute effects of BPA and for this reason the rats were repeatedly behaviorally assessed in the same tasks using different BPA and E2 doses. Each behavioral assessment (and drug treatment) was separated by 10 days and, for each assessment, there were 6-12 subjects/group. Thus, each subject may have received up to 6 treatment injections. Two types of object tasks were performed: novel object placement (NOP) and novel object recognition (NOR). For each of

these, the subject is placed into an empty open field for 6 minutes for habituation (day 1). The subsequent 7 days contain 2 trials each. On trial 1 (T1), the subject is placed into the open field containing two identical objects and the duration of exploration toward both objects is recorded for 3 minutes. Following an inter-trial interval of 2 or 4 hours, the subject is placed back into the open field (trial 2 or T2) in which one of the previous objects is placed in a different location (NOP) or is replaced with a novel object (NOR) and exploration toward the novel placement/object is recorded for 3 minutes. T1 and T2 were videotaped; however, it is not clear if the exploration duration was assessed blind to treatment conditions.

To test the effects of BPA alone on memory consolidation (Study 1), OVX rats were subcutaneously (sc) injected immediately after T1 with vehicle, 1, 4, 40, 120, 240, or 400 µg/kg BPA (Sigma-Aldrich). Subsequently (Study 2), OVX rats were sc injected with vehicle, 0.4, 1, 4, 40, 120, 240, or 400 µg/kg bw BPA before T1 (a different treatment than the prior BPA alone study) and then sc injected with vehicle or 5 (for NOR) or 20 (for NOP) µg/kg 17ß-E2 immediately after T1. Here, the authors adopt strict criteria for determining significance: First, the vehicle+vehicle group (negative control) must significantly differ from the vehicle+17ß-E2 (positive control) group; secondly, each BPA group must significantly differ from both the vehicle+vehicle group and the vehicle+17ß-E2 group. Following those BPA and 17ß-E2 tests, the effects of 17α-E2 and BPA were assessed (Study 3). Here, OVX rats were sc injected with vehicle, 0.4, 1, 4, or 40 µg/kg bw BPA before T1 and then sc injected with vehicle, 1 (for NOR) or 5 (for NOP) µg/kg bw 17α-E2 immediately after T1. The OVX rats were next assessed in a standard elevated plus maze assessment (Study 4). Intact rats were assessed in the NOP and NOR tasks (Study 5) after being sc injected with vehicle or 40 µg/kg bw BPA immediately after T1. Estrous phase was determined immediately after T2. Ten days after the final behavioral test (Study 6), OVX rats received a pre-T1 sc injection (vehicle or 40 µg/kg bw BPA), a T1 sample trial, and a post-T1 sc injection (vehicle or 20 µg/kg bw E2) and were killed at 0.5 or 4 hours later for the spine density and serum E2 level analysis; however, this study did not include a BPA only treated group. To measure spine density, defined as the number of dendritic spines divided by length of dendrite, brain blocks (anterior and posterior) were stained using Golgi Stain kit (which stains some of the neurons) and the apical and basal dendritic spine density for specific regions ("the medial PFC (layers II/III) and the CA1 region of the hippocampus") were analyzed microscopically in a blind manner. Serum E2 was measured using a commercial radioimmunoassay (RIA) kit.

Study 1 (BPA alone in OVX rats): T1 exploration times did not differ among groups (as expected, since this was prior to BPA treatment). BPA treatment had no effect on T2 exploration ratios in either task (NOP or NOR).
Study 2 (BPA and 17ß-E2 in OVX rats): For both NOP and NOR T1 exploration durations, there were no significant BPA or 17ß-E2 effects, but there were significant effects in T2 exploration times. Post-hoc tests revealed that the NOP exploration ratio (calculated as T2/T1+T2) increased for 17ß-E2 treated rats compared to vehicle controls. However, groups treated with both 17ß-E2 and 4, 40, 120, 240, or 400 µg/kg bw BPA did not show this improvement: the ratios returned to vehicle only levels, but differed significantly from 17ß-E2 group. The same effect was observed in T2/(T1+T2) ratios for NOR: 17ß-E2 rats had better memory retention than those receiving

vehicle while groups treated with both 17ß-E2 and 40, 120, 240, or 400 µg/kg bw BPA did not differ from the vehicle controls but had significantly lower exploration ratios compared to the 17ß-E2 group.
Study 3 (BPA and 17 ß-E2 in OVX rats): There were no significant BPA effects on exploration times during T1 for either NOP or NOR. NOP exploration ratios were increased by 17ß-E2 compared to vehicle, but exploration ratios of the 17ß-E2+ 0.4, 1, 4, or 40 µg/kg bw BPA groups were similar to the vehicle group. Exploration ratios of the 17ß-E2+ 1, 4, or 40 µg/kg bw BPA groups were significantly lower than the 17ß-E2 group. For T2 NOR exploration times, there were no significant BPA effects.
Study 4 (BPA effects on elevated plus maze behavior of OVX rats): There were no significant BPA effects.
Study 5 (BPA alone in intact rats): For NOP T2 exploration times, there were no significant effects of BPA. For NOR T2 exploration times, the significant interaction of treatment and estrous phase indicated that the control and BPA-treated groups differed significantly only during proestrus (when E2 levels are highest) during which the BPA-treated rats exhibited a lower exploration ratio than controls.
Study 6 (spine density and E2 levels in OVX rats): In OVX rats which received E2 only, spine density was significantly increased after 30 minutes in the basal area of CA1 and in the apical and basal areas of the medial prefrontal cortex. The same increases were still apparent after 4 hours in the basal areas of CA1 and the prefrontal cortex. Relative to the E2-treated group, BPA treatment further significantly increased spine density after 30 minutes in the apical and basal areas of CA1. Relative to the vehicle+vehicle group, BPA treatment further increased spine density after 30 minutes in all four areas. After 4 hours, relative to the E2-treated group, BPA treatment further increased spine density in the apical area of CA1 only, but decreased it in the basal area of CA1. There were no BPA differences in the medial prefrontal cortex relative to the E2-treated group after 4 hours.

Statistical methods were appropriate. This study has significant limitations: (1) Although described as an acute study, the rats were not naïve to the test articles or the memory assessments. E2 or BPA or both were administered repeatedly, every 10 days for 2 months. (2) For 83 OVX rats, there were 24 dosing regimens and two memory tests (NOP and NOR) performed at each dose. This means that subjects were tested multiple times and the results for each dose group are pooled. (3) It is not clear if the changes in measured outcomes are due to a cumulative effect of the BPA doses given (if indeed long-lasting changes in neuronal morphology do occur following BPA treatment as the authors conclude), due to the sequence of the test articles administered, due to the timing of the tests performed, or due to chance alone given the multiple measures using the same animals. (4) BPA-induced changes in memory and spine density were only seen after administering E2 in OVX rats, an artificial scenario that may applicable to specific populations (*e.g.*, a model for post-menopausal women). In addition, it is not clear how these doses of 17α-E2 and 17ß-E2 injected sc might relate to the physiological levels of intact rats or those of adult women. (4) There may be environmental BPA exposure through caging and water bottle material. (5) Many groups contain less than 10 subjects/group. **Given the limitations, this study has no utility for HI and no utility for RA.**

High dose bisphenol A impairs hippocampal neurogenesis in female mice (Jang *et al.*, 2012)
The objective of this study was to evaluate the effects of transgenerational exposure to BPA on hippocampal neurogenesis and neuronal damage, markers of neuronal signaling, and learning and memory in F2 female mice. C576BL/6 mice (Daehan Biolink) were maintained under a 12:12 h light-dark cycle at 20-23°C, in accordance with an Institutional Animal Care Committee review. Caging and water bottle material are not stated, nor is the type of diet specified. Although a negative control was included in the study (corn oil vehicle), an experimental positive control was not. BPA (purity 99.7%) (Sigma Chemical) at doses of 0.1, 1, or 10 mg/kg or corn oil was injected intraperitoneally in the initial (F0) generation of females from gestational day (GD) 6 to GD 17. No justification for those doses was provided. Female mice from those litters (F1 control and BPA-treated) were mated with control males to produce an F2 generation. At 6-8 weeks of age, F2 females were divided into four groups of 20-22 mice/group for the evaluation of learning/memory; hippocampal histology; cellular markers of neurodegeneration; phosphorylation, activation of proteins involved in cell growth or differentiation, cell proliferation, and gene expression. Assays included (a) the Morris Water Maze for spatial learning and the step-through protocol (passive avoidance) for short term memory; (b) neuronal degeneration determined by Nissl staining and Glial Fibrillary Acidic Protein (GFAP) (astrocyte) and Iba-1 (microglia) staining of hippocampal tissue sections; (c) the density of hippocampal neuronal progenitor cells determined by bromodeoxyuridine (BrdU) staining, (d) immunoblotting of hippocampal and cortical brain homogenates for cell signaling proteins (phospho-ERK, phospho-p38, phospho-JNK, all normalized to β-actin), and the growth factor, brain derived neurotrophic factor (BDNF); (e) immunostained hippocampal slices for phospho-CREB and BrdU, or GFAP and Iba-1; (f) DNA methylation. Results were analyzed using analysis of variance (ANOVA) with Fisher's protected least significant difference post-hoc comparisons.

Statistically significant effects were reported at the high dose (10 mg/kg bw/day) BPA exposure in F2 female mice, resulting in (a) decreased numbers of hippocampal BrdU positive cells; (b) decreased levels of hippocampal phospho-ERK (but not phospho-p38 or phospho-JNK), BDNF, and phospho-CREB (hippocampal phospho-ERK was also decreased in the 1 mg/kg BPA group); (c) an increase in DNA methylation of CREB regulated transcription coactivator 1 (Crtc1); and (d) a decreased cross-over passive avoidance latency. However, there was no neuronal loss or damage in the hippocampal area of the F2 female mice, nor was there any change the GFAP or Iba-1 markers. Morris water maze performance was not altered by BPA exposure; however, F2 female mice of the 1 and 10 mg/kg bw/day BPA groups exhibited significantly shorter cross-over latencies in the passive avoidance test, indicating impaired memory. The authors suggest a link between transgenerational BPA exposure, impaired hippocampal neurogenesis, and memory functions, without significant involvement of neuroinflammation or neurodegeneration.

Problematic to this study were the lack of controls for exogenous exposure to BPA or estrogenic substances and the lack of a positive experimental control. *In vivo* exposures were not verified through analysis of blood for levels of BPA, and no justification was provided on use of these BPA doses. The lowest BPA dose here (100 µg/kg bw/day) did not produce any significant effects. Although the number of subjects/group is adequate (n=20-22/group), it is not at all clear that the litter is the unit of analyses. It is not clear why only females were assessed in this study since all endpoints could have been assessed in F2 males as well. Finally, some of these

endpoints could easily be affected by estrous phase and this was not measured at sacrifice. **This study has no utility for HI and no utility for RA.**

Effects of perinatal exposure to low dose of bisphenol A on anxiety like and dopamine metabolites in brain (Matsuda *et al.*, 2012).

The purpose of this study was to determine the effects of perinatal BPA exposure on locomotor activity (anxiety) and dopaminergic metabolism in offspring of mice exposed to BPA from gestational day (GD) 10 through lactational day 20 via injection. Female C57BL/J6 mice (age not specified) were purchased at GD 8 and subcutaneously injected with 250 ng/kg bw/day BPA (Wako Pure Chemicals) or corn oil vehicle daily from GD 10 to offspring postnatal day (PND) 20. Although dams were weighed every 3 days, it appears the authors used a standard weight of 30 g to calculate the BPA dose, rather than each dam's actual body weight. Dams were fed a soy-based diet (CE-2, CLEA Japan Inc.). Caging and water bottle material are not specified. Blood levels of free or conjugated BPA were not determined in serum or urine of dams or offspring. At PND 2, litters were culled to 3/sex/litter and weaned on PND 21 to group-housing in same-sex groups (3-5/cage). At PNDs 28 and 56 or 63 (text states both ages in different portions), male and female offspring were assessed for open field locomotor activity. It is not clear if the same subjects were tested at both ages. After sacrifice at approximately PND 70, the hippocampus, amygdala, and medulla were dissected and stored at -85°C. Those tissues were analyzed for dopamine and DOPAC levels (standard HPLC methods) and monoamine oxidase (MAO) A and B activity using traditional methods. Two-way (sex x treatment) ANOVAs with Bonferroni post-hoc testing were used to analyze behaviors and dopamine and DOPAC levels. It is not clear why a three-way ANOVA (sex x treatment x age) was not used to analyze the open field data (especially since in a later publication from this same lab in which mice were assessed at the same ages, the factor of age was included in analyses). Since only males were assessed for MAO activity, a one-way ANOVA was used to analyze those data.

Female open field activity was not affected by BPA treatment. However, post-hoc comparisons of the significant treatment x sex interactions at PND 28 and PND 63 indicated that BPA-treated males exhibited a significantly shorter duration of time in the center of the open field at both ages. The authors interpret this as increased anxiety. Neither total distance nor time in the center area indicated sex differences. Post-hoc comparisons of the significant interaction of treatment x sex on dopamine levels in the dorsal hippocampus indicated that BPA-treated males had increased dopamine levels compared to control males. There were no treatment effects on dopamine or DOPAC levels in the amygdala. Dopamine levels in BPA-treated males were also significantly increased in the medulla. Dopamine turnover (measured as DOPAC/dopamine ratio) was decreased in BPA-treated males relative to control males in the dorsal hippocampus, amygdala, and medulla. In males, MAO-A and –B activity in the hippocampus and amygdala were not affected by BPA treatment; however, MAO-B activity was decreased in BPA-treated males in the medulla (decreased ≈ 40%). Thus, all reported significant effects occurred in males.

Even though the levels of serotonin (5-HT) or its metabolite 5-HIAA are closely linked to anxiety and depression, these were not measure in the current study. 5-HT and 5-HIAA levels were reported in a subsequent manuscript from this lab (Matsuda *et al.*, 2013) but at PND 28 only. In

this report, they failed to measure DOPA or HVA levels which are also dopamine metabolites and are as good or better measures of dopamine release/turnover. Although the amygdala is a key region in anxiety and emotional control, the hippocampus and even more so the medulla is not nearly as important. Frontal cortex regions, such as the cingulate and orbital cortex, are also key brain regions for modulation of anxiety and emotion. Amygdalar levels of dopamine, DOPAC, and MAO-A and –B levels were unaffected by BPA, and this area is where one might have expected alterations, if there were behavioral changes in anxiety. Use of duration in the center area of an open field is not the best measure of rodent anxiety. Behavior on the elevated plus and zero maze are much better validated measures.

This study has little or no utility for HI and no utility for RA. The study has almost no use for HI in humans and of no use for RA because: 1) only one BPA dose was evaluated, 2) the control group (and some of the BPA endpoints) contains less than 10 subjects, 3) the diet was a soy-based diet, and 4) there does not appear to be any control for exogenous estrogen exposure via caging or water bottle material (note: the later publication from this same lab states polycarbonate caging and water bottle material). This is particularly problematic given the single, very low BPA dose used (250 ng/kg bw/day). The authors did not state if litter was used as the unit in the statistical analysis.

Perinatal exposure to bisphenol A enhances contextual fear memory and affects the serotonergic system in juvenile female mice (Matsuda *et al.*, 2013)

The purpose of this study was to determine effects of perinatal exposure to BPA on anxiety and dopaminergic metabolism in offspring of mice exposed to BPA from gestational day (GD) 10 through lactational day 20. Dams were injected with 250 ng/kg/day BPA during this time. Female C57BL/J6 mice (age not specified) were purchased at GD 8 and subcutaneously injected with 250 ng/kg BPA (Wako Pure Chemicals) or corn oil vehicle daily from GD 10 to offspring postnatal day (PND) 20. Although dams were weighed every 3 days, it appears a standard weight of 30 g was used to calculate the dose, rather than each dam's actual body weight since they state they used the same injection protocol as their previous study. Dams were fed a soy-based diet (CE-2, CLEA Japan Inc.). Caging and water bottle material were polycarbonate, but the authors state that they did not detect BPA in the drinking water. At PND 2, litters were culled to 3/sex/litter and weaned on PND 21 to group-housing in same-sex groups (3-5/cage). Litters with less than 6 pups were fostered to a different dam, although it is not clear what this means. At PNDs 27-29 and 63, male and female offspring were assessed for contextual fear conditioning (3 sessions) with endpoints of freezing behavior prior to, immediately after, and 24 hr after footshock. It is not clear if the same subjects were tested at both ages. After sacrifice at PND 28 or 63, the hippocampus, striatum, midbrain, pons and medulla were dissected and stored at -85°C. Tissues were analyzed for serotonin (5-HT) and 5-HIAA metabolite levels (standard HPLC methods). In a separate experiment (but likely using female siblings of the subjects above), BPA-treated and control female mice were tested for fear conditioning on PNDs 27-29 using identical methods as previously. However, on PNDs 25-28, ½ of the BPA-treated females received subcutaneous injections of 5 mg/kg sertraline, a selective 5-HT reuptake inhibitor. It is not clear how soon after testing that the females were sacrificed; however, the hippocampus was assessed, using standard RT-PCR methods, for mRNA expression levels of three 5-HT receptors

(*Htr1a, Htr2a and Htr2c),* 5-HT plasma membrane transporter *(Slc6a4*), tryptophan hydroxylase (*Tph2*) and monoamine oxidase A (*Maoa).* 2-,3-, and 4-way ANOVAs with Bonferroni post hoc testing were used to evaluate freezing behavior and 5-HT, 5-HIAA, and 5-HIAA/5-HT ratios. Gene expression levels were analyzed via Student's t-tests.

Although the treatment x sex x age x session interaction was not significant (*i.e.*, PRE, POST, and TEST), a post-hoc test compared juvenile female freezing during the TEST session and reported increased freezing levels in BPA-treated females than control females; there were no other BPA effects on fear conditioning. BPA-treated juvenile males had increased striatal 5-HIAA/5-HT ratios and higher 5-HT levels in the pons. BPA-treated juvenile females were more affected, exhibiting significantly increased hippocampal, striatal, midbrain, pons, and medulla 5-HIAA levels and 5-HIAA/5-HT ratios. There were two effects reported in adult mice: a decreased 5-HIAA/5-HT ratio in BPA-treated females and an increased 5-HT level in the medulla of BPA-treated males. In the subsequent sertraline fear conditioning test, sertraline had no effects on freezing. However, BPA-treated females (sertraline or vehicle injected) exhibited significantly more freezing during the TEST session than control females. With one exception (decreased striatal levels of *Htr2c*), all significant gene expression effects were increased in BPA-treated females (increased levels of *Tph2* in hippocampus and striatum, increased levels of *Htr1a* in hippocampus and medulla, increased levels of *Slc6a4* in the hippocampus, increased levels of *Htr2a* in the midbrain, increased levels of *Maoa* in the hippocampus, pons, and medulla).

BPA exposure to rodent offspring indirectly through lactation from dams exposed to 250 ng/kg bw/day BPA by sc injection would likely be undetectable. Thus, any effect observed would have no plausible mechanism of action. Additionally, the lack of control of additional sources of exposure to BPA and estrogenic compounds is problematic, given the single BPA dose used in the study. In comparing the results here with a previous study from the same lab, it seems the authors may have selectively published data in 2 separate papers from the same study. For example, they did not report 5-HT and 5-HIAA levels in adults in the 2012 paper. The second part of this paper is based on a single behavioral effect in females (not detected in the overall ANOVA); however, their earlier 2012 study did not detect any significant effects in females identically treated with BPA. They misstate their effects from the prior study in this paper: In the Discussion, they note that "we previously showed that BPA tended to enhance anxiety-like behavior in juvenile female mice in an open-field experiment"; however, this significant effect was in males only. They acknowledge their prior discussion indicating that the effects seen here at PND 28 may disappear at PND 63 (2012 paper). Another example is that striatal levels of dopamine and metabolites are not reported in the 2012 paper but striatal 5-HT and 5-HIAA levels are described here. They report modest 15-30% increases in 5-HIAA levels in all brain areas of females which is consistent. Although the amygdala is a key region in anxiety and emotional control, the hippocampus and other measured brain regions are not as important. Frontal cortex regions are also key regions for anxiety and emotion modulation.

This study has no utility for HI and no utility for RA. The study is of no utility for RA because: 1) only one BPA dose was evaluated, 2) several treatment groups contains less than 10

subjects, 3) the diet was a soy-based diet, 4) there does not appear to be any control for exogenous estrogen exposure via caging or water bottle material and, 5) the BPA dose (0.25 µg/kg bw/day) would result in negligible BPA levels in the offspring likely far below estrogen receptor binding levels and almost all known physiological receptor Kd.

Prenatal and lactational exposure to low-doses of bisphenol A alters adult mouse behavior (Nakamura *et al.*, 2012)

The purpose of this study was to assess the effects of prenatal and lactational BPA exposure on pre-adolescent and adult activity and anxiety levels, and adult spatial learning and memory in male and female mice. Sperm plug positive female ICR/Jcl mice (n=8/treatment group) were subcutaneously injected with sesame oil (vehicle) or 20 µg/kg BPA (from Wako, Osaka, Japan) dissolved in sesame oil from gestational day 0 until lactational day 21 (*i.e.*, postnatal day (PND) 21 for the offspring). Litters were culled to 5/sex and weaned on PND 21. Type of caging and water bottle material are not specified. Diet is not specified. Separate subjects were used for the pre-adolescent and adult assessments. A standard 10 minute open field test was conducted at PND 21 or 22 and PND 70. A standard 10 minute elevated plus maze session was conducted at PND 24, 25, or 26 and PND 77. A standard water maze assessment was conducted at PND 84 for females and PND 91 for males, consisting of 4 trials/day for 5 consecutive days followed by a probe test on the sixth day. All data were automatically collected via computer software, except number of rears in the open field. It is not stated if that endpoint was collected blind to treatment. Data were analyzed via two-way ANOVAs (with factors of treatment and sex); however, it is not clear if the repeated measure(s) of trials and/or days were included in the water maze analysis.

There is no information on gestational/lactational body weight or food/water intake. There is no information on litter endpoints (*e.g.*, birth weight, litter size, sex ratio). PND 70 body weight was unaffected by BPA treatment; however, at PND 20-21 (table states PND 21-22, but text states PND 20-21), male and female BPA-treated mice weighed less than same-sex controls. It is not clear if this was a significant interaction of BPA and sex or a significant main effect of treatment. There were no significant effects on any open field endpoint in pre-adolescents; however, females exhibited a significantly longer duration in the central area. In the elevated plus maze analyses, pre-adolescent BPA-treated male and female mice exhibited a decreased distance traveled, indicating hypoactivity. Other elevated plus maze endpoints were unaffected by treatment and there were no significant sex effects. Analyses of adult open field activity indicated significantly decreased distance traveled by male and female BPA-treated mice and no significant sex effects. Adult elevated plus maze activity was not significantly affected by treatment; however, females exhibited a shorter distance traveled than males. Water maze performance was not significantly affected by treatment or sex.

Although stated to be statistically significant, the BPA effect on weaning body weight was mild (3.5% and 5.1% decrease in males and females, respectively) and the calculated effect size is small for males (using the authors' incorrect sample sizes of 39-40, Cohen's d effect size of 0.26). Similarly, the BPA effect on distance traveled in the adult open field assessment was also mild, at least in males (5.7% and 20.9% decrease in males and females, respectively and using the authors' incorrect sample sizes of 19, Cohen's d effect size for males is 0.34). Distance

traveled in the elevated plus maze at pre-adolescence was also mildly decreased by BPA treatment (12.8% and 7.8% decrease in males and females, respectively; however, those result in larger effect sizes). It is not clear why the pre-adolescent activity assessment did not indicate significant BPA effects, but the adult assessment (conducted over 7 weeks since the last BPA exposure) did. Similarly, the anxiety assessment was affected by BPA treatment at pre-adolescence but not at adulthood. Only one measure was collected in the water maze (*i.e.*, latency to the platform). Swim speed and a measure of thigmotaxis and/or floating may have proven more sensitive. Both sexes were evaluated; however, there was no positive control group. Although the number of subjects/group is adequate (n=15-40/sex/group), the litter is not the unit of analysis. There were 8 control litters and 8 BPA-treated litters. Thus, when analyzed correctly, this study has n=8/sex/group. Only one BPA dose was evaluated. Finally, there was no positive control group, and it is not specified that exposure to exogenous estrogens or BPA were controlled in caging material, water bottle material, or diet.

This study has no utility for HI and no utility for RA. The litter is not the unit of analysis. It is not clear if there was adequate control for environmental exposure to estrogens or BPA via caging and water bottle material or if the diet was a low phytoestrogen chow.

Maternal and fetal exposure to bisphenol A is associated with alterations of thyroid function in pregnant ewes and their newborn lambs. (Viguié *et al.*, 2013)

The study aim was to characterize the internal fetal and maternal exposures to BPA and BPA-glucuronide (Gluc) and to determine to what extent it might be associated with thyroid disruption in sheep. BPA was dissolved in ethanol and then in corn oil to the intended concentration. Adult (2-5 year old) ewes were mated or artificially inseminated and then randomized (balanced by weight and insemination modality) to receive SC injections as follows: n=6 and n=5 were treated with BPA at 5 mg/kg/d or vehicle, respectively, from GD28 until GD145, at which point C-section was performed. Animals received vegetable pellets and were kept indoors during the duration of pregnancy. Total and free T4, total T3, and/or TSH analysis was performed in serum samples from blood collected twice a week and prior to delivery for ewes and in the cord blood, within the first hour of birth and at 2 months for the lambs using RIA kits. Also, blood for PK analysis as well as amniotic fluid, placenta, cord blood, and colostrum samples were collected and analysis was performed to determine BPA and BPA-gluc concentration. Only samples from artificially inseminated ewes were included in the PK analysis and thyroid hormones during pregnancy. PK analyses were done using WinNonlin software. For the ewes, a two-way ANOVA with treatment and sampling time and their interactions as fixed-effect factors and animal nested within treatment as a random-effect factor. For the lambs: TH concentrations in samples collected at birth was analyzed using a two-way ANOVA with sex, treatment, and their interactions as fixed-effect factors. Note that one lamb with very high BPA in amniotic fluid was determined to be an outlier and excluded from analysis.

Unconjugated BPA did not accumulate in pregnant ewes, and its concentration was similar in plasma from the newborns and their mothers. The majority of BPA present in amniotic fluid and cord blood was its conjugated form (BPA-Gluc), which was 30-50X higher than in maternal blood. In addition, placental levels of unconjugated BPA were higher than in maternal blood.

Although the study authors hypothesized that there was a placental / fetal cycle of deconjugation / conjugation that resulted in these higher levels, the role of ex vivo deconjugation and partitioning from blood into tissue were not considered as contributing to the apparent partition ratio. Total T4 and free T4 plasma concentrations in the BPA treatment were reported as decreased 30% in the newborns (hypothyroidism). These changes seem to have been reversed at 2 months of age. The author hypothesized that the finding could be explained by decreased TSH secretion due to disrupted neuroregulation of thyroid function.

This is a study is primarily a PK analysis of BPA in pregnant ewes. It measures both free and conjugated BPA. The study results are in agreement with findings in other species that BPA is rapidly conjugated and eliminated from circulation, and that fetal and maternal levels are similar. The authors noted the very high exposure compared to humans. Some limitations include lack of dosing during early organogenesis, lack of control for phytoestrogen exposure which could confound results, and low sample size. **The study has utility in HI but no utility in RA**

Reproductive and Developmental Studies

Summary

Twenty-five studies were reviewed in this area: twelve used rat models (Holtzman [3], Sprague-Dawley [5], Long-Evan [1], Wistar [3, including a transgenic—enhanced fluorescent protein linked to the GnRH promoter]); eleven used mice (CD-1[7], C57BL/6[2], ICR[2], and Balb/c[1]; one study used two stains); one used pregnant female monkeys (rhesus); and another used pregnant ewes (Suffolk). One additional study examined the environmental contaminants present in studies of BPA and how these factors might confound interpretation of the study results. Four rat studies, five mouse studies, and the ovine study utilized the subcutaneous (SC) route of administration. Implanted osmotic pumps were used in one mouse study. The rest of the rodent studies used the oral route of administration (diet, gavage or drinking water). The monkey study used both oral (fruits) and Silastic tubing implants. Eleven studies used, either entirely or partially, *in utero* exposure (monkey [1], sheep [1], rat [4], and mouse [5]). Ten of twenty-five studies focused on effects in females, primarily ovarian functions, *i.e.* follicular and oocyte development, hormone production, puberty onset or mammary gland function, and eleven studies focused on effects in males, primarily on testicular development/function and spermatogenesis. Three studies evaluated effects on reproductive parameters in both sexes. Only ten of twenty-five studies included BPA at three or more dosing levels (SC [2] and diet [8]); thus, no dose response could be assessed in the rest of studies.

In general, the studies reviewed here were exploratory, mechanistic, or focused on specific endpoints or biomarkers. The main issues raised in the current discussion of the studies were similar to the last two cycles of review. None of the studies was guideline-compliant using common or validated toxicological endpoints for regulatory purposes. Although this does not mean that the studies were inadequate, had poor data quality, or lacked potential important findings, a full regulatory RA is not possible. Some improvements in study reporting have been noticed in this cycle of review; however, lack of complete reporting of details in study design and procedures is still considered as a main problem in most of the studies reviewed here. As delivery vehicle, several commonly used oils have been used in the reviewed studies, *i.e.* sesame oil (6

studies), olive oil (2 studies), corn oil (6 studies), and peanut oil (1 study). There are literature reports of potential biological activity of components of the oils; however, it is not clear whether the oils used as vehicles in the studies have any impact on the measured endpoints.

While the overall conclusion of the review group was that none of the studies as presented were useful for RA, there were some findings identified as potential hazards. Studies reviewed here report effects of BPA on follicle and oocyte development in ovary (Chao *et al.*, 2012, SC; Hunt *et al.*, 2012 implant vs. oral; Karavan *et al.*, 2012, SC; Signorile *et al.*, 2012, SC), the HPG axis or puberty onset (Lee *et al.*, 2013, oral; Kendig *et al.*, 2012, oral; Nah *et al.*, 2011, SC; Losa-Ward *et al.*, 2012, SC), mammary gland function (Kass *et al.*, 2012, drinking water), and estrous cyclicity in females (Nah *et al.*, 2011, SC; Lee *et al.*, 2013 oral), and on spermatogenesis and gonadal hormone production in males (Jin *et al.*, 2013, oral; Liu *et al.*, 2013, oral; Nanjappa *et al.*, 2012, oral; Qiu *et al.*, 2013, oral; Tiwari et al, 2013, oral). No additional hazard has been identified in this review; reported findings described herein complement previous reported effects of BPA. A 90-day study, deemed relevant for RA, evaluated many endpoints similar or related to those stated in this paragraph for HI. The lack of positive effects in RA relevant studies further lowers confidence in the HI endpoints listed here.

Most importantly, the data from the studies described in this review do not affect the oral exposure NOAEL of 5 mg/kg bw/day. Moreover, as earlier FDA reviews have discussed, mechanistic studies have reported that BPA potentially induces molecular or cellular level changes that might affect reproductive functions or development in tested animals, *i.e.* spermatogenesis, folliculogenesis or oogenesis. However, those data are sometimes conflicting and generally inconclusive. For example, both decreases (Karavan *et al.*, 2012) and increases (Chao *et al.*, 2012) in the number of primary, secondary, and antral follicles in ovary of CD-1 mice are reported as the effect of neonatal BPA treatment (SC). In this review, effects on mammary gland milk production (Kass *et al.*, 2012, drinking water), femur development (Pelch *et al.*, 2012, implant), and spermatozoa-related post-implantation loss (Doshi *et al.*, 2012 & 2013, SC) are noted. It is also worthy to note that the effects of BPA may be quite different upon comparison of continuous dosing and intermittent dosing (Hunt *et al.*, 2012, implant vs. oral daily). One study (Thigpen *et al.*, 2013) reviews and summarizes the effects of environmental contaminations which can confound the results of studies attempting to evaluate potentially endocrine active compounds including BPA and provides valuable considerations for interpretation of studies and suggestions for improving the value of future BPA studies. Below are the reviews of the individual studies. The comments relating to the value of the data, limitations, and concerns are contained in each review.

Individual Study Reviews
Oral exposure

Low dose bisphenol A impairs spermatogenesis by suppressing reproductive hormone production and promoting germ cell apoptosis in adults rats (Jin *et al.*, 2013)
The study authors conducted this study to explore possible mechanisms which could explain how bisphenol A (BPA) may impair spermatogenesis. Adult male Sprague-Dawley rats (12 weeks

old) were divided into five treatment groups (n=10 rats/group). The rats were obtained from Oriental Bio Service Inc., Nanjing, Jiangsu, China. BPA was administered orally (>99% purity) in olive oil. Rats were gavaged with BPA at 2 µg/kg bw/day for 14 consecutive days, but the paper did not state how much time elapsed between the sacrifice and the last dose. Control groups received an equal volume of the carrier. There were three BPA treated groups: BPA only, BPA and testosterone-propionate (TP), and BPA and dimethylsulfoxide (DMSO). The TP was given by subcutaneous injection dissolved in DMSO at a dose of 0.1 mg/rat per day. There was a baseline control group and the experimental control group.

Measurements include epididymal sperm counts and testicular germ cells at stage VII of the seminiferous cycle. Serum levels of luteinizing hormone (LH), follicle stimulating hormone (FSH), and testosterone (T) were obtained from blood samples by jugular venipuncture. Intratesticular testosterone was measured from the testis after obtaining the testicular weight. In the preoptic area (POA) of the brain, the gonadotropin-releasing hormone (GnRH) immunoreactive cells were counted using a conventional light microscope. In the testicular tissues, TUNEL staining was used to quantitate the apoptotic seminiferous tubules. Real time-PCR (RT-PCR) was used to measure GnRH, androgen receptor (AR), Fas, FasL, and caspase-3 mRNA in the testes and POA of the brain. Statistical significance was determined using one-way ANOVA followed by Bonferroni's post-hoc tests or by Students *t*-test when only one group was being compared to the control.

The study authors reported no change in the testes weight, but the sperm counts and the number of stage VII sperm cells were reported as decreased in the BPA group. Rats treated with TP and BPA showed partial recovery of these effects. In the BPA group there was a reported significant decrease in the serum FSH and testosterone levels, increase in serum LH, and decrease in testicular testosterone. The number of GnRH immunoreactive cells and the levels of GnRH mRNA in the preoptic area of the BPA-treated rats were reported as significantly lower than the control rats. In the seminiferous tubules, the apoptotic index was higher in the BPA treated rats and RT-PCR showed that the levels of Fas, FasL, and caspase-3 mRNA in the testis were significantly higher in the BPA- treated rats.

This study is primarily a mechanistic study. Potential sources of environmental contamination in the study were not addressed, *i.e.* diet, water, cage, etc. The study only used one dose level and thus could not identify a dose-response for the parameters measured. Based on the mechanistic nature of the study, other limitations cited, and use of one dose level, **this study has no utility for HI and no utility for RA.**

<u>Perinatal exposure to xenoestrogens impairs mammary gland differentiation and modifies milk composition in Wistar rats (Kass *et al.*, 2012)</u>

This study assessed the effects of perinatal (gestation and lactation) exposure to BPA and diethylstilbestrol (DES) on F_1 female mammary gland differentiation and milk protein expression during their gestational and nursing periods. Wistar-derived (not clear if it is an in-house strain) female rats (90-day old) were bred with fertile males. BPA (99%) and DES were dissolved in ethanol. Pregnant female rats (F_0 dams, 10-12/dose) were orally dosed by drinking water with

estimated 0, 0.5 (0.7±0.05, BPA0.5) and 50 (63.87±2.84, BPA50) µg BPA/kg bw/day or 5 (5.87±0.2) µg DES/kg bw/day from gestational day (GD) 9 (GD1= vaginal sperm positive) through lactation day (LD) 21 (parturition = LD0). The daily dosage was estimated by averaging water consumption over the body weight during the entire treatment period. Environmental parameters were partially controlled, *i.e.* bedding, water bottles. The source of drinking water and housing conditions were not described. Phytoestrogens in the diet (16-014007 Rat-Mouse diet, Argentina) were not analyzed. The authors suggest that the diet is not a factor since all animals had similar levels of food intake. At parturition, four F_1 pups/sex/litter were selected if it was feasible. After weaning at LD21, F_1 females were transferred to a "BPA- and DES-free environment" without further treatment until the end of the experiment. Ninety day old F_1 females were bred with normal untreated fertile males. One pregnant F_1 female per F_0 litter from each treatment group was assigned to each time point groups (*i.e.* GD18, GD21 and LD14). Mammary gland differentiation of pregnant F_1 dams was histologically analyzed on GD18 and GD21 with Hematoxylin and Eosin stain (2 slides/dam/time point). However, no details on scoring methods were given in the paper, except some parameters. On GD18 and GD21, both blood and mammary gland samples were collected (10-12 F_1 dams/dose/GD) for serum hormone analysis and mammary gland gene/protein expression measurement. Serum 17β-estrogen (E_2) and progesterone (P_4) were measured by RIA. The number of mammary gland cells containing progesterone receptors (PR), estrogen receptors (ERα & ERβ) and phosphorylated Stat5a/b protein were examined and quantitated with immunochemistry (peroxidase or fluorescence stains). Prolactin receptor (PRLR) mRNA levels in F_1 mammary gland were quantified by RT-PCR. The protein and mRNA levels of α-lactalbumin and β-Casein in F_1 mammary gland on GD18 and GD21 were measured by Western blot/immunochemistry and RT-PCR, respectively. Some reproductive parameters (F_1 dams, n=20; F_2 pup, n=10-12 litter) were also examined in the study, *i.e.* numbers of corpora lutea, implantation sites, resorption sites, F_2 pup body weight, etc. The third group of F_1 dams completed their pregnancy. Their milk yield (g/litter/h) was measured every other day during LD1 to LD13 by measuring the difference in the pre-fasted pups' body weights before and after suckling for one hour. On LD14, 1 hour after nursing, oxytocin-induced milk samples (10-12 dams/dose) were collected for quantifying lactalbumin and β-casein levels in the milk by Western blot. ANOVA and Dunnett's post hoc test were used for statistical analysis and REST 2009 software was used for the analysis of real time RT-PCR to find differences among groups.

No reproductive or developmental toxicity was observed in either the F_0 or F_1 female generations, except a higher rate of resorption sites was reported in F_1 BPA50 and DES groups. On GD18, no difference in mammary gland differentiation scores was reported between control and any treated groups. Lower mammary gland differentiation scores were reported for all treated F_1 dams, with the glands showing less distended lumens in alveoli compared with controls, thus resulting in a smaller lobule size on GD21. A significantly higher serum P_4 level in F_1 BPA50 dams was also reported on GD21. A lower count of mammary gland cells expressing ERα and PR was observed in F_1 BPA50 on GD21, but not on GD18 or among other treated groups. A lower level of PRLR mRNA in all treated F_1 mammary glands with similar magnitude across dose groups was observed on GD18, but not on GD21. The percentage of alveolar cells expressing pStat5a/b protein paralleled with PRLR expression in the same animals. Both α-

lactalbumin mRNA and protein levels in all treated F_1 mammary glands were lower ($P<0.05$) on GD18, but not on GD21. On LD14, no change was observed for α-lactalbumin level in milk samples among all groups. However, β-casein mRNA and protein levels in all treated F_1 mammary gland were lower ($P<0.05$) on both GDs. β-Casein levels in milk samples were lower in all treated F_1 groups on LD14. Moreover, reduction in milk production (g/litter/h) was observed in all treated F_1 groups from LD1-LD7, but gradually disappeared toward LD14, except BPA50 F_1 females. Although reductions of the milk yield and milk β-Casein content were reported in these F_1 dams, the body weights of their F_2 pups among all groups were apparently the same on LD14.

Strengths of this study include large sample size, oral administration, apparent attention to litter effects in the statistical analysis, and justified dose level for DES as a positive control. The use of a single time point for milk protein content measurement and the use of only two BPA doses in the study limit the result interpretation. It is also worth noting that the administration of exogenous oxytocin can confound interpretation due to the effect of oxytocin on milk secretion and composition (Deis *et al.*, 1971; Linzell *et al.*, 1975) and the protein contents between human and rat milk are quite different. Based on pharmacokinetic studies in rodents, the oral administration of this dose (50 µg/kg bw/day or lower) of BPA to adult animals (pregnant or lactating dams) would be expected to result in extremely low or undetectable exposures of the pups to the presumed active agent, BPA aglycone (Doerge et al, 2010; Doerge *et al.*, 2011). **this study has no utility for HI and no utility for RA.**

Estrogen-like disruptive effects of dietary exposure to bisphenol A or 17α-ethinyl estradiol in CD1 mice (Kendig *et al.*, 2012)

This study was designed to assess the potential for disruptive actions of dietary bisphenol A (BPA) exposure in CD1 mice, while controlling for known confounders. CD1 mice (F_0) were fed defined phytoestrogen-free diet containing BPA (0.03, 0.3, 3, 30, and 300 ppm or 0.004-40 mg/kg bw/day) or 17α-ethinyl estradiol (EE: 0.0001, 0.001, and 0.01 ppm or 0.00002-0.001 mg/kg bw/day) for two-weeks before mating (mating procedure was not described) and through gestation and lactation period. The progeny (F_1) were maintained through adulthood on the same diet as the parents. Reproductive parameters, plasma hormone levels and lipids, glucose tolerance, and body composition were examined. All measures and endpoint assessments were made by an observer blinded to treatments, litter, and sex when appropriate. The statistical unit was the litter or breeding pair for all analyses. Analysis of weight data was performed using one-way analysis of variance followed by Dunnett Multiple Comparison test. Analysis of timed data was performed using the Kaplan-Meier method and estimates of food consumption per mouse per day were analyzed by linear regression.

The authors reported moderate increases in body weights were observed in both F_1 males and F_1 females treated with 3, 30, and 300 ppm BPA as well as with 0.0001 ppm EE. But this change was reported as significant at PND28 only. In F_1 females, uterine weights were significantly increased in all EE groups, a non-significant increase was seen at the 30 ppm BPA group, but not in the 300 ppm group. In BPA-exposed females, no treatment-related differences were observed in parental reproductive function, or in the timing of puberty and metabolic functions in F_1

females. In F_1 males, a modest decrease in fasting blood glucose was measured in males exposed to 0.03 and 3 ppm BPA and non-significant changes were seen in adiposity. These changes were sometimes isolated and non-consistent between treatments. According to the authors, there is an increase in prolactin (PND36) and testosterone (PND70) circulating levels associated with accelerated balanopreputial separation at 0.03 and 3.00 ppm BPA and increased in anogenital distance seen at 0.03, 30, and 300 ppm BPA at PND21. Sperm counts were increased with 3.0 ppm BPA. The authors concluded that overall, BPA was found to have modest, sex specific endocrine disruptive effects on a variety of endpoints.

this study has no utility for HI and no utility for RA. The strength of this study includes controlled environments, large samples size, positive controls, and litter as the unit for statistical analysis. However, in some case only 6 samples were analyzed, *i.e.*, organ weight, glucose tolerance test, etc. Other limitation includes single dose level and/or single time point measurement in some cases, *i.e.*, testosterone and prolactin, respectively. The BPA effects in this study were found to be non-dose-dependent, non-existent, modest, and random (not associated) sex endocrine disruptive effects on a variety of endpoints. Most of the effects were seen at a single dose and did not show a dose-response.

Strain specific induction of pyometra and differences in immune responsiveness in mice exposed to 17α-ethinyl estradiol or the endocrine disrupting chemical bisphenol A (Kendziorski *et al.*, 2012)

The study investigated the potential induction of pyometra and effects on reproductive parameters by dietary BPA and 17α-ethinyl estradiol (EE_2) in two different mouse strains. C57BL/6 or CD1 mice, 5 animals (6-7 week age)/sex/strain/dose were housed 5/cage (BPA free) with a 14/10h light/dark cycle and provided food and water ad libitum. Distilled water and Sani-chip bedding were used to avoid exposure to environmental estrogens. BPA was added to the standard diet (defined composition, but source is not described) at 0, 0.03, 0.3, or 30 ppm (equivalent to 4.0, 32.8, or 4168 µg BPA/kg bw/day). 17α-ethinyl estradiol (EE_2) was also given at 0.01, 0.1 and 1.3 ppm (equivalent to 1.4, 15 and 164 µg EE_2/kg bw/day) as positive controls. Food consumption, body weights, and estrous cycles (vaginal cytology) in females were measured. Males and females in the same dose level groups were randomly mated. Fertility and fecundity were evaluated thereafter. At necropsy (19-23 week age, 1-2 weeks earlier for C57BL/6 EE_2 groups due to morbidity), organs were collected and weighed. Uterine morphology was examined by microscopy with hematoxylin and eosin (H&E) stain and macrophage infiltration was determined by immunohistochemistry using a macrophage specific F4/80 rat monoclonal antibody in animals treated with 0.1 ppm EE2 and 0.3 ppm BPA groups only. Analysis of weight and cell count data was performed using one-way ANOVA followed by Tukey's multiple comparison test.

In all BPA-treated groups, no dose-dependent effects on food consumption or caloric intake, body weight, organ weight, or fertility/fecundity were observed in either strain. A significant increase in uterine weight was noted at 0.3 ppm BPA in C57BL/6, but not at higher dose (30 ppm) or any dose groups in CD1 mice. Uterine morphological examination revealed that pyometra was only evident at 0.1 ppm EE_2 (4/5) and 0.3 ppm BPA (1/5) groups in C57BL/6

strain, but not in other C57BL/6 groups or in any CD1 groups. Sporadic cystic endometrial hyperplasia (CEH) was reported in both strains treated with 0.1 ppm EE_2 or 0.3 ppm BPA. BPA (0.3 ppm) seemed to significantly increase the number of F4/80 immunoreactive macrophages in the uterus of C57BL/6 mice, but not in CD1 mice. Thus, the authors concluded that these observations indicate a potential difference in susceptibility to developing pyometra which is related to the immune response and that pyometra in C57BL/6 mice may serve as a responsive endpoint for estrogenic chemicals on immune system.

The study authors have suggested a potential strain difference in the immunologic sensitivity of the female reproductive tract to EE_2 and BPA between the C57BL/6 and CD1 strains. However, the supporting evidence in this study is weakened by small sample size ($n \leq 5$) and the limited statistical power. Another limitation is single BPA/EE_2 dose observation/comparison in immunohistochemistry. **This study has no utility for HI and no utility for RA.**

Lack of effects for dietary exposure of bisphenol A during *in utero* and lactational periods on reproductive development in rat offspring **(**Kobayashi *et al.*, 2012)

The purpose of the study was to investigate the effects of low-dose exposure to BPA on reproductive development in F_1 male and female rat offspring. Pregnant Sprague-Dawley rats (Crj: CD (SD) IGS, Charles River Japan) were fed a diet (CE-2, Japan) containing doses of bisphenol A (BPA: 0, 0.33, 3.3, or 33 ppm; purity >99.6%, dose certification/homogeneity data not provided; authors estimate 0.017, 0.17, and 1.7 mg/kg bw/day) from gestational day (GD) 6 through postnatal day (PND) 21. The time mated females were obtained from the supplier at GD3, acclimated, and randomly assigned to a control diet or one of the three BPA treatment groups. On PND5 the litter size was culled to 8 pups (male/female=4/4, when possible). Reproductive outcomes measured included: dam weight gain, gestation length, sex ratio, and live births per litter. At weaning, 20 male and 20 females from each group were given a normal diet (CE-2, Japan), and the remaining weanlings were sacrificed. The F_1 offspring were subjected to necropsy at 5 weeks (n=10/group/sex) or 3 months (n=10/group/sex). Body weights were measured weekly until sacrifice. Anogenital distance (AGD) was measured and expressed as the ratio of the AGD to the cube root of body weight (AGD index). Blood was collected before sacrifice for hormone analysis (testosterone, dihydrotestosterone, estradiol, and progesterone). Reproductive organs (testis, epididymis, prostate, ovary, vagina, and uterus) were removed and weighed. Sperm motility was determined in 3-month old F_1 males. Although the study methods indicated that the sperm count was measured, the data were not shown or discussed in the publication.

There were no effects on the parameters used to measure the reproductive outcomes for the F_0 dams or F_1 pups. The authors stated that no BPA related changes were observed in body weight or weight of the major reproductive organs in F_1 male and female rats. The only effect detected was a reported significant reduction in epididymis weight in the 3-month old F_1 males exposed to 33 ppm BPA. The authors stated that the toxicological significance was not known. The AGD index was reported as significantly lower and the relative ovary weight as significantly higher in the 5 week old F_1 females exposed to 3.3 and 33 ppm BPA, but significant differences were not observed in the 3 month old F_1 females. Thus, the authors stated that these effects were not

considered to be biologically relevant. The AGD index in 5 week and 3 month old F_1 males was not affected by BPA exposure. Plasma reproductive steroid hormones were not significantly altered among groups of either sex. BPA treatment did not result in significant changes in the percent motile or progressive sperm in the 3 month old F_1 male rats.

The study design has strengths and limitations. The strengths of the study include the use of three BPA treatment groups, two time points for the parameters as well as n values of at least 10, and appropriate statistical methods. One weakness of the study methods is that there was no discussion of control for environmental BPA exposure in the cages or phytoestrogen level in the diet. Another weakness of the study is that the methods did not clearly state how the offspring were selected from the litters for the parameters measured. **This study has limited utility for HI and none for RA.**

Bisphenol A exposure during adulthood causes augmentation of follicular atresia and luteal regression by decreasing 17β-estradiol synthesis via downregulation of aromatase in rat ovary (Lee *et al.*, 2013)

The authors hypothesized that the adult ovary is susceptible to BPA *in vivo* and that long-term exposure to low concentrations of BPA disrupts17β-estradiol (E_2) production by granulosa cells via alteration of steroidogenic proteins in ovarian cells. Adult female Sprague Dawley (Sam Tako Bio-Korea) rats (8 weeks of age) were allowed unlimited access to rat chow (unspecified). Rats received BPA (0.001 or 0.1 mg/kg bw, n=30/dose), estradiol benzoate (EB; 0.001 mg/kg bw; estrogenic control, n=30/dose), or vehicle (0.5% dimethylsulfoxide [DMSO] in corn oil, n=30/dose) daily for 90 days via oral gavage. After 90 days, 18 rats per dose were euthanized via carbon dioxide asphyxiation on the day of normal estrus. Right ovaries and uterine horns were removed and fixed in Bouin's fixative. Left ovaries were placed in cold PBS for collection of granulosa cells, and the left uterine horns were snap frozen for biochemical analysis. The remaining 12 rats per dose were continuously examined for estrous cycle staging. The estrous cycle analysis was started the day after BPA treatment was completed (day 1) and was done for a total of 30 days. The data were collected by vaginal smears on days 1-15 and 31-45, with no vaginal smears on days 15-30. Serum testosterone (T), E_2, follicle stimulating hormone (FSH), and luteinizing hormone (LH) levels were determined by ELISA. Granulosa cells were collected by follicular puncture. Ovarian sections were probed for caspase-3 or stained for collagen fibers with trichrome stain. Western blot analyses were done to determine expression levels of P450arom, StAR, P450scc, and 3β-HSD in the ovaries. Data were analyzed using ANOVA followed by Duncan's post hoc test.

Serum levels of T and E_2 were reported as significantly decreased, whereas serum LH concentration and pituitary protein levels were reported significantly increased at both BPA doses. The duration of days in estrus was significantly increased at both BPA doses. The number of caspase-3 positive follicles and corpora lutea were significantly increased at both BPA doses. The amount of StAR protein and the P450arom/actin ratio were significantly decreased at both BPA doses. The authors suggest that BPA exposure during adulthood disturbs the maintenance and normal ovarian functions by reducing E_2, and that StAR and P450arom are the definitive steroidogenic proteins that are targeted by BPA.

This study has no utility for HI and no utility for RA. A dose-response could not be determined because the study methods used a limited dose range. Sample size was small; 3-5 animals per analysis. Certain study details which could impact the results of the estrous cycle analysis were not specified. For example, the study methods did not state the number of shipments of animals, how the animals were assigned to treatment groups, and did not evaluate cycling status of each animal before treatment to ensure that regularly cycling animals were assigned to control and treated groups. BPA purity and potential environmental sources of contamination were not addressed.

Exposure to bisphenol A disrupts meiotic progression during spermatogenesis in adult rats through estrogen-like activity (Liu *et al.*, 2013)

This study aimed to evaluate the effects of orally administered BPA on spermatogenesis in adult rats and determine if any observed effects were due to estrogenic activity. Wistar rats from the investigators institution were acclimatized for one week and started on experiment at 90 days of age. A diet with undetectable phytoestrogens (analytical methodology details not provided) was used, and water was dispensed *ad libitum* from glass bottles. There were 8 animals per dose group for the sperm counts and body and organ weights. A second study was conducted for mechanistic work that included only the high BPA dose and 17β-estradiol (E_2), with an antiestrogen (ICI 182780) control and a BPA plus ICI group. These mechanistic studies had 6 animals per dose group. BPA was administered at 2, 20, and 200 μg/kg bw/day by gavage and E_2 at 10 μg/kg bw/day by subcutaneous (SC) injection. Test articles were dissolved in absolute ethanol and diluted in corn oil for administration, and treatment was for 60 consecutive days to cover a full cycle of spermatogenesis. Animals were anesthetized with 20% urethane and blood sampled from the right ventricle for measurement of serum hormones (follicle stimulating hormone (FSH), luteinizing hormone (LH), testosterone (T)). Left epididymis and testis were removed and weighed, epididymal sperm analyzed, and the testis used for meiotic chromosome spread analysis and alkaline comet assays. Right epididymis and testis were removed after perfusion fixation, and sections were stained with hematoxylin and eosin and Periodic acid Schiff stain, respectively, for histology, including staging of seminiferous epithelium. Sperm apoptosis was assessed by Annexin V staining and flow cytometry for epididymal sperm and by TUNEL in testis sections. The statistical analysis described was an unpaired Student's t-test. No correction for multiple comparisons was indicated.

Only the high BPA dose and E_2 were reported to reduce sperm counts, and no BPA dose affected body or organ (epididymis or testis) weights relative to body weight. E_2 significantly reduced relative epididymal weight compared to vehicle controls. The mechanistic studies thus focused on the high BPA dose group. There were no effects of BPA on epididymal sperm motility, morphology, or apoptosis or serum hormone levels. E_2 did not affect these parameters either, except for serum T, which was reduced. The authors focused their mechanistic studies on possible direct effects on the testes. Fluorescence activated cell sorting was used to determine the proportion of 1C, 2C, and 4C testicular germ cells, and chromosome spreads were stained with SYCP3, γH2AX, and DAPI to evaluate the stages of meiosis I (leptotene, zygotene, pachytene, and diplotene). Similar effects were reported for high dose BPA and E_2 such as decreasing 1C

and increasing 4C cell populations, decreasing the proportion of leptotene and zygotene cells, and increasing the proportion of pachytene cells. ICI treatment fully or partially blocked the effects of BPA. γH2AX was used as a measure of meiotic double strand breaks and was reported to be increased by BPA and E_2. Similar results were reported for an alkaline comet assay on a partially purified (elutriation and Percoll density gradient) pachytene spermatocyte population. ATM and p-Chk2 were also elevated by these treatments. Testicular apoptotic cells, as evaluated by TUNEL staining and Western blot analysis of active caspase 3, were reported as elevated by BPA and E_2. In all cases, pretreatment with ICI completely or partially blocked the effect of BPA. Histological evaluation indicated an increased proportion of Stage VII tubules and a decreased proportion of Stage VIII tubules. The study authors conclude that BPA, administered by gavage to Wistar rats at 200 μg/kg bw/day, adversely affects spermatogenesis by delaying spermiation (release of mature spermatids from Sertoli cells) and inducing a meiotic delay produced by persistent double strand breaks. Because these effects are also produced by E_2 and blocked by the antiestrogen ICI, they concluded that BPA produces these effects by an estrogenic mechanism.

This study has no utility for HI and no utility for RA. Earlier FDA reviews have discussed inconclusive data concerning effects of BPA on spermatogenesis. Uncertainties and Limitations include: The statistical analysis described consists of multiple t-tests, without correction for multiple comparisons. The animal numbers are limited, and the mechanistic data are limited to a single dose group. The authors note differences in the actions of E_2 and BPA (*e.g.* serum T) and discuss the differences in receptor affinities of the compounds and possible differences in receptor type and location distributions at various seminiferous epithelium stages, but they do not discuss the likely differences in active compound that reach the target organ given the differing routes of administration used for E_2 (SC) and BPA (gavage). An E_2 plus ICI group would have been useful to support the mechanistic similarities of BPA and E_2.

<u>The effects of different endocrine disruptors defining compound-specific alterations of gene expression profiles in the developing testis (Lopez-Casas *et al.*, 2012)</u>

The purpose of this study was to evaluate differences in the transcriptome in the testes of mice exposed to various concentrations of different endocrine disruptors over different exposure scenarios. Bisphenol A (BPA), estradiol, zearalenone, mono (2-ethylhexyl)phthalate, and lindane were administered via drinking water to CD-1 mice. Only the results with BPA are included in this review. Adult female CD-1 mice (N at least 3/group) were obtained from an in-house colony. BPA (source and purity not described) was dissolved in ethanol and diluted with drinking water. There is no mention of analytical confirmation of doses. Intake was estimated from water consumption (presumably bottle weight) and body weights; *in utero* and neonatal exposures were assumed to occur via the placenta and through lactation. Intake of BPA was estimated to be 0.16, 16, or 64 mg/kg/d. The doses selected were below doses that had adverse effects on pregnancy outcome in a preliminary experiment. Three different exposure conditions were used; exposure of dams for 2 weeks prior to mating only (exposure A); exposure continued through mating and gestation ending at birth (exposure B), or exposure continued until 4 weeks after birth (exposure C). All male pups were sacrificed 4 weeks after birth (n=4-6, each from different litters), and testes were removed. The rationale for evaluating the male pups at this age is not stated.

Endpoints evaluated included testis weight (absolute and relative to body weight), testis histology, including apoptosis analysis by TUNEL assay, and transcriptome analysis. Expression of twenty-three genes randomly selected from those identified by microarray was confirmed by TaqMan low density arrays. Statistical analysis of histological data was done by ANOVA; there was no description of the analysis for body weight data. Statistical analysis of transcriptome data was done using bioinformatic software (Alma Bioinformatica, Tres Cantos, Spain).

The authors reported no clear effect of BPA on body or testis weight across doses and exposures; there were statistically significant increases in body weight at the low and high doses in exposure A and in testis weight relative to body weight at the mid dose in exposure B. There were no differences in seminiferous tubule diameter, the percentage of tubules with epithelial abnormalities or the percentage of tubules with diploid spermatids across doses of BPA and across exposure conditions. However, there was a reported increase in the number of apoptotic cells at the mid and high doses of BPA under exposure C conditions. The gene expression profiles for BPA and estradiol suggested that these compounds could act through similar mechanisms.

This study has no utility for HI and no utility for RA. Small numbers of animals were used in each exposure scenario. The exact number of dams treated per group is not directly stated, but since all males were presumably from different litters, it seems that 4-6 female mice were treated per group. There was little description of environmental conditions (diet, caging, etc.). Intake of BPA was estimated to be the same across all exposure scenarios; this can be problematic since as the animals age during lactation in exposure condition C, they could have been drinking the dosed water thereby getting direct exposure in addition to exposure via lactation. There was also no indication of the frequency of obtaining body weights and water bottle weights and no indication of the age of weaning or the number of males per cage after weaning.

<u>The industrial chemical bisphenol A (BPA) interferes with proliferative activity and development of steroidogenic capacity in rat Leydig cells (Nanjappa *et al.*, 2012)</u>

This study was designed to evaluate whether BPA alters Leydig cell numbers and testosterone production after perinatal exposure to BPA. Mechanistic studies were done to understand how BPA may act on Leydig cells at the molecular and cellular level. Pregnant Long Evans rats (Harlan-Teklad, n=14/group) were dosed by oral gavage with 2.5 or 25 µg/kg bw/day BPA (source Sigma-Aldrich, purity not stated) in olive oil from gestation day (GD) 12 to postnatal day (PND) 21. To reduce the environmental exposure to estrogenic compounds, Soy Protein free diet (#2020X; Harlan-Heklad), polypropylene cages and glass water bottles were used in the study. Parameters measured at birth included litter size, birth weight, and sex ratio. Pup body weights were assessed on PND7, 21, 35, and 90. The paired testes were weighed on PND21, 35, and 90. The PND21 rats (pooled from 28-30 rats per control and BPA treatment groups) were used to obtain progenitor Leydig cells (PLCs) for the following assessments: 1) measurement of PLC proliferative activity using [^{3}H] thymidine incorporation and 2) Western blot analysis of PLC lysates for molecular targets affecting cell division such as cell cycle proteins (*e.g.*, PCNA, cyclin D3), p-MAPK3/1, hormone transcription factors (*e.g.*, LHCGR, ESR1, AR), and growth response factors (IGF1RB, EGFR). Leydig cell numbers were assessed in testes of control (n=3)

and 90 day old male BPA treated rats (n=3) using stereological techniques. The amount of testosterone was analyzed by RIA in Leydig cells isolated and pooled on PND21 (n=28-30), PND 35 (n=14) and PND90 (n=8) in the control and BPA treated male rats. The serum testosterone was measured on PND21, 35, and 90 (N value not specified) from blood collected at the time of death. The PND90 rats (pooled from 7-10 rats per control and BPA treated group) were used for Western blot analysis of lysates of adult Leydig cells (ALCs) for the following: LHCGR protein levels, steroidogenic acute regulatory protein (STAR), and steroidogenic enzymes (CYP11A1, HSD3B, CYP17A1, and HSD17B3). The study authors stated that the experiments performed *in vitro* were repeated at least three times using Leydig cells isolated from groups of 35 prepubertal rats at 21 days of age on each occasion. Statistical analysis was done by the unpaired t-test for two groups or one-way ANOVA followed by Dunnett test for multiple group comparisons

There were no effects on litter size, birth weights of pups, and postnatal testes and body weights. BPA (2.5 and 25 µg/kg bw/day) was reported to increase proliferative activity of Leydig cells on PND21. Using *in vitro* techniques to assess proliferative activity, PLCs were isolated from PND21 BPA-free animals and then incubated for 18 hours in culture media containing BPA and ovine LH (10 ng/ml). The *in vitro* study reported an increase at 10 nM BPA in [^{3}H] thymidine incorporation (*i.e.*, proliferative activity) and protein levels of IGF-1RB and EGFR. On PND21, the study reported increases in the following protein levels at 2.5 and 25 µg/kg bw/day of BPA: PCNA, cyclin D3, p-MAPK3/1, ESR1, LHCGR, AR, IGF-1RB, and EGFR. Leydig cell numbers on PND90 were increased at both BPA doses (2.5 and 25 µg/kg bw/day). On PND21, 35, and 90, both *in vivo* doses of BPA were reported to significantly decrease Leydig cell testosterone production and non-significantly decrease serum testosterone level. A decrease in the protein levels of LHCGR and HSD17B3 was reported at both *in vivo* dose levels of BPA on PND90. The study authors hypothesized that BPA may target the Leydig cells to act as a mitogen and decrease Leydig cell androgen secretion, both of which may be mediated by BPA induced changes in expression of specific proteins as reported in the study.

This study has no utility for HI or for RA because of limitations in the experimental design and methods**.** Although the study protocol used soy protein free diets and polypropylene cages, the vehicle was olive oil, which has been reported to have 5-alpha reductase inhibition activity. Dose response could not be assessed because three or more dose levels were not used. Although tissues were pooled from multiple F_1 offspring, in the description of the study methods it is not clear if n values of 10 or more were used. The study protocol did not describe what method was used when male pups were assigned to litters within treatment groups, selected for measurements, or pooled for the molecular studies.

Decreased androgen receptor expression may contribute to spermatogenesis failure in rats exposed to low concentrations of bisphenol A (Qiu *et al.*, 2013)

The purpose of this study was to investigate the effects of low bisphenol A (BPA) concentration-induced reproductive and endocrine disorders in male adult rats. Adult male Sprague-Dawley rats were randomly divided into four groups (14 rats per group, 3 per cage) and fed rat chow (unspecified) and water *ad libitum*. BPA (purity >99%, Sigma, Aldrich) was dissolved in ethanol and diluted in corn oil. Rats were given 0.0005, 0.5, or 5 mg/kg bw/day by oral gavage for 8

weeks at a volume of 0.1 mL/100 g bw. Control rats were given the same volume of corn oil. At the end of the study, rats were anesthetized with ethyl ether inhalation, blood was collected *via* left ventricle puncture, and organs were collected and weighed. Intra-testicular testosterone (T) was measured by radioimmunoassay (RIA), sperm count and motility were analyzed *via* computer-assisted sperm analysis (CASA), expression of genes related to hormone synthesis and spermatogenesis were measured via qPCR, and protein expression via immunoblot assay. Statistical significance was assessed using ANOVA followed by the Duncan post hoc test for multiple comparisons.

The majority of the significant changes that were reported occurred at the highest dose (5 mg/kg bw/day) of BPA. These included increased intra-testicular T and significant decreases in sperm count, epithelial height (also decreased in the 0.5 mg/kg bw/day group), number of round spermatids per tubule, and round spermatids/Sertoli cell ratio. The expression of genes involved in spermatogenesis such as ODF1 and TNP1 were reported to be altered by the high dose of BPA. ODF1 mRNA and protein expression were significantly decreased at 5 mg/kg bw/day. TNP1 mRNA expression was significantly decreased at both 0.5 and 5 mg/kg bw/day; however, protein expression was only significantly decreased at 5 mg/kg bw/day. The expression of hormone synthesis related genes such as StAR, Cyp450scc, 3β-HSD, 17β-HSD, and Cyp450arom were altered with high doses of BPA. The expression of 3β-HSD mRNA, 17β-HSD mRNA, CypP450arom mRNA, and AR mRNA and protein were significantly decreased at both 0.5 and 5 mg/kg bw/day, whereas expression of StAR mRNA and protein and CypP450scc mRNA were significantly increased at 5 mg/kg bw/day. The authors conclude that low BPA concentrations induced spermatogenesis failure by down-regulating androgen receptor (AR) expression and by significantly down-regulating the genes related to spermatogenesis, such as ODF1 and TNP1.

This study has limited utility for HI but no utility for RA. As previous FDA reviews have discussed, inconsistent or even conflicting effects of BPA on spermatogenesis have been reported. Potential sources of contamination in caging, water, and food were not addressed. The widely spaced doses of BPA provide limited dose-response information.

<u>Bisphenol A differentially activates protein kinase C isoforms in murine placental tissue (Tan *et al.*, 2013)</u>

Based on previous work reporting that BPA could suppress aromatase and up-regulate corticotropin-releasing hormone (CRH), the current study examined aromatase and *crh* expression in an *in vivo* model. Female ICR mice were fed a modified AIN93 diet. Pregnant mice were treated with BPA (0, 2, 20, or 200 mg/kg bw/d) in ethanol/corn oil (1:9 v/v) by gavage from gestational day (GD) 13 to GD16. Gavage volume was limited to 150 µl per animal. Mice were euthanized on GD17 between 9:00-11:00 am. Serum was collected by cardiac puncture, and placentas were excised and pooled from individual mothers. Delivery prior to GD18.5 was considered premature. The expression of *crh* was analyzed via qPCR. Immunoblots were performed to detect protein kinase C isoforms and CREB. Serum testosterone (T), 17β-estradiol (E_2), and CRH were measured via ELISA. Data were analyzed by one-way ANOVA followed by Bonferroni's multiple comparison test if significant differences were observed.

Serum levels of T, E_2, and CRH were reported as significantly increased following exposure with 20 or 200 mg/kg bw/d BPA. In addition, placental expression of *crh* mRNA was significantly increased at the 200 mg/kg bw/d exposure. CREB protein levels were significantly increased at all BPA exposures. Placental PKC ζ/λ protein was significantly increased at all BPA exposures, and PKC δ protein was significantly increased at the 200 mg/kg/d exposure. The study authors conclude that Bisphenol A could be a multi-targeted endocrine disrupter.

This study has no utility for HI and no utility for RA. Sample size was small, 3-5 animals per analysis. Potential sources of contamination in caging, water, and food were not evaluated. In the statistical analysis, the standard deviations are small and uniform in each analysis which is unexpected for the endpoints measured.

Mutagenic effect of Bisphenol A on adult rat male germ cells and their fertility (Tiwari and Vanage, 2013)

The purpose of this study was to evaluate the effect of BPA on germ cell development during spermatogenesis and to assess possible genotoxicity in male germ cells using the dominant lethal assay. Holtzman strain rats (source not described but presumably from an in-house source) were housed in cages with autoclaved paddy husk and fed soy-free chow pellets; these pellets were prepared in-house. BPA (~99% purity from Sigma Chemical Co. in US) was dissolved in 99% ethanol and diluted with sesame oil to obtain doses of 10 µg/kg bw and 5 mg/kg bw; the ethanol was allowed to evaporate overnight. Doses were administered to adult male rats (8 weeks old) orally for 6 consecutive days. On the next day, each male (n=7) was mated with two untreated females; GD0 was defined as the day the vaginal lavage was sperm positive. Each week additional untreated females were added to the cage in an effort to evaluate effects on different stages of spermatogenesis; mating continued for 8 weeks. Pregnant females were sacrificed on GD15, and the following endpoints were evaluated: mating index, gestation index, number of corpora lutea, number of implantations, number of live fetuses, pre- and post-implantation loss and dominant lethal mutation rate. When the mating study was completed, treated males were sacrificed, and several sperm parameters were determined, including sperm number and motility, morphology and daily sperm production (n=5 males/group). The comet assay was also used to determine damage to sperm DNA (n=4 males/group). Statistical analyses included Bartlett's test for homogeneity, ANOVA with Dunnett's post-hoc *t*-test; non-parametric tests such as the Kruskal-Wallis test were used for count data.

The authors reported no differences in the mating index [(number of females mated/number of females paired) X100] or the gestation index [(number of pregnant females/number of females mated) X 100] during the 8 week mating period, although there were slight decreases observed in some weeks, especially for the high dosed animals. There was a decrease in the total number of implantations only at week 4 at the high dose (5 mg/kg) as well as decreases in live implants and increases in resorptions only at week 4; this corresponds to the mid-spermatid stage of spermatogenesis. In the text in the Results section, the authors indicate that there was also a decrease in the number of live implants and an increase in the number of resorptions at week 6; however, these changes were not noted to be significant. An increase in post-implantation loss at

week 6 was noted. After the mating study was completed, treated males were sacrificed, and sperm parameters were determined (including the comet assay). Both doses of BPA were reported to decrease daily sperm production, efficiency of sperm production and sperm count; the high dose also decreased sperm motility. The comet assay showed increases in DNA damage at the high dose of BPA.

This study has no utility for HI and no utility for RA. The number of males treated in each group is marginal (n=7), and sperm parameters and the comet assay were done in fewer animals (n=4-5). The dose spacing and number of doses in all assays is inadequate. The results in the comet assay may be secondary to toxicity to the sperm (due to apoptosis/necrosis and not a direct effect of BPA on DNA). Also the comet assay did not include untreated controls or a positive control, and arbitrary units are given rather than absolute percent tail DNA. No justification was presented for the length of the treatment period. The authors took some care to avoid environmental exposure to BPA with regard to diet and water; although soy-free chow was used, it was prepared in-house, and quality control measures were not described. Additionally, caging and the possible presence of arsenic, which has adverse effects on sperm, in the paddy (rice) husk bedding is possible, were not described.

Effects of perinatal exposure to bisphenol A and di(2-ethylhexyl)-phthalate on gonadal development of male mice (Xi *et al.*, 2012)

The purpose of this study was to determine the effect of prenatal and lactational exposure to the combination of BPA and di(2-ethylhexyl)-phthalate (DEHP) on the sex ratio at birth as well as the development and function of the male reproductive system in male mice. Adult CD-1 mice were housed in polypropylene cages with sterilized bedding and fed standard rodent diet 5002 (Lab diet). Males and females were bred, and the presence of a vaginal plug was defined as GD0. Pregnant mice were dosed daily by gavage from GD1-PND21 (day of birth defined as PND0). Mice (n=5 per group although different Ns were presented in the Results section) were dosed with vehicle (corn oil), 0.1mg BPA + 0.1 mg DEHP per kg body weight, 1 mg BPA + 1 mg DEHP per kg body weight, 10 mg BPA + 10 mg DEHP per kg body weight. Endpoints examined included litter size, sex ratio at birth, testes weight on PND15 or PND42, AGD (determined on PND5, PND10, PDN15 and PND20), serum hormone levels (FSH, progesterone, testosterone) determined on PND42, epididymal sperm count determined on PND42, and gene expression in hypothalamus, pituitary and testes on PND15 and PND42. **This study has no utility for HI and no utility for RA.** No animals were dosed with BPA alone; animals were either dosed with the vehicle or with the combination of BPA and DEHP; therefore, no effects could be ascribed to BPA alone.

Subcutaneous exposure

Bisphenol A exposure modifies methylation of imprinted genes in mouse oocytes via the estrogen receptor signaling pathway (Chao *et al.*, 2012)

The purpose of this study was to evaluate potential effects of BPA on the methylation status of several genes to determine if alterations in gene expression influenced oocyte development. The authors evaluated the effect of “hypodermical injection” of BPA (purity and source not

described) to CD-1 female mice (source not described) at 0, 20, or 40 µg/kg bw/day on PND7-14 (day of birth was not defined); the vehicle was physiological saline with 0.1% DMSO. The mice were sacrificed on PND15, and the methylation status of the maternally imprinted genes, *Igf2r* and *Peg3* as well as the paternally imprinted gene, *H19*, was evaluated in oocytes. Additionally, the expression of several DNA methyltransferase genes as well as ERα and ERβ was determined by qRT-PCR; Western blots were used to determine the protein expression for ERα. The authors stated that a second experiment was performed to more closely mimic human exposure; in this experiment 73 female CD-1 mouse pups were dosed with either 0, 20, or 40 µg/kg bw/dayBPA by hypodermic injection on PND5, PND10, PND15, and PND20 and sacrificed on PND21. The endpoints evaluated were generally the same as those determined in the first experiment. Finally, the authors also evaluated the numbers and types of follicles (on PND21) in the ovaries, the diameter of all oocytes (on PND15 and 21) and the oocyte spindle assembly (PND21) to determine if BPA altered follicular development. Differences between groups were analyzed by t-tests or by one-way ANOVA followed by Tukey's tests.

BPA was reported to decrease the methylation of the maternally imprinted *Igf2r* and *Peg3* genes but had no effect on the paternally imprinted *H19* gene in both experiments. BPA also decreased expression of several DNA methyltransferase genes. *ERα* gene expression was increased at the high concentration of BPA in the first experiment, but there were no differences between groups in the second experiment. ERα protein expression was increased by both doses of BPA in both experiments. An *in vitro* experiment was conducted to examine the role of ER signaling in BPA-induced hypomethylation. Ovarian cultures from untreated PND7 female pups were treated with a single dose of BPA and, in some cases, an ER inhibitor was added; the endpoint analyzed was the methylation status of *Igf2r* and *H19*. BPA decreased methylation in the *Igf2r* gene; this decrease was normalized by the inhibitor. Additionally, the expression of several DNA methyltransferase genes was decreased by BPA, and this decrease was also normalized by addition of the ER inhibitor. These results suggest that BPA hypomethylation may be regulated, in part, by the ER signaling pathway. The authors reported increases in the diameters of oocytes following BPA treatment. They also reported a decrease in the number of primordial follicles with subsequent increases in primary, secondary, and antral follicles; there were no differences in the total number of follicles. There was also an increase in the number of oocytes with abnormalities in the spindle apparatus during meiosis I.

This study has no utility for HI and no utility for RA due to a lack of clarity in the description of the experimental design and methods**.** The exact number of mice per group was not presented, but 110 mice were utilized in Experiment 1 and 73 mice in Experiment 2. The study protocol did not describe how female pups were grouped with the dams nor did it describe how many female pups were assigned to treatment groups, selected for measurements, or pooled for the molecular studies. Thus the study methods stated that oocytes from 10-12 ovaries were used in the analysis, but the number of F1 female pups or number of litters from F0 parental females was not stated. From the description in the study methods, it is not clear if N values of 10 or more were used in the statistical analysis and how pooled tissues were used for statistical analysis. Because only two treated groups were used, a dose response could not be assessed. The route of administration was not described completely, and no information was presented on housing conditions, the type of

chow administered, the source and purity of BPA and the source of the animals.

Effects of neonatal exposure on male rats to bisphenol A on the expression of DNA methylation machinery in the postimplantation embryo (Doshi *et al.*, 2012)
The purpose of this study was to examine the expression of genes involved in embryonic development to discern their role in post-implantation loss induced by BPA in a previous study by this group. Randomly bred Holtzman strain female rats (source not described) were used for this study, and pregnant rats naturally delivered their offspring. Day of delivery was considered to be postnatal day (PND) 0 and the litter size was adjusted to 4–5 male pups. The animals were fed soy-free chow (prepared in-house), but caging was not described. BPA (purity not described; from Sigma Chemical Company in US) was dissolved in 99% ethanol and diluted in sesame oil to obtain the dose of 400 µg/kg body weight which was administered by subcutaneous (SC) injection to male rat pups on PND1-5. On PND75, control and BPA-treated male rats (N=12 per group) were mated to untreated normally cycling female rats; one male was mated with two female rats. Pregnant rats were sacrificed on GD20 (day of sperm- positive smear defined as GD0). Endpoints included the time taken for copulation (the interval between the first day of cohabitation and gestation), number of live implants, number of non-live implants and number of corpora lutea. Additionally, RNA was collected from whole embryos, and qRT-PCR was done to examine expression of DNA methyltransferase genes (*Dnmt1*, *Dnmt3a*, *Dnmt3b*) and transcription factors (*Sp1* and *Sp3*) relative to ribosomal *L19* gene expression. One-way ANOVA followed by Bonferroni's post hoc test was the statistical method used to analyze for differences between groups.

The authors reported that BPA increased the length of time for copulation, had no effects on the numbers of corpora lutea or implantations and increased post-implantation loss. The authors also reported that BPA decreased expression of the DNA methyltransferase and transcription factor genes in resorbed embryos compared to treated viable embryos. There was no difference in gene expression between control embryos and BPA-treated viable embryos. The authors suggested that these results indicated that BPA may have altered the transcriptome in the sperm which was transmitted to the embryos and led to post-implantation loss.

This study has no utility for HI and no utility for RA. Although a reasonable number of animals were examined, only a single SC dose of BPA was utilized in this study, so no dose-response could be determined. This dose was chosen based on previous work which suggested an effect on fertility. The time taken for copulation was defined as the "interval between the first day of cohabitated and the gestation day"; the gestation day was not defined but was assumed to be the day the vaginal smear was sperm positive. It appears that data on number of corpora lutea, number of implantation sites and post-implantation loss were presented on a litter basis, but this was not indicated in the methods. Additionally, ANOVA was the only statistical test described; only two groups were used in the study and many of the variables are not normally distributed. For these reasons, ANOVA may not have been the correct statistical test to use. The authors used soy-free chow; however, this chow was prepared in-house, and no quality control information was presented.

Aberrant DNA methylation at Igf2-H19 imprinting control region in spermatozoa upon neonatal exposure to bisphenol A and its association with post implantation loss (Doshi *et al.*, 2013)
The stated purpose of the study was to evaluate the molecular effects of neonatal exposure of male rats to BPA. The authors focused on methylation of H19 imprinting control region (ICR) in resorbed embryos (F_1) and compared that with the ICR of viable embryos as well as in the spermatozoa of the respective F_0 parental males. This study is one in a series by these authors to explore potential effects of BPA on male fertility. A strain of Holtzman rats (source not described) was dosed subcutaneously (SC) on postnatal days 1 through 5 with 2.4 µg/30 µl. Exact dosing (mg/kg bw/day) was not determined, but assumed to be 400 µg/kg bw/day based on previous paper (Doshi *et al.*, 2012). BPA (purity not stated) was dissolved in a small volume of ethanol (99% pure) and diluted in sesame oil. The diet was soy-free, in-house prepared rat pellets. On PND 75, BPA treated and control male rats (n=12/group) were cohabitated with normal cycling non-treated female rats (1 male: 2 females). Pregnant females were sacrificed on gestation day 20 and the uterine contents were examined for implantation sites, numbers of live and dead fetuses, and resorbed embryos. Viable and resorbed embryos were dissected out and frozen at -80ºC until DNA and RNA extraction. BPA treated and control male rats were sacrificed following the mating studies; the epididymis was dissected out and caudal sperm were collected and processed for DNA extraction. Studies on the embryos included the following: RNA isolation, first strand cDNA synthesis, and quantitative real time polymerase chain reaction (qPCR) for *Igf2* and *H19*. Studies in the embryo and sperm DNA included the following: DNA preparation, bisulfite modification of sperm and embryo DNA, bisulfite genomic sequencing PCR (BSP), cloning, and sequencing for CpG island at *Igf*2-H19 ICR locus in sperm and embryo.

The incidence of post-implantation loss (%POL) was reported as 24.8 in the females (n=23) mated with BPA exposed males and 0.04 in the females (n=22) mated with non BPA exposed males. The authors reported a significant down regulation in the transcript expression of *Igf2* and *H19* genes in BPA resorbed embryos (F_1) as compared to control viable embryos. A significant hypomethylation was observed at the H19 ICR in the spermatozoa as well as in resorbed embryos sired by rats exposed neonatally to BPA. The authors concluded that the perturbation in the expression of *Igf2* and *H19* lead to post-implantation loss and this resulted from aberrant methylation at ICR in spermatozoa which were transmitted to the embryo.

This study has no utility for HI and no utility for RA. Exact dosing of BPA was not determined on a mg/kg bw basis. The dose was administered subcutaneously (SC) at only one dose level in sesame seed oil. Although consistent within this lab, post-implantation loss has not been observed in large multigenerational studies in Sprague-Dawley rats when BPA is administered orally in the diet equivalent to intakes ranging from 0.001 to 500 mg/kg bw/day (Tyl *et al.*, 2002) or given orally by gavage at doses ranging from 0.2 to 200 µg/kg bw/day (Ema *et al.*, 2001). A 90 day subchronic study in Sprague-Dawley rats did not report post-implantation loss at oral gavage doses ranging from 0.0025 to 300 mg/kg bw/day (Delclos *et al.*, 2014).

Effects of transplacental 17-α-ethinyl estradiol or bisphenol A on the developmental profile of steroidogenic acute regulatory protein in the rat testis (Horstman *et al.*, 2012)

The purpose of the study was to establish the intratesticular spatial distribution and temporal expression pattern of steroidogenic acute regulatory (StAR) gene, a key gene involved in steroidogenesis. Sprague-Dawley rats (Charles River) were housed in metal cages. No mention was made regarding the quality of diet or water. Beginning on gestation day (GD) 11, pregnant dams were dosed daily with 17-α-ethinyl estradiol (EE: 0.001, 0.1, or 10 µg/kg bw/day; 98% purity) in peanut or sesame oil, or bisphenol A (BPA: 0.02, 0.5, and 400 mg/kg bw/day; > 99% purity) in dimethyl sulfoxide *via* subcutaneous (SC) injections. Controls received equal volumes of vehicle. At least 6 dams were used per dose level and time point (actual number unspecified). Fetal tissues were harvested two hours post administration of the final dose, at GD16, 18, or 20. Fetal reproductive tissues or male pelvic halves (containing the reproductive tissues, dorsal root ganglia or adrenal glands) were harvested for either quantitative reverse transcriptase (QRT-PCR) or immunohistochemistry analyses, respectively. Analysis of QRT-PCR BPA and EE GD20 values was conducted by Kruskal-Wallis one way ANOVA on ranks followed by multiple comparisons using Dunn's method. EE GD16 and GD18 QRT-PCR values were analyzed by t-test followed by Mann-Whitney rank sum. Samples within litters were pooled and the litter was used as the unit of statistical analysis.

The QRT-PCR values demonstrate that pre-natal exposure to 0.02 or 0.5 mg BPA/kg/day SC and 0.001 or 0.1 µg EE/kg/day did not affect StAR gene expression in the developing male rat reproductive tract at GD20. Similarly, there were no gross abnormalities and changes in StAR protein levels in the male rat testis following BPA exposure and changes at the highest dose of EE were seen at GD20. No significant differences in StAR transcript levels present at GD16 were demonstrated by QRT-PCR following EE exposure. However at GD18, StAR transcripts were significantly decreased following transplacental EE exposure. Immunohistochemistry demonstrated similar StAR protein levels in interstitial region of GD16 testes and a decrease in StAR protein levels in the interstitial region of GD18 testes. Moreover, starting at GD11 additional dams were dosed with 0.001 or 0.1 µg/kg/day EE or 0.02, 0.5, 400 mg/kg/day BPA, *via* subcutaneous injections. QRT-PCR validated previous microarray dose-related decreases in StAR transcripts at GD20; whereas immunohistochemistry results demonstrated decreases in StAR protein levels in the interstitial region at the highest EE and BPA doses only. Neither EE nor BPA exposure caused morphological changes in the developing seminiferous cords, Sertoli cells, gonocytes, or the interstitial region or Leydig cells at GD16-20. High levels of estrogens decrease StAR expression in the fetal rat testis during gestation. The authors considered that gestational exposure to BPA did not cause alterations in fetal intratesticular spatial distribution or temporal expression of steroidogenic acute regulatory (StAR). In contrast, high doses of EE caused decreased expression of StAR in fetal rat testis during gestation.

This study has no utility for HI and no utility for RA. Potential sources of contamination in water and food were not evaluated. The distribution of the doses was skewed, with two doses being fairly close to each other (0.02 and 0.5 mg/kg/day) and one dose being substantially higher (400 mg/kg bw/day). Samples within the litters were pooled and the litter was used as the statistical unit. However, the actual group sizes were unspecified (at least 6 litters used per group). Because an effect was seen in the QRT-PCR with the highest dose of BPA at GD20, the authors only examined that dose at the other time points.

Effects of estrogenic compounds on neonatal oocyte development (Karavan *et al.*, 2012)
The purpose of the study was to determine if exposure to diethylstilbestrol (DES), ethinyl estradiol (EE), or BPA affected perinatal oocyte development. Female mice were mated and allowed to deliver normally. The method of selecting postnatal female pups for the study was not described. The CD1 outbred mice (Charles River Laboratories) were subcutaneously (SC) injected with 50 µl of either peanut oil or a test compound dissolved in peanut oil from postnatal day (PND) 1 to PND4. The test compounds (purity not identified) were DES, EE, and BPA and were given at either 5 or 50 mg/kg bw/day. On PND5, ovaries were collected and fixed for immunocytochemistry. Phytoestrogen content in the diet and vehicle were not reported. Ovaries were analyzed using confocal microscopy. Programmed cell death was examined using the percent of poly ADP-ribose polymerase (PARP) positive labeled oocytes in ovaries of mice. For the programmed cell death measurements, mice were treated on PND1 and 2 with peanut oil (control) or with 50 mg/kg bw/day DES, EE, or BPA and ovaries were collected on PND3. Statistical analyses of cyst breakdown, total number of oocytes, and follicle development were done using one-way ANOVA and the Tukey *post hoc* test.

All treatment groups showed a significant decrease in the percent of single oocytes except for the lower dose level of BPA. The number of oocytes per confocal section was significantly increased at the higher concentrations of DES and EE and both concentrations of BPA. The high dose treated DES, EE, and BPA groups had a decrease in the percent of PARP positive oocytes as compared to the control group. Animals treated with the higher dose of DES and EE and both doses of BPA showed a significant increase in the percent of primordial follicles from 62 % in the control to over 80% in the treated groups. Follicle activation was reduced in these treated groups resulting in more primordial follicles and fewer primary and secondary follicles on PND5. The authors concluded that cyst breakdown and oocyte survival were altered in the treated groups, and follicle activation was delayed.

this study has no utility for HI and no utility for RA. The exact number of mice per group was not presented. The study protocol did not describe how female pups were grouped with the dams nor did it describe how many female pups were assigned to treatment groups, selected for measurements, or pooled for the parameters measured. Thus, the study methods state that 8-12 ovaries per group were used in the analysis, but the number of F_1 female pups or number of litters from F_0 parental females was not stated. From the description in the study methods, it is not clear if n values of 10 or more were used in the statistical analysis. A dose-response could not be determined because the study methods used a limited dose range (only two doses of each compound). BPA purity and phytoestrogen content in the diet and vehicle were not reported. It is worth noting that different findings, in regard to the effects of BPA on primordial and growing follicle populations, have been reported in rats (Rodriguez *et al.*, 2010).

Disrupted organization of RFamide pathways in the hypothalamus is associated with advanced puberty in female rats neonatally exposed to bisphenol A (Losa-Ward *et al.*, 2012)
This study tests the hypothesis that bisphenol A (BPA) accelerates puberty (*i.e.*, vaginal opening) in female Wistar rats by either accelerated maturation of kisspeptin pathways or accelerated

decline in RFRP3 (RFamide-related peptide-3) control of GnRH release. Transgenic Wistar rats with enhanced green fluorescent protein linked to the gonadotropin releasing hormone (GnRH) promoter were used to facilitate assessment of contacts between kisspeptin or RFRP3 neurons and GnRH neurons. Housing (polysulfone cages, glass water bottles, woodchip bedding) and feed conditions (AIN-93G) were such as to attempt to limit phytoestrogen and exogenous BPA exposure. A total of 17 dams were used in the study divided among the 4 dose groups. Pups were dosed subcutaneously with vehicle (sesame oil), 10 μg 17β-estradiol (E_2), or BPA at 50 μg/kg bw/day, or 50 mg/kg bw/day for 4 consecutive days from the day of birth (PND0) until PND3. (*If the dose of E_2 indicated in the paper is not a typographical error, the dose translates to greater than 1 mg/kg bw/day.*) Female pups were euthanized on PND17, 21, 24, 28, or 33 and males (controls only) on PND21 or 33 between 0930 and 1400 by transcardial perfusion. After weaning, pups were housed in same sex littermate groups of up to 4. To control for litter effects, no more than 2 pups per litter were used at each age, and pups from at least 3 litters were used at a given age. The percentage of animals having achieved vaginal opening at a given age was recorded along with terminal body weights. Plasma estradiol was measured by ELISA at all-time points, except PND28 vehicle controls due to lack of volume, and luteinizing hormone (LH) was measured by RIA at PND28 and 33. Sections were prepared from the perfused brains containing the regions of interest (rostral borders of the arcuate nucleus (ARC), organum vasculosum of the lamina terminalis (OVLT) through the caudal border of the medial preoptic area (MPOA), rostral to caudal borders of the dorsal medial nucleus (DMN)). Kisspeptin staining was conducted for all ages in sections from the first two regions, while RFRP3 staining was conducted for PND28 and PND33, due to limited antibody, in sections from the latter two regions. Quantitation was conducted by two independent observers blinded to treatment and averaged values were used for analysis. The statistical method applied for analysis of vaginal opening is not mentioned, but other endpoints were analyzed by ANOVA and pairwise comparisons were conducted if the omnibus test was significant using the Fisher least significant difference test. For analysis of contacts between kisspeptin or RFRP3 neurons and GnRH neurons, one-sided, hypothesis-driven, Dunnett's tests were used to compare treated groups to controls. In the case of sex comparisons involving vehicle control males conducted at PND21 and PND33, t-tests were used.

The study authors report that a higher percentage of animals had earlier vaginal opening than vehicle controls in the low BPA and E_2 groups; high BPA did not have an effect. E_2 and LH levels were unchanged by treatments. Body weights of PND33 females were significantly higher than vehicle controls in the high BPA and E_2 groups. BPA did not affect kisspeptin neuron density or contacts between kisspeptin and GnRH neurons. E_2 did reduce the kisspeptin neuron density on most of the days examined, but not the number of contacts. Low BPA and E_2 reduced the RFRP3 fiber density on PND28 and PND33 and low BPA, but not E2, reduced the number of RFRP3 neurons on both days. Low BPA and E_2 reduced the number of contacts between RFRP3 and GnRH neurons on PND28, but not PND33. The authors conclude that their data support the hypothesis that BPA accelerates puberty in female rats by reducing RFRP3 inhibition of GnRH release. This acceleration of puberty was not associated with increased body weight.

This study is a hypothesis-driven mechanistic study that provides data to support a possible mechanism whereby accelerated vaginal opening might be produced by BPA. For reasons

discussed below, **this study has no utility for HI and no utility for RA.** The acceleration of vaginal opening has been reported in some studies (cited in this manuscript) but, as discussed in previous FDA reviews of the BPA literature, has not been a consistent finding.
Uncertainties and Limitations: A primary limitation of this study for use in HI or RA is the low animal number, with 3 or 4 litters represented at each time point. The two doses of BPA used are widely spaced and provide limited dose-response information. The statistical method used in the analysis of the apical endpoint measured, vaginal opening, is not described and it is not clear how litters were taken into account in that analysis. As noted and discussed by the authors, the explanation for the observed dose-response (low dose effective but not high BPA dose) is not clear. A high dose of E_2 is reported to be effective here in accelerating vaginal opening, and there are differences between the reported BPA and E_2 effects. It is pointed out that mechanisms of BPA and E_2 may differ.

Effects of early prepubertal exposure to bisphenol A on the onset of puberty, ovarian weights, and estrous cycle in female mice (Nah *et al.*, 2011)
This study examined the effects of early prepubertal BPA exposure on the onset of puberty and reproductive parameters such as estrus cycle and reproductive organ weights in female mice. Female ICR mice (5/dose group) were injected subcutaneously (SC) at postnatal day (PND) 8 with BPA (0.1, 1, 10, 100 mg/kg) in sesame oil (100 µl/kg) or with sesame oil alone. Body weight was measured from PND10 to 70. Vaginal opening and estrous cycle were monitored from PND20 to 29. Animals were sacrificed at PND25, 30, and 70, and the ovary and uterus weights were measured. Statistical analysis was carried out by one-way analysis of variance followed by Tukey's test for multiple comparisons among means.

As reported by the authors, BPA (10 and 100 mg/kg) significantly decreased body weight from PND18 to 30. All BPA treated mice were reported to show early opening of the vagina compared to control group, *i.e.,* 27.7 ± 0.61 days for control group, 26.4 ± 0.43 for 0.1mg/kg group, 26.2 ± 0.28 for 1 mg/kg group, 26.2 ± 0.57 for 10 mg/kg group, and 25.9 ± 0.56 for 100 mg/kg group. The number of estrous cycles and days of estrus were significantly decreased in high dose (100 mg/kg) BPA treated mice. The weight of the ovaries was significantly decreased at PND25 and 30 in all BPA treated animals. The authors concluded that early prepubertal exposure to a single SC injection of high dose of BPA slightly accelerates the onset of puberty.

This study has no utility for HI and no utility for RA. The use of SC route, rather than oral route, in these PND8 animals would be expected to lead to higher internal doses of free BPA and should be a consideration in evaluating the reported effects (Doerge *et al.* 2011). As noted in previous FDA reviews, inconsistent findings on vaginal opening and ovarian weight changes have been reported in mice treated with BPA. The estrous cycle measurement in this study is inadequate since mice, not like rats, have not established their normal cycle yet at PND20-29 (Nelson *et al.*, 1982). The stage of estrous cycle in the animals at sacrifice was not described, which can confound the ovarian morphology and weights profoundly. Other limitations include the lack of environmental control (diet, water, cage, etc.) and few animals (n=5) used for statistics.

Endocrine disruptors *in utero* cause ovarian damages linked to endometriosis (Signorile *et al.*, 2012)

This study investigated the effects of BPA on ovarian primordial, developing, and atretic follicles and the relationship between potentially BPA-induced endometriosis-like phenotype and ovarian alterations in mice. It is worthy to note that the observations reported in this paper are extended end-points from a previously published experiment, which has been reviewed and evaluated by the Agency 2011 BPA WG. Briefly, groups of 6 pregnant female BALB-C mice from Regina Elena Cancer Institute of Rome were administered by subcutaneous (SC) injection either 2% ethanol (ETOH) in saline, or 100 or 1000 µg BPA/kg bw/day in 2% ETOH in saline, from gestational day (GD) 1 until postnatal day (PND) 7. All F_1 pups within the same treatment group were pooled, separated by sex, and then fostered to dams in the same treatment group. Five males and 5 females were retained per litter. Pups were weaned at PND21 and held until 3 months of age (5/sex/cage). Twenty pups per treatment group (the authors stated that total 6 litters/group) were examined. The authors have reported that mouse chow tested negative for estrogenic activity by the E-screen assay, tap water was provided in glass bottles, and cages and bedding tested negative for estrogenic activity (test method not given). Hematoxylin and eosin stained ovarian histology was conducted in F_1 females.

As reported by the authors, no macroscopic defects were found in the female reproductive tract, but a significant decrease in the number of primordial (2.65 and 3.8 *vs.* 7.75) and developing (4.5 and 5.5 *vs.* 11.8) follicles were observed in mice treated with 1000 and 100 µg BPA/kg bw/day compared to the control. A significant increase in the number of atretic follicles (3.3 and 3.4 *vs.* 1.55) was evident in both BPA treated groups. In a comparison of follicular development between BPA-treated mice (pooled in both BPA groups) with and without endometriosis-like phenotype, significantly lower numbers of primordial (2.63 *vs.* 3.76) and developing (4 *vs.* 5.9) follicles were observed in BPA-treated mice with an endometriosis-like phenotype but not atretic follicles (3.32 *vs.* 3.38). The authors suggest that ovarian disruption and endometriosis should be considered as disorders caused by *in utero* expose to BPA.

This study has no utility for HI and no utility for RA because of the statistical analysis, which did not address litter effects. The limitation also, as indicated previously, includes the use of an isolated mouse colony and unusual diet. However, it is worthy to note that effects on follicular development have been reported in another BPA study utilizing subcutaneous administration in neonatal rats (Rodriguez *et al.*, 2010).

Developmental programming: gestational bisphenol-A treatment alters trajectory of fetal ovarian gene expression (Veiga-Lopez *et al.*, 2013)

This study investigated the impact of gestational exposure to BPA on developmental changes in the ovarian transcriptome in ovine fetus. Pregnant Suffolk ewes (2-3 years old, n=4-5) were treated with 0 or 0.5 mg BPA (purity≥99%) /kg bw/day in corn oil by SC from gestational day (GD) 30 through GD90. The ewes were fed with shelled corn (0.5 kg/day) and alfalfa hay (1.0-1.5 kg/day) and given chlortetracycline 250 mg/day to prevent abortion. Fetal ovaries were collected from both groups on GD65 (Control, n=4 and BPA, n=5; dam was used as the experimental unit) and GD90 (Control & BPA, n=5). Gene expression of steroidogenic enzymes, hormone receptors, growth

factors and their receptors, and signal transduction factors, microRNA regulators, and micro RNAs were measured by quantitative RT-PCR. On GD90, the level of BPA in umbilical arterial blood was measured by HPLC coupled with mass spectrometer. All RT-PCR data were analyzed by 2-way ANOVA followed by a Bonferroni post hoc test, and Student's *t*-test was used for internal BPA concentration.

No difference was reported in number of fetuses per dam between control and BPA groups at either GD. Concentration of free BPA in the umbilical artery was measured on GD90 only, which was significantly higher in BPA treated group than control (2.672 *vs.* 0.43 ng/ml). Age-dependent changes in ovarian mRNA expression were observed in steroidogenic enzymes, hormone receptors, growth factors and insulin-related biomarkers in both control (most up-regulated, except *17βHSD*) and BPA (most up-regulated, except *17βHSD* and *Cyp19*) groups. No differences in expression of these genes were observed between control and BPA groups at either GD, except *Cyp 19* (upregulated by BPA at GD 65 only) and *SRD5A1* (up-regulated by BPA at GD65 only). Age-dependent (both treated and untreated) and BPA treatment-related (both GDs) changes were also noticed in fetal ovine ovarian miRNAs. BPA down-regulated all measured miRNAs (from 2 to 275 fold) on both GDs, but had no effect on gene expression of miRNA regulators (endoribonuclease enzymes, *Drosha* and *Dicer*) at either GD.

In this study, the environmental conditions were not properly controlled. High phytoestrogen containing (alfalfa) hay was fed to the pregnant ewes through the entire experiment, and the content of phytoestrogen in the hay was not analyzed. Other activities of chlortetracycline are not known in addition to its antibiotic effect. Other limitations include a single dose treatment, and one time point blood sample collection and measurement. Although BPA seemed to down-regulate expression of a number of miRNAs, no changes in the gene expression of miRNA regulators, ovarian steroidogenic enzymes, receptors or growth factors were observed, except a transient up-regulation of *Cyp19* and *SRD5A1* on GD65, which returned to normal levels on GD90. At the present time, it is not clear whether those observed changes are due to BPA. Moreover, the biological significance of these transient changes in expression of aromatase, 5α-reductase, and various miRNAs to fetal ovarian development and function remains to be determined. **this study has no utility for HI and no utility for RA.**

Exposure to bisphenol A results in a decline in mouse spermatogenesis (Zhang *et al.*, 2013)
This study evaluated the effects of BPA, administered subcutaneously, on spermatogenesis in CD-1 mice. In the first experiment, BPA at 0, 20, or 40 μg/kg bw/day was administered SC to CD-1 male pups from PND3 to PND21, 35, or 49 (3, 5, and 7 weeks, respectively). The vehicle was 0.1% DMSO in saline. In the second experiment, males were similarly dosed from PND3 to PND49 and then mated with untreated females on PND49. For the first experiment, endpoints examined included germ cell counts and stage of development; seminiferous tubule diameter; sperm number, motility, morphology; number of cells undergoing meiosis measured by immunohistochemistry (synaptosomal complex protein 3(SCP3)); methylation of selected imprinted genes (Igf2, Igf2r, Peg3, H19); serum estradiol; and estrogen receptor (ER) alpha and beta expression. In the second experiment, data on the body weight and appearance of offspring of BPA-treated males are reported up to PND35. Statistical analyses described include t-tests or

one way ANOVA with Tukey's test for all pairwise comparisons.

Germ cells in testes were reported to be increased at 3 weeks at high dose BPA and decreased at both BPA doses at 5 and 7 weeks. The authors indicate increased germ cell development at 3 weeks in the high BPA group and decreased development at 5 and 7 weeks, but data (Fig 1c of the study) appear to show a differential response at 5 and 7 weeks (increased spermatogonia and decreased spermatids at both doses at 5 weeks, increased spermatogonia at high dose at 7 weeks with no effect on spermatids). Seminiferous tubule diameter was reported to be increased at 3 weeks with high BPA, decreased by both BPA doses at 5 weeks and by high BPA at 7 weeks. Sperm number was decreased in the high dose group at 7 weeks, and morphology and motility defects are reported at both doses at 7 weeks. Germ cells entering meiosis, as measured by SCP3 staining, were increased at 3 weeks in the high BPA group, decreased in the high dose group at 5 weeks, and in both dose groups at 7 weeks. There were no effects on methylation patterns of the imprinted genes that were evaluated. Testicular ER alpha was increased by both BPA doses at all ages examined. Data for ER beta are not shown, but indicated to be unchanged. Finally, serum estrogen was reported to be increased in the high dose BPA group at 3 weeks and in both dose groups at 5 and 7 weeks.

For the second experiment that evaluated offspring of BPA-treated males, several effects are reported for the high BPA group: decreased pup number and increased birth weight at high BPA (appears to be less than 10%); decreased body weight and "lower quality pelage" at later ages. A higher rate of dystocia was reported in the high BPA group. How the investigators evaluated dystocia is not clearly defined. No significant effects on the offspring of males treated with the low BPA dose are reported. The authors conclude that BPA at 20 and 40 μg/kg bw/day (SC) inhibits spermatogenesis and that adverse effects are produced in the offspring of males treated with the 40 μg/kg bw/day dose level.

This study has no utility for HI and no utility for RA because of a lack of clarity in the description of the experimental design and methods. Previous FDA reviews of the BPA literature have noted conflicting data on effects on spermatogenesis.
Uncertainties and Limitations:
Interpretation of this experiment is made difficult by an inadequate and confusing description of experimental details.

1) No specifics of BPA purity and source, diet, or housing conditions are given. It is not clear how the pups (30 per treatment) were grouped with dams prior to wean or whether this was taken into account in the statistical analyses. How pups were selected at the 3 time points examined is not specified.
2) It is not clear how the testes were sampled for the analyses on fixed and frozen tissue, or how multiple measurements were used in the statistical analysis. For the immunohistochemical analysis, for example, it is indicated that "…a total of 60 testes from 30 mice in each group" were examined. Does this mean that 20 animals from each of 3 groups were selected? It is indicated that "Each testis was sliced into 5 μm sections, with representative sections taken every 10 sections for evaluation"; was the average computed for each animal for analysis? In the Statistics section, it is stated that "For each set of results, independent experiments were

performed at least 3 times." Were the results averaged for analysis?
3) Specifics of germ cell quantification on testis sections (number of sections, area evaluated, etc.) are not provided.
4) In Materials and Methods, under "sperm death and semen quality," it is indicated that: "Mice with a normal sperm concentration were used and were divided into 2 groups (n=27 in each group)." It is not clear how this fits in the experimental design. This experiment appears to be what is referred to in Results (study page 6, right column under Figure 2), where it is stated that BPA and estrogen reduced sperm survival, with a greater effect of BPA. No doses are specified in the text, and the Supplemental Material available on the publisher's website does not include information on such a study.
5) For the statistical analyses, there is no mention of evaluation of examining normality or equal variance assumptions for the count and percentage data examined.

Oral-implant and Implant exposure

Bisphenol A alters early oogenesis and follicle formation in the fetal ovary of the rhesus monkey PNAS (Hunt *et al.*, 2012)

This study was intended to determine if BPA affects the onset of meiosis in the developing fetal ovary and formation of the ovarian follicles in the perinatal ovary. Adult rhesus monkeys, age 6-13 years were used. They were housed individually in stainless steel cages and fed Purina monkey chow *ad libitum*. Supplements consisted of seasonal produce, seeds, and cereal. Pachytene oocytes from ovaries in the "early treatment" group were scored for synaptic defects. MLH1 (*mutL* homolog 1) protein foci in pachytene cells were counted. Follicles from ovaries in the "late treatment" group were staged and the number of oocytes per follicle was recorded.

A. Single oral exposure per day: There were 2 cohorts: early treatment and late treatment:
The early treatment consisted of groups of 5 treated and 6 controls administered 400 µg deuterated bisphenol A (dBPA) in small pieces of fruit from GD 50-100. Fetuses were removed by C-section at the end of the period. The late treatment consisted of groups of 6 treated and 6 controls administered 400 µg dBPA each day in small pieces of fruit from GD 100-term.

B. Silastic capsules intradermally implanted to release dBPA and produce serum levels from 2.2 to 3.3 ng/ml unconjugated dBPA: There were 2 cohorts: early treatment and late treatment:
The early treatment consisted of groups of 6 treated and 2 controls from GD 50-100. Fetuses were removed by C-section at the end of the period. The early treatment consisted of groups of 6 treated and 2 controls from GD 100-term.

Effects on meiotic chromosome behavior: Due to technical difficulties, cells suitable for MLH1 staining could only be obtained on two exposed and one control animal from the group given a single daily exposure only. For continuously exposed females, the mean values for MLH1 were "significantly "different" for 33 cells from continuously exposed animals compared with 70 cells from placebo treated animals (50.4 +/- 7.0 *vs.* 42.2+/- 7.9.) However, there were a lot of overlapping values, and the range was wide. Synaptic defects (non-homologous chromosome associations) identified by SYCP3 staining, reportedly previously seen in mice, were not seen at significantly increased incidences in either single exposure or continuous exposure groups. The authors reported that the percentage of oocytes with centromere associations was not different

between exposed and control animals in the group given the single daily dose, but that there was a significant increase in centromere associations in the continuous exposure group. It is noted that the control values for centromere associations were notably different for fetuses between the study with a single exposure per day and that with continuous exposure.

Effects on follicle formation: The authors reported that they were unable to count single and multioocytes per primordial follicle, so they focused on counting secondary and antral follicles in the medullary region of the ovary. In females exposed continuously, the overall distribution of follicles containing 1 to >5 oocytes was not statistically different between exposed and control females, whereas for females exposed once per day there was a statistically significant result. For both groups, the number of follicles with >5 oocytes was significantly increased but the absolute number/incidence is a few percent. It is noted again that the control values for the number of oocytes per follicle were notably different between the single exposure per day fetuses and continuous exposure fetuses.

The authors conclude that BPA disrupts key events of meiotic prophase and follicle formation. The effects were less pronounced than in the mouse. **This study has no utility for HI and no utility for RA.** Uncertainties and Limitations include the following: The intradermal implant route of exposure with silastic capsules are of questionable utility or relevance. The vehicle in the slow release implant capsule and other chemical components are not reported. The estrogenic content of the diet is unclear. For MHL1 counts; cells from fetuses were counted. Small numbers of animals (only two control dams) were used for some analyses, and only one dose was analyzed. The timing mimicked the developmental windows purportedly "showing effects for mice". However timing in primates would be different. Because of design deficits, it is not clear that a biologically meaningful difference has been identified to be a BPA hazard. Extrapolation of effects of BPA from rhesus to humans regarding any fetal effects in this study is confounded by differences between rhesus monkeys and humans:

A. "The differences in metabolism of progesterone and pregnenolone in the placenta and fetus of the rhesus monkey and human" (Leung *et al.*, 1972). "In the ovary, only weak β-subunit (of inhibin) immunoreactivity was detected in granulosa cells of a few primary follicles from midgestational human fetal ovaries. In contrast, all three subunits were found in granulosa cells of numerous primary and secondary follicles in the late gestation rhesus monkey ovary" (Rabinovici *et al.*, 1991).
B. Rhesus monkeys are seasonal breeders (only 11 days per year, usually fall-winter) thus hormonal control of ovulation differs markedly for rhesus monkeys and humans (Riesen *et al.*, 1971).

That the single daily dosing and continuous dosing experiments were conducted in two different breeding seasons cannot be ruled out as a confounding factor in interpretation of the results since the control values for the number of oocytes per follicle were notably different between the single exposure per day fetuses and continuous exposure fetuses.

<u>Developmental exposure to xenoestrogens at low doses alters femur length and tensile strength in adult mice (Pelch *et al.*, 2012)</u>

This study examined the effects of developmental exposure to 17-ethinyl estradiol (EE_2),

diethylstilbestrol (DES), and bisphenol A (BPA) on femur geometry and biomechanical strength in adult mice. C57BL/6J mice were housed in polysulfone cages, fed Rodent Chow (Purina product no. 5008), and received acidified water ad libitum from polysulfone bottles. The phytoestrogen content of the diet was not reported, but it is a soy-based diet. Mice were time-mated and on GD11 were implanted with a mini-osmotic pump. The pumps had an average release rate of 0.2 µl/hr. Experiment 1 mice received EE_2 (0.01, 0.1, or 1.0 µg/kg/day) or vehicle control (80% polyethylene glycol, 20% dimethylsulfoxide). Experiment 2 mice received DES (0.1 µg/kg/day), BPA (10 µg/kg/day), or vehicle control. F_1 mice were exposed through lactation until PND12. For analysis, 2-3 females per litter and 1 male per litter were used for Experiment 1, 1-2 females per litter and 1 male per litter were used for Experiment 2. Males were used only from those litters with at least two males in an attempt to reduce the influence of the intrauterine hormone environment. F_1 mice were euthanized by carbon dioxide asphyxiation and cervical dislocation at 10 weeks of age (Experiment 1 females), 13 weeks of age (Experiment 2 females), or 23 weeks of age (Experiment 2 males) and the left femur was excised. Femur length, periosteal major diameter (D_p) and minor diameter (d_p), endosteal major diameter (D_e), marrow cavity diameter, cortical bone width, q (derived from D_e/D_p), and polar moment of area (K) were measured or calculated based on the micro-computed tomography scan. Femur torsional strength was tested by torsional loading to failure. Statistical analysis was a two-sided Student t-test for Experiment 1 and a one-sided Student t-test for Experiment 2.

Femur length was reported as significantly increased in male mice receiving BPA. No other statistically significant effects ($p<0.05$) of BPA exposure were reported. Femur length was significantly increased in female mice receiving either 0.01 EE2, 0.1 EE2, or DES. Male mice had significantly decreased marrow cavity diameter, cortical bone width, and tensile strength when exposed to DES. Female mice also had significantly decreased tensile strength with exposure to 0.01 EE_2. Femurs of female mice exposed to DES had significantly lower energy to failure. The study authors conclude that humans exposed to xenoestrogens while in the womb may have an increased risk of fracture due to alterations in bone geometry and material strength. **This study has no utility for HI and no utility for RA.** A single dose of BPA at a single time point was used. A soy-based, high phytoestrogen content diet was used. Statistical analysis was not robust and did not account for litter effects (more than one female per litter was used for analysis of both experiments).

Accessory study

Estrogenic Content of Rodent Diets, Bedding, Cages, and Water Bottles and Its Effect on Bisphenol A Studies (Thigpen *et al.*, 2013)

This study compared the estrogenic content of rodent diets, bedding, cages, and water bottles to evaluate their impact on the estrogenic activity of bisphenol A (BPA) and review the literature on BPA to determine the most frequently reported diets, bedding, cages and water bottles used in animal studies.

Zearalenone was measured in corncob bedding and rodent diets. Also measured was the amount of BPA that leached from various types of animal cages and water bottles. Previously reported

data were presented regarding the effects of rodent diets on the time of vaginal opening in CD1 mice, and in F344 and Sprague-Dawley rats. Dietary phytoestrogens (daidzein, genistein, formononetin, biochanin A, and coumesterol) were analyzed by HPLC. Mass spectrometry was also used. Total metabolizable energy was also calculated for various diets. A number of BPA studies by routes other than oral was collected and summarized. Corn and corn cob bedding contain zearalenone, which has greater potency than BPA *in utero*trophic assays and vaginal opening assays. Different mill dates of the same diet can vary by more than 3-fold in phytoestrogen content. The diet at the animal supplier may be highly estrogenic with high amounts of metabolizable energy levels. The amount of methyl donors in the diet (*e.g.*, folic acid, vitamin B12, choline chloride, L-methionine, and betaine) can also affect the outcome of studies with BPA. A large number of BPA studies were conducted by routes other than oral.

The study authors concluded that many studies of BPA in the literature are confounded by unwanted and uncharacterized estrogenic activity in the diet and bedding. Such levels should be measured and amounts minimized. The authors concluded that rodent diets and corncob bedding are more important contributors to unwanted estrogen exposure than water. They suggested that studies of BPA only be conducted by routes relevant to human exposure.

This paper has valuable suggestions for improving the value of future studies of BPA in animals. Uncertainties and limitations include the following: since no phytoestrogen in the rodent diet at all may result in some adverse effects, it is not clear that no phytoestrogen in the diet is the best goal. However, the minimal amount of phytoestrogen necessary for normal development is not known.

Carcinogenesis Studies

Summary

Several papers were reviewed that attempted to address the potential for carcinogenicity of BPA with regard to mammary gland and prostate in various models. The Acevedo *et al.* study evaluated pregnant Sprague Dawley rats administered 4 doses of BPA in 50% DMSO subcutaneously by pump. Data did not support any finding of mammary carcinogenicity in offspring; the data actually supported no mammary carcinogenicity effect at the four doses tested. In the Betancourt *et al.* study, Sprague Dawley rats were administered BPA in sesame oil by gavage during pregnancy. The study reported protein expression but no structural changes in mammary glands of offspring, possibly measured after DMBA treatment. The DMBA model of carcinogenicity was referenced but no actual carcinogenicity data are reported. The gavage vehicle was sesame oil, which may be a confounder, and only two doses 500x apart were tested. In Castro *et al.*, adult male Wistar rats were administered four different doses of BPA by sc injection in sesame oil. In this mechanistic study, no carcinogenicity endpoints were examined, but plasma testosterone and 5-alpha reductase gene expression levels in the rat ventral prostate were measured. The authors reported effects on the ventral lobe but not on the dorsal or lateral lobes, which are the lobes homologous with humans. However, no effects on prostate histopathology were observed in other studies reviewed herein and for previous FDA reviews. The Tharp *et al.* study administered only one dose level of BPA in fruit to pregnant female

Rhesus monkeys. The study reported a change in number of mammary buds per number of ductal units, however, a link from this endpoint to carcinogenicity is neither clear nor demonstrated in this study. In Vandenberg *et al.*, pregnant female CD-1 mice were administered 50% DMSO or BPA in 50% DMSO via an implanted osmotic pump day 8 of gestation thru day 16 of lactation. While mammary gland morphometry and ER immunohistochemistry were evaluated in male offspring, no link to carcinogenicity was provided by this study. The study reported positive effects on gland development at the lower but not higher doses tested.

None of the 5 studies (three in rats, one in mice, and one in rhesus monkeys) demonstrated a carcinogenic effect of BPA, and one study referred to a previous study, but that model - initiation with DMBA- is not relevant to humans. The one study that did examine carcinogenicity did not demonstrate a carcinogenic effect. Two of the studies were with osmotic pumps sc in 50% DMSO, one sc study was by injection, one route was in fruit, and one study was by gavage in sesame oil. Four of the studies had design limitations that limited their ability to influence RA of BPA. The negative findings in the carcinogenicity do not support mammary gland carcinogenicity as a potential hazard. The negative findings in prostate histopathology do not support prostate carcinogenicity as a potential hazard.

Individual Study Reviews

Perinatally Administered Bisphenol A as a Potential Mammary Gland Carcinogen in Rats (Acevedo *et al.*, 2013)

The objective of the study was to determine if *in utero* exposure to BPA through dams during gestation only or throughout lactation affects the incidence of mammary gland neoplasia in female offspring. Groups of 9-12 per dose/exposure Pregnant Sprague Dawley rats were administered 0.25, 2.5, 25 or 250 µg BPA/kg bw/day in 50% DMSO subcutaneously, with an implanted Alzet pump (implanted on day 9 of pregnancy), continuously up to 14 days for exposure only through gestation or 28 days for dams exposed through gestation and lactation. Cages, water bottles, and bedding were reported to have negligible estrogenicity by E-screen assay. Feed (Harlan Teklad 2018) was reported to have 8-15 fmol of estrogen equivalents per gram, but neither BPA nor phytoestrogens were measured analytically. After normal delivery, litters were culled to 10 individuals on PND2. Mammary gland tissue was harvested on PND 50, 90, 140, and 200. The fourth left inguinal abdominal mammary gland was fixed and processed for paraffin embedding and the contralateral gland was whole mounted and stained with carmine. Serum samples from dams were treated with beta-glucuronidase/sulfatase to estimate total BPA and processed without enzymatic treatment to estimate concentrations of unconjugated BPA.

Serum levels of BPA were analyzed for control and 250 µg BPA/kg bw/day dams and pups at GD21 and PND10. At PND10, total BPA was detected in 2/6 dams that received no BPA. The incidence and range was similar to that of the pups from mothers that received 250 µg BPA/kg bw/day, and the mean for both groups was below the level of quantitation. The range for unconjugated BPA reported in pups was below LOQ for PND 10 and included samples below LOQ for GD10. Single incidences of microfibroadenomas or adenocarcinomas occurred randomly, including in controls. The groups with the single incidences did not overlap between

the gestation only and the gestation plus lactation. The authors concluded that sc exposure to BPA during gestation to Sprague Dawley rats or during gestation plus lactation induces mammary neoplasms in offspring, as a complete carcinogen. However, the conclusions are not supported by the data.

This study has no utility for HI or RA for BPA as a carcinogenic hazard. This study suffered from a number of limitations. The data (the single incidences and the distribution of the single incidences) do not support the author's conclusion of BPA causing mammary neoplasms. The choice of 50% DMSO as a vehicle is undesirable. The contribution of DMSO in this study as the control vehicle is unknown, but DMSO is biologically active in embryonic stem cells, human pluripotent stem cells, and in mammary cell lines, as well as *in vivo* in mice for affecting secretion of growth hormone and prolactin. The diet manufacturer's webpage indicates that the typical isoflavone concentrations (daidzein + genistein aglycone equivalents) range from 150 to 250 mg/kg diet, a relatively high concentration. Mammary adenocarcinomas have been reported in control female Sprague Dawley rats in less than 6 months (*e.g.*, see Kuzutani *et al.*, 2012). Extrapolating from rats to humans regarding any fetal mammary effects, such as nonneoplastic effects, may also be confounded with regards to an endogenous human fetal-specific estrogen, estetrol, which has been reported to be antagonistic to estradiol effects in breast tissue. It could be presumed that estetrol (E4) may protect the human from adverse mammary effects due nonpotent exogenous estrogenic substances at low levels or that the rat may be more sensitive than human to estrogenic substances.

Altered Carcinogenesis and Proteome in Mammary Glands of Rats after Prepubertal Exposures to the Hormonally Active Chemicals Bisphenol A and Genistein (Betancourt *et al.*, 2012)
The objective of the study was to evaluate BPA-induced changes to the postnatal day 50 (PND 50) female rat (Sprague-Dawley) mammary gland proteome that might enhance susceptibility to challenge with a carcinogen. Limited experimental details are provided in this paper. The authors refer to Jenkins *et al. (*2009) [# 9 in the reference list for this paper] for the dosing regimen as well as the tumor and cell proliferation data that provided the rationale for the protein analysis studies that are the focus of this article. Jenkins *et al.* (2009) was covered in a previous CFSAN review (CFSAN/DFCN, Bisphenol A: Review of Low Dose Studies, 2009). It is not clear if the pups examined in the present study were littermates of a subset of the Jenkins *et al.* (2009) study litters or are from a separate study that followed the same dosing protocol. In Jenkins *et al.* (2009), animals treated as described above received a single dose of the carcinogen dimethylbenz[a]anthracene (DMBA) at PND 50 and tumor development was followed until sacrifice at 12 months of age. An increase in mammary tumors over the control was found only in the high BPA dose group (250 μg/kg bw/day), so this group and the vehicle control were selected for analysis of proteome changes. There were no morphological changes in the mammary glands noted, but there was a reported increase in cell proliferation, as measured by KI-67 in the terminal end buds. Apoptosis, as evaluated by the TUNEL assay, was also reported to be decreased in Jenkins *et al.* (2009).

In Jenkins *et al.* (2009), attention was paid to control of background phytoestrogens (AIN-93G diet) and BPA (polypropylene cages, glass water bottles), and litter effects were controlled by

using one female pup per litter. Sprague-Dawley (Charles River) dams received daily gavage doses of vehicle alone (sesame oil), 25 or 250 μg BPA/kg bw/day from PND 2 through PND 20. The exposure regimen used in this study relies on transfer to the pups through the dams' milk. The authors note that there is a significant attenuation of dose due to maternal metabolism (stated in error as "fetal metabolism") and secretion into the milk. The authors estimate that 0.01% of the dose administered to the dam would be transferred to the pups, so that the high BPA dose pups would receive, by their estimate, approximately 25 ng BPA/kg bw/day. The exact dose received by the pups is unknown. There were 6 – 8 female pups evaluated at PND 50. Two-dimensional gel electrophoresis/mass spectrometry (2-DGE/MS) was used to evaluate differential protein expression in the high dose BPA and vehicle control groups. Specific proteins were also quantitated by Western blot.

Eighteen proteins were reported as being differentially expressed relative to vehicle controls, with 7 being down-regulated and 11 up-regulated. The fold-changes vary from 1.2 to 2.1, although it is not clear how these values are related to biological activity. The authors selected specific proteins, some affected by 2-DGE/MS and some not, involved in the vascular endothelial growth factor receptor 2 pathway (VEGFR2, annexin2, Phospho-Akt), the regulation of cell proliferation, apoptosis, and angiogenesis, or in cancer cell survival (GRP-78, HSP-70, selenium binding protein) for Western blot analysis. All of these proteins were reported as significantly changed relative to vehicle controls (all increased except selenium binding protein, which was decreased) in the direction predicted for increased proliferation and cancer cell survival. The authors suggest that the protein expression changes reported could be involved in increasing susceptibility to mammary cancer induction in this rodent model, and possibly humans, at low BPA exposures.

This study has no utility for HI and no utility for RA. The possible utility of this paper is in identifying potential pathways affected by BPA that could alter susceptibility to carcinogenesis, although the mechanism whereby such effects would be elicited at this dose are not clear. This is a meeting report, which has very limited information on experimental details and statistical analysis. It provides mechanistic information on a single dose of BPA. Based on the experimental design described in the manuscript, there was disagreement among the reviewers as to whether the animals in this study received DMBA prior to tissue harvest and analysis. The above comments assume that animals did not receive DMBA, based on the wording of the figure legends. If, in fact, the animals did receive DMBA, additional groups receiving DMBA and BPA alone should have been included. Although sesame oil has been commonly used over the years as a dosing vehicle for many compounds that are poorly soluble in water, there are indications in the literature that components of sesame oil have various biological activities. A saline gavage or untreated control group would thus be useful for comparison. The long term consequences of the reported changes without DMBA challenge are unknown. Studies using the DMBA model of mammary carcinogenesis have been previously reviewed by FDA and/or CFSAN as part of the BPA safety assessment. Those reviews have pointed out that this model has little relevance to human RA in terms of the carcinogenic risk of BPA.

<u>Bisphenol A exposure during adulthood alters expression of aromatase and 5α-reductase</u>

isozymes in rat prostate (Castro *et al.*, 2013)

The objective of this study was to evaluate the effects of BPA on 5α-reductase isozymes and aromatase in the rat ventral prostate. Four consecutive daily subcutaneous injections [vehicle (sesame oil), 25, 50, 300, and 600 μg/kg bw/day] were administered to adult male Wistar rats (260-280 g, age not specified, 8 per dose group). Rats were maintained on a phytoestrogen-containing chow diet (phytoestrogen levels not measured) and maintained in stainless steel cages with tap water administered *ad libitum* from glass water bottles with rubber stoppers. The authors acknowledge that phytoestrogens were present but argue that all groups had the same exposure. Thirty minutes after the final dose (time of day not specified), the animals were sacrificed by decapitation and blood (for plasma) and prostate were removed. Some of the prostates were apparently frozen for gene and protein expression analysis and others were fixed in formalin, although how these differently processed samples were collected (whole prostate from a single animal split for differential processing or whole prostates from a subset of the total of 8 animals either frozen or fixed) is not described. While it is indicated that the whole prostate was collected and both lobes (presumably ventral and dorsolateral) were evaluated by immunohistochemistry (IHC), RNA and protein were extracted from the ventral prostate and results are reported only for ventral prostate.

Plasma testosterone (T) and estradiol (E2) and the estradiol: testosterone ratios were reported. Gene expression of 5α-reductases R1, R2, and R3 (hereafter abbreviated R1, R2, and R3) and aromatase were measured by Q-PCR at all doses. R1, R2, and aromatase were evaluated by IHC at all doses and by Western blot at the 50 μg/kg bw/day dose level. Because there were no antibodies available for 5α-reductase R3, this isozyme was not measured by IHC or Western. Statistical analysis for all endpoints consisted of one way ANOVA and all pairwise comparisons by the Tukey test. As noted above, the numbers of samples used for fixed (IHC) and frozen (Q-PCR and Western blots) were not clear. Plasma T was reported as reduced relative to control at all dose levels with a similar depression at the 2 lowest doses and a dose-responsive decline for the highest 3 doses. Plasma E2 was reported as elevated relative to control to a similar degree by all 4 doses. The E2/T ratio was reported as elevated at all doses, with a marked difference between highest dose level and others. mRNAs for R1 and R2 were reported as reduced by all doses, and mRNAs for R3 and aromatase were increased at all BPA doses. No dose response was apparent for R1, R3, or aromatase, with all doses showing a similar effect. R2 showed a dose responsive reduction for lowest 3 doses, with the 2 highest doses showing a similar depression relative to the vehicle control. A similar response for R1, R2, and aromatase to the responses shown by Q-PCR and IHC was reported with Western blots at the 50 μg/kg bw/day dose, the only dose group compared to the vehicle control by this method. The authors suggest that the hormonal and enzyme changes reported are consistent with the production of adverse consequences in the prostate, including benign prostatic hyperplasia and carcinogenesis.

This is a mechanistic study and has no utility for HI or RA. Previous studies that have been reviewed by FDA and CFSAN have identified the prostate as a potential target tissue for BPA, although results are not consistent across studies. The present study does not resolve the uncertainty, but suggests a possible mechanistic basis for potential prostate effects that requires confirmation. However, although the changes reported are of interest from a mechanistic

standpoint, all measurements were made 30 minutes after the last of 4 exposures. There was no evaluation of apical endpoints to demonstrate adverse effects resulting from the changes in hormones and enzyme expression evaluated. It is not clear why results are only reported for the ventral prostate. While any treatment-related differences are of interest, it is notable that the dorsal and lateral lobes of the rat prostate are considered to be more homologous to the human prostate. The authors note the lack of dose response (mentioned above) over a 24-fold dose range for many of the endpoints but do not discuss the possible reasons for these observations. While sesame oil has been commonly used as a vehicle, there are literature reports of biological activity of components of sesame oil that could impact the endpoints measured.

Bisphenol A alters the development of the rhesus monkey mammary gland (Tharp *et al.*, 2012)
The objective of this study was to assess the effects of BPA on fetal mammary gland development in nonhuman primates. Pregnant adult female rhesus macaques were given a daily dose of 400 µg/kg bw/day of BPA or 100 mL of ethanol (vehicle) in fruit from gestational day 100 thru 165. Offspring were delivered around day 165 by natural birth. Tissues were collected within 1-3 days after birth from the female offspring. One neonatal mammary gland was whole mounted on a slide; the other was processed for histological analysis.

The mammary glands of neonates from BPA treatment were reported as more developed for every parameter assessed including terminal buds, terminal ends, branching points, bifurcating ends as well as the total mammary gland area including the ductal area and the number of ducts, although many endpoints were reported as not statistically different (it was not clear in the article which were and which were not). The number of animals was low, 5 in control and 4 treated. No differences were reported in both the expression of ER alpha and beta between control and treated animals. The authors concluded that the mammary glands of nonhuman primates are sensitive to BPA exposure during gestation as manifested by increasing complexity in the ductal system of the gland as compared to controls.

This study has no utility for HI and no utility for RA. A link to carcinogenicity was not discussed, and it is not clear that a more complex ductal system at birth leads to cancer or if this effect persists. Very few animals were used so differences in endpoints between treated and controls may not necessarily be statistically significant.

The Male Mammary Gland: A Target for the xenoestrogen bisphenol A (Vandenberg *et al.*, 2013)
The objective of this study was to characterize the development of the mammary gland in the male CD-1 mouse and to examine the effects of BPA administered in a range of doses on this development. Pregnant female CD-1 mice were exposed to either 50% DMSO or BPA in DMSO via an implanted osmotic pump day 8 of gestation thru day 16 of lactation. BPA was administered at 0.25, 2.5. 25 or 250 µg BPA/kg bw/day. Litters were culled to eight pups/mother at day 1 after birth. Control male pups were sacrificed at E18, PND1, PND5, PND 10, 3-4 months, 7-9 months, and 12-15 months. For BPA exposed animals, a single male offspring from each litter was sacrificed at 3-4, 7-9, and 12-15 months. The number of litters used was not given. One fourth inguinal mammary gland was dissected from skin, spread on a glass slide and

stained and examined. When no visible epithelium was seen, another male from the litter was sacrificed. Paraffin sections were processed for immunochemistry. Development of the male mammary gland was outlined.

The reported immunohistological data indicated that 10-20% of epithelial cells in the 7-9 month gland were ER-alpha positive in the control male tissues. At 3–4 months of age, a non-monotonic dose response to BPA was reported, where animals exposed to 0.25BPA and 2.5BPA showed more advanced gland development than the controls, but 25BPA and 250BPA were statistically indistinguishable from controls. At 7–9 months of age, a non-monotonic relationship between dose and mammary gland morphology was reported as still present but had shifted such that animals in the 2.5BPA and 25BPA groups were significantly different from controls; however, lower doses (0.25BPA) and higher doses (250BPA) were statistically indistinguishable from controls.

The study reported that the male CD-1 mouse retains its mammary epithelium throughout its lifetime and the gland is responsive to early life exposure to BPA. The study authors suggest that this may have some implications for the development of gynecomastia in humans. Overall, the study had limited utility. **This study has no utility for HI and no utility for RA.** A link to carcinogenicity was not discussed. Replacing male animals with no epithelial tissue with other animals without determining if it was a preparation artifact or if a certain number of male animals did not retain the tissue added uncertainty to the conclusions about male CD-1 mammary epithelium development.

Other Endpoint Studies

Summary

Thirteen studies were reviewed. Six studies used the rat as the animal model [Wistar (3), F344 (1), Charles Foster (1), and Sprague Dawley (1)], while the remaining seven studies used a mouse model [C57BL/6 (4), Swiss Albino (1), MF-1 (1), and Avy (1)]. Three different routes of exposure were used: intraperitoneal injection (1), subcutaneous injections (4), and oral route [gavage (4), drinking water (2), diet (1), and pellet (1)]. Nine studies used a vegetable oil as vehicle, including castor, peanut, olive, and corn (tocopherol-stripped or not). In addition, several studies used diets with considerable levels of phytoestrogens, and one study used corn cob bedding, which is possibly contaminated with mycoestrogens. Approximately half of the studies dosed the dam (gestational and/or lactational exposure) and assessed the potential for effects of BPA in the F_1 pups, but many failed to account for litter effect. Only two studies, which report data that seem to have been collected from a common animal cohort, used a reference estrogen control (17β-estradiol) to compare its effects with those reported to be induced by BPA. In many studies, critical rodent housing information (including cage and water bottle material, bedding, and/or diet used) were not specified, which complicates data interpretation. With the exception of one study, no BPA internal dosimetry data were collected. In addition, no study quantified BPA in housing materials (diet, bedding, drinking water), and only one study certified the dosing solutions.

One study assessed the effect of BPA on inflammatory colitis in rat and found no effects. The remaining studies assessed endpoints related to cardiotoxicity, hepatotoxicity, testicular toxicity, and/or glucose homeostasis. None of the thirteen studies reviewed were found to be useful for RA. Three studies (Cagampang *et al.*, 2012; D'Cruz *et al.*, 2012a; and Batista *et al.*, 2012) were considered to have some degree of utility for HI, and all three reported effects of BPA on glucose homeostasis. In addition, Cagampang *et al.* (2012) and Batista *et al.* (2012) reported that 100 µg BPA/kg bw/day sc decreased locomotor/ambulatory activity in mice. However, in all cases, deficiencies in study design and/or lack of complete report of study details limited the data utility. D'Cruz *et al.* (2012a) reported statistically significant effects of BPA on plasma glucose and insulin at oral doses as low as 5-50 ng BPA/kg bw/day, but failed to properly describe the animal housing conditions and did not mention whether the rats were housed under BPA-free conditions, the phytoestrogen content of the diet, or whether environmental BPA contamination was taken into account in the study. Reproducibility and accuracy of such low doses is also a question based on issues identified in Churchwell *et al.* (2014) and the 90 day study (Technical Report E2176.01). The effect of BPA on glucose homeostasis was already considered in the previous FDA assessment. A 90-day subchronic study reviewed herein and considered of utility for RA did not find an effect of a wide dose of oral BPA (2.5 – 300,000 µg/kg bw/day) in the circulating glucose and insulin levels.

Individual Study Reviews
Oral exposure

Neither direct nor developmental exposure to bisphenol A alters the severity of experimental inflammatory colitis in mice (Roy *et al.*, 2012)
The goal of this study was to determine whether oral exposure to BPA affects inflammatory bowel disease (IBD), an immune-mediated disease of the colon, using a mouse model of inflammatory colitis. Adult C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed in pre-washed polysulfone microisolator cages under pathogen-free conditions. Mice received phytoestrogen-free irradiated rodent chow (AIN-76 A, Test Diet, Richmond, IN), and drinking water that was purified by reverse osmosis and provided in glass bottles. Colitis was experimentally induced in adult (8-week-old) mice by instilling 4 mg of 2,4-dinitrobenzene sulfonic acid (DNBS) dissolved in 50% ethanol using catheters placed in the colon, 3.5 cm from the anus of the anesthetized animals. For direct exposure to BPA, 6–8-week-old male mice were given BPA (50 µg/kg bw/day) or vehicle control (peanut oil) by oral gavage every day for 7 days. On the 7th day of BPA treatment, DNBS was rectally instilled to induce colitis and daily oral BPA administration was continued for 21 more days. For developmental exposure to BPA, adult mice were paired for breeding, and females were checked daily for vaginal plugs as an indication of pregnancy. Singly housed pregnant females began receiving 50 µg/kg bw/day, or vehicle (peanut oil) orally starting on gestational day (GD) 6 and continuing daily through post-natal day 21. Each pregnant or lactating mouse received BPA dissolved in 20 µl of peanut oil absorbed to an additive-free puffed wheat cereal. On post-natal day 21, offspring were weaned and housed in same sex groups until ready for experimental procedures.

Administration of DNBS consistently generated an inflammatory response in the colons of all

mice regardless of BPA exposure history. Exposure to BPA either via direct oral route or through maternal sources (*i.e.*, developmental exposure) did not significantly alter gestation length, litter size, offspring sex ratio, body weight, survival, or colonic pathology. Oral BPA exposure at this dose and for this exposure duration has minimal influence on aspects of the inflammatory response that regulate immune mediated diseases of the gastrointestinal tract. **This study does not suggest colitis as a BPA hazard and has no other utility for HI or RA.**

Epigenetic Responses Following Maternal Dietary Exposure to Physiologically Relevant Levels of Bisphenol A. (Anderson *et al.*, 2012)

The goal of this study was to examine the effects of developmental BPA exposure (50 ng BPA/kg diet, 50 μg BPA/kg diet, and 50 mg BPA/kg diet through maternal diet) on global and candidate gene methylation at postnatal day 22 in the viable yellow agouti (Avy) mouse. Avy mice were obtained from a colony that has been maintained with sibling mating and forced heterozygosity for the Avy allele for over 220 generations (Waterland and Jirtle, 2003). Animals were fed low-phytoestrogen AIN-93G diet with 7% corn oil substituted for 7% soybean oil, were housed in polycarbonate-free cages [cage material not specified], and provided ad libitum access to diet and BPA-free water [no water or feed BPA quantification data provided]. BPA was supplied by the National Toxicology Program. Virgin a/a dams, 6 weeks of age, were randomly assigned to four exposure groups: (1) standard diet (n = 11 litters, 86 total offspring, 39 Avy/a offspring); (2) standard diet plus 50 ng BPA/kg diet (n = 14 litters, 107 total offspring, 48 Avy/a offspring); (3) standard diet plus 50 μg BPA/kg diet (n = 9 litters, 67 total offspring, 32 Avy/a offspring); and (4) standard diet plus 50 mg BPA/kg diet (n = 13 litters, 91 total offspring, 45 Avy/a offspring). The 50 mg/kg dosage, previously used by this research group in a similar study design (Dolinoy *et al.*, 2007), was formulated to be "an order of magnitude lower than the dietary administered maximum nontoxic threshold in rodents (200 mg/kg BW/ day, Takahashi *et al.*, 2003)", whereas the ng and μg BPA dosages were used to "potentially capture the physiologically relevant range of human exposure." Following two weeks of acclimation, dams were mated with Avy/a males. The dams remained on the assigned diets throughout pregnancy and lactation. At postnatal day 22 (PND 22), a/a and Avy/a offspring were weighed and their tails tipped. In addition, a single observer visually classified Avy/a offspring coat color phenotype into one of five categories based on proportion of brown fur: yellow (<5% brown), slightly mottled (between 5 and 40% brown), mottled (~50% brown), heavily mottled (between 60 and 95% brown), and pseudoagouti (>95% brown). Genomic DNA was isolated from the a/a and Avy/a tail tips and global methylation was quantified using the Luminometric Methylation Assay (LUMA). In addition, the DNA methylation of four CpG sites of the A^{vy} and *Cabp*^{IAP} alleles was quantified. Finally, the levels of free and total BPA (*i.e.*, before and after deconjugation with β-glucuronidase) were quantified in the liver of d22 Avy/a mouse liver (n = 8– 11 per exposure group) and fetal human liver (n = 51, gestational age 74-120 days) by LC-ESI-MS/MS. The influence of gestational BPA exposure on litter size, survival, wean weight, genotypic ratio, and sex ratio was evaluated using ANOVA with Bonferroni post-hoc analysis. The distribution of the five coat color phenotypes between each exposure group was analyzed using a Chi-square goodness-of-fit test, with the control coat color distribution representing the expected distribution. All comparisons resulted in cell counts with no more than 20% of cells containing fewer than five observations. The influence of sex, genotype, and coat color on mean CpG

methylation was measured using ANOVA with Bonferroni corrections. Average CpG methylation within an amplicon, site-specific CpG methylation, and global methylation among the three BPA exposed groups and the control group were evaluated by two-sample hypothesis analysis of means and ANOVA with Bonferroni correction as post-hoc analyses. Normality of percent methylation was evaluated using histograms and Q-Q plots. Two samples of the 50 mg/kg exposure group were identified as outliers for A^{vy} allele methylation analysis by a studentized residual greater than 2.0. Statistical significance was defined as P-value < 0.05.

As reported in the study, gestational body weight was not affected by BPA exposure, while the low dose BPA (50 ng BPA/kg diet) significantly decreased the mean wean weight by ~7% versus control. All three doses of BPA induced significant shifts in the coat color distribution of A^{vy}/a offspring: 50 mg BPA/kg shifted towards yellow, 50 µg BPA/kg towards brown (pseudoagouti), and 50 ng BPA/kg towards slightly/heavily mottled. Global DNA methylation was significantly higher in all BPA doses tested compared to the control, although there were no statistical differences between the three BPA exposed groups. The average methylation of the A^{vy} allele CpG sites 6-9 was lower in the 50 mg BPA/kg exposure group than control; when analyzed individually, CpG sites 6, 7, and 8 were less methylated than control. These endpoints were not affected by the two lower doses of BPA tested. The methylation status of the $Capb^{IAP}$ allele CpG sites 6-9 was not affected by 50 mg BPA/kg, but was increased by the 50 µg BPA/kg dose versus control (combined sites 6-9 and individual sites 6, 8, and 9). The 50 ng BPA/kg dose did not affect the average methylation of the $Capb^{IAP}$ CpG sites, but sites 8 and 9 were significantly hypermethylated compared with controls. Measured levels of total BPA ranged between 9.46 and 870 ng/g liver (mean free BPA = 164 ng/g; mean conjugated BPA = 278 ng/g) in 50 mg/kg rats; < LOQ and 11.3 ng/g liver (mean free BPA = 1.8 ng/g; mean conjugated BPA = 0.3 ng/g) in 50 ng/kg rats; < LOQ and 13 ng/g liver (mean free BPA = 1.8 ng/g; mean conjugated BPA = 1.0 ng/g) in 50 ng/kg rats; and < LOQ and 96.8 ng/g liver (mean free BPA = 7.6 ng/g; mean conjugated BPA = 3.2 ng/g) in human fetus.

Strengths of the study include the use of a low phytoestrogen diet and polycarbonate-free cages, the partial reproducibility of previously reported effects of the 50 mg BPA/kg diet dose (shift of color coat towards yellow, hypomethylation of A^{vy} allele; Dolinoy *et al.*, 2007), and assessment of both free and total BPA levels in the liver of study animals and human fetuses. Three BPA doses were used, however, they were spaced 1000x apart. Other weaknesses of the study include the lack of accountability for litter effect in the statistical analysis, the lack of determination of the BPA dose per unit of body weight, and the lack of liver histopathology data. In addition, the high ratio of free:conjugated levels of BPA in liver samples is indicative of post-sampling contamination and/or deconjugation of BPA while processing the liver samples, thus compromising the interpretation of these data. **The study has no utility for HI or RA.**

<u>Hepatic DNA methylation modifications in early development of rats resulting from perinatal BPA exposure contribute to insulin resistance in adulthood (Ma *et al.*, 2013)</u>
The goal of this study was to determine the relationship between perinatal BPA exposure and hepatic DNA methylation status in rats exposed to BPA perinatally. Pregnant Wistar rats (Hubei Research Center of Laboratory animals, Hubei, China) were housed in “special pathogen-free

conditions" (SPF) in an environmentally-controlled room under a 12-h light/dark cycle with feed [feed not specified] and water ad libitum. On GD 0, pregnant rats were assigned to two groups: gavage with 50 µg/kg bw/day of BPA in corn oil or corn oil alone from GD 0-PND 21. At PND 21, randomly selected male pups from each group were weighed and decapitated, and livers were snap-frozen. After weaning, pups were provided standard diets without addition of BPA and 'treated as described above', which may indicate that they were gavaged with BPA or vehicle as were their dams, until the pups were 21 weeks-old. The following parameters were determined for offspring at PND 21 and at 21 weeks of age: body weight, serum glucose and insulin, homeostasis model assessment (HOMA)-insulin resistance, and insulin sensitivity index. The details of how these parameters were measured were not provided in the study report; however, Fig 1 in the text indicates that 6 pups/group were assessed for each endpoint. Histopathology was performed on liver sections stained with periodic acid Schiff and hepatic glycogen content and global DNA methylation were measured in 6 pups/group. Additional endpoints included: bisulphite sequencing of the *Gck* promoter from liver tissue and mRNA and protein quantification from liver samples using quantitative real-time PCR and Western blotting, respectively. Statistical analyses were performed using one-way ANOVA and Student's t-test at a significance level of $P < 0.05$. Regarding accounting for the effect of liver, the authors stated "To control for litter effects, offspring from different litters were chosen for experiments described below in the BPA-treated and control groups. Three analyses per pup were carried out for each experimental condition."

BPA did not induce clinical signs of toxicity in the dams nor alterations in feed consumption during gestation, gestation length, malformation incidence, litter size or sex ratio, or postnatal survival. At PND 21, the only *in vivo* parameter significantly different from control was serum insulin, which was increased in pups from BPA-treated dams. At week 21, the following significant differences with respect to control were noted in BPA-exposed pups: increased body weight, increased serum insulin and HOMA-insulin resistance, and decreased insulin sensitivity index. Hepatic glycogen content was significantly decreased at 21 weeks of age, and global DNA methylation in hepatic tissue was significantly decreased at both time points.

The strengths of the study were the dosing (direct via the dam), the numbers of pups/endpoint for the *in vivo* measurements, and the *in vivo* endpoints measured (insulin tolerance, serum glucose and insulin, hepatic histology). The basis for randomization of the dams was unstated, and only a single dose of BPA was used in the study. The statistical analyses did not account for multiple measurements. It is unclear from the description of the study methods whether more than one pup/sex/litter was used for each endpoint, and whether the litter of origin or the use of different litters per endpoint were properly accounted in the statistical analyses. The study did not appear to use positive controls for the *in vivo* glucose and insulin tolerance tests, and the study report did not describe how the HOMA-insulin resistance tests of assessment of serum insulin and glucose were conducted; in particular, the study report did not specify whether serum insulin and glucose were assessed under fasting conditions. Moreover, it is unclear from the study report as to whether pregnant dams and postweaning pups were individually-housed. In addition, the description of the housing conditions did not mention whether the rats were housed under BPA-free conditions, the phytoestrogen content of the diet, or whether environmental BPA

contamination was taken into account in the study. A single dose of BPA was tested. **Due to the limitations noted above and lack of accounting for litter of origin in the statistical analyses of data, this study has no utility for HI and no utility for RA.**

Bisphenol A exposure increases liver fat in juvenile fructose-fed Fischer 344 rats (Rönn *et al.*, 2013)

The goal of this study was to test if exposure of juvenile rats to BPA in combination with 5% fructose affects fat mass or liver fat content. The secondary aim was to investigate whether the obesity parameters and the liver were affected by fructose feeding alone, using water-fed rats as a control group. Female F344 rats, 3 weeks of age, were purchased from Charles River International (Salzfeld, Germany) and housed 3 rats/cage at Uppsala University Hospital animal facility in a temperature-controlled and humidity-controlled room with a 12-h light/dark cycle. Polysulfone IV cages (Eurostandard IV), glass water bottles, and a "low-phytoestrogen" (100-200 μg/g diet) standard pellet RM1 diet (ad libitum, from NOVA-SCB, Sollentuna, Sweden) were used. Animals were acclimatized for two weeks before being assigned to five dose groups (12 rats/group): water containing 1% ethanol (water control), 5% fructose solution containing 1% ethanol (fructose control), 5% fructose/1% ethanol plus 0.025 mg BPA/L, 5% fructose/1% ethanol plus 0.25 mg BPA/L, and 5% fructose/1% ethanol plus 2.5 mg BPA/L. Bisphenol A (BPA, ≥99% purity) and fructose (≥99% purity) were purchased from Sigma–Aldrich (St. Louis, MO). 100x BPA stock solutions were prepared in 100% ethanol [incorrectly reported as 1% in manuscript] and diluted 1:100 in 5% fructose solution. Dosing solutions were certified by HPLC-MS/MS; the BPA concentrations were: water control – 0.00020 mg/L; fructose control – 0.00011 mg/L; BPA 0.025 mg/L – 0.029 mg/L; BPA 0.25 mg/L – 0.25 mg/L, and BPA 2.5 mg/L – 2.7 mg/L. Before the treatment start, animals were given water or 5% fructose solution, both containing 1% ethanol, for ten weeks. To avoid unnecessary stress, no cage-mates were separated, but the cages were allocated to the different groups to achieve equality in weights in all groups. Food and liquid consumption in each cage and individual body weights were determined once a week. Groups given fructose solution drank more than the water control rats and also raised their liquid consumption during the experiment but ate less. The water control group had an almost constant food and liquid intake. Magnetic resonance imaging was conducted in anesthetized rats to quantify the volume of total, visceral, and subcutaneous adipose tissue, lean tissue, and liver fat content. At termination, plasma triglycerides and cholesterol were quantified using an Architect C 8000 analyzer, plasma apolipoprotein AI (apo A-I) was quantified by Western blot, liver and left perirenal fat pad were weighed, and the liver somatic index (LSI, liver weight × 100/body weight) was calculated. Differences between the fructose control group and the three fructose plus BPA exposed groups were evaluated by factorial ANOVA. When the three BPA groups were analyzed versus the fructose control group one by one, a Bonferroni adjustment for three tests was used and a P-value < 0.0167 considered significant (P-value = 0.05/3 = 0.0167). In the secondary analysis, when the water control group was compared with the fructose control group, a P-value < 0.05 was considered as significant.

Body weight, body weight gain, liver weight, fat pad weight, and adipose tissue volumes (total, visceral, and subcutaneous) were not affected by fructose + BPA versus fructose control. Liver fat content was significantly higher in the fructose + 0.25 and 2.5 mg/L groups, but not fructose +

0.025 mg/L. The R2* analysis of the liver showed a significant increase in all three fructose + BPA doses compared with the fructose control; however, this increase can be due to iron infiltration in the liver and all doses of fructose + BPA induced the same fold-change versus fructose control. The LSI was significantly increased in the fructose + 0.025 and 0.25 mg BPA/kg bw/day dose group, but not in the fructose + 2.5 mg BPA/kg bw/day, although the fructose + BPA effects were not significant following Bonferroni adjustment. Plasma apoA-I was increased by fructose + 0.25 and 2.5 mg/L, but not fructose + 0.025 mg/L. Fructose + BPA had no effect on plasma cholesterol and triglycerides, blood glucose, AST or ALP versus fructose control. The only endpoints significantly different between the water control and fructose control were the plasma triglycerides and LSI (both higher in the fructose control).

Strengths of this study include the use of polysulfone cages and glass water bottles, the use of three BPA doses (10-fold apart), the large sample size (n = 12 per dose group), the certification of the dosing solutions, the use of multiple technologies to assess similar endpoints, the assessment of multiple related endpoints, and the statistical correction for multiple comparisons. Weaknesses include the use of a diet with a relatively high phytoestrogen content (100-200 ppm), multiple housing of animals (3 per cage), the use of a single sex (female), the lack of histopathology data, the lack of BPA internal dosimetry data. Since there is no BPA only dose group, it is not possible to discern between BPA and/or fructose effects. **The study has no utility for HI or RA.**

Bisphenol A induces oxidative stress and decreases levels of insulin receptor substrate 2 and glucose transporter 8 in rat testis (D'Cruz *et al.*, 2012a)

The goal of this study was to assess the effects of BPA on glucose metabolism in testes and whether oxidative stress played a role in BPA's effects in this tissue. Male Wistar rats (Sri Ragavendra Enterprises, Bangalore, India; 90 days-old) were maintained in an environmentally-controlled animal care facility at 24 ± 2°C on a 12-h light/dark schedule, with standard lab chow [diet not specified] and tap water provided ad libitum. No further specifics were provided regarding housing conditions, number of animals per cage, or habituation period. Animals were divided (randomization procedure unstated) into the following treatment groups (n=6/group): 0, 0.005, 0.5, 50, or 500 µg/kg bw/day BPA in olive oil by daily gavage in a volume of 1 mL/kg bw for 45 days. A positive control group was gavaged with 50 µg/kg bw/day 17β-estradiol for the same period of time. Body weights were monitored 'regularly' during dosing. Fasting plasma glucose and insulin were assessed after overnight fasting just prior to sacrifice. Testes were removed, weighed, and homogenized. The following assays were conducted on the homogenate supernatants: testes glucose, hydrogen peroxide (H_2O_2) generation ex vivo, hexokinase and phosphofructose kinase activity ex vivo, and immunoblot analyses of IRS-2 and GLUT-8. Bouin's-fixed whole testes were also stained with immunofluorescent anti-GLUT-8 and analyzed microscopically. Data were analyzed via one-way ANOVA with post-hoc testing using Tukey's test. The significance level was set at $P < 0.05$.

Significant dose-related increases in fasting plasma glucose and insulin levels and decrease in testes glucose levels were noted in rats administered BPA at all doses as well as in the positive control, with the greatest effects noted in the 17β-estradiol group. These effects on testes glucose

were associated with concomitant significant increases in H_2O_2-generation, decreased activities of hexokinase and phosphofructokinase, and decreased levels of IRS-2 and GLUT-8 proteins in testicular lysates. The dose-response curves for these ex vivo effects mirrored the curves for the *in vivo* effects.

The strengths of the study were the numbers of rats/endpoint, the *in vivo* endpoints measured (fasting serum glucose and insulin), the large number of doses used, and the use of a positive control. The statistical analyses appear to be appropriate, although the study authors did not appear to adjust for multiple comparisons. The method for randomization of the rats was unstated. Several details of the housing arrangements during the study were missing from the study report, including whether the animals were individually-housed during the study and the type of cage used to house the animals. In addition, the description of the housing conditions did not mention whether the rats were housed under BPA-free conditions, the phytoestrogen content of the diet, or whether environmental BPA contamination was taken into account in the study. **The numbers of animals used during the study and the *in vivo* endpoints measured give this study utility for HI; the numbers of animals used make this study of no utility for RA**. However, the apparent lack of accounting for environmental contamination by BPA or dietary phytoestrogens may introduce complications in the interpretation of the study results, particularly at the lower doses used in the study.

Bisphenol A impairs insulin signaling and glucose homeostasis and decreases steroidogenesis in rat testis: an *in vivo* and in silico study (D'Cruz *et al.*, 2012b)

The goal of this study was to assess the effects of BPA on insulin-signaling molecules and steroidogenesis in the testes. Male Wistar rats (Sri Ragavendra Enterprises, Bangalore, India; 90 days-old) were maintained in an environmentally-controlled animal care facility at 24 ± 2°C on a 12-h light/dark schedule in polypropylene cages with autoclaved paddy husk with standard lab chow [diet not specified] and tap water provided ad libitum. The study report did not state whether there was a habituation period. Animals were divided (randomization procedure unstated) into the following treatment groups (n=6/group): 0, 0.005, 0.5, 50, or 500 µg/kg bw/day BPA in olive oil by daily gavage in a volume of 1 mL/kg bw for 45 days. A positive control group was gavaged with 50 µg/kg bw/day 17β-estradiol for the same period of time. Body weights were monitored 'regularly' during dosing. Testes were removed at sacrifice, weighed, and homogenized. The following assays were conducted on the homogenate supernatants: antioxidant enzyme levels (SOD, catalase) and lipid peroxidation; levels of insulin receptor, IRS-1, PI3-kinase, StAR, and GLUT-2 proteins by immunoblot; and activities of steroidogenic enzymes (3β-HSD, 17β-HSD). Bouin's-fixed whole testes were also stained with immunofluorescent anti-GLUT-2 and analyzed microscopically; spermatogenesis was also assessed in H&E-stained sections. Plasma testosterone and in silico analyses of BPA-binding to GLUT-2 and GLUT-8 were also performed. Data were analyzed via one-way ANOVA with post-hoc testing using Tukey's test. The significance level was set at $P < 0.05$. The data presented in the paper state that each data point presented in graphical form represents the average values from 4 independent experiments normalized against the control (testes protein levels and steroidogenic activity levels). The tabulated data for antioxidant enzyme activity and lipid peroxidation in testes homogenates appear to be from a single experiment.

Dose-dependent, significant changes in testes were noted in the following parameters in all BPA-treated groups and the positive control: decreased antioxidant enzyme activity; increased lipid peroxidation levels; decreased insulin, insulin receptor, IRS-1, and PI3-kinase; and decreased steroidogenic enzyme activity. Testicular GLUT-2 levels were significantly decreased in doses equal or above 0.5 µg/kg bw/day BPA and positive control groups, and plasma testosterone was significantly and dose-dependently decreased in all BPA-treated groups as well as the positive control. Germ cell loss was noted in the testes of rats treated with 500 µg/kg bw/day BPA or the positive control. In silico modeling demonstrated that BPA and 17β-estradiol could both bind to GLUT-2 and -8.

The strengths of the study were the numbers of rats/endpoint, the large number of doses used, and the use of a positive control. The statistical analyses appear to be appropriate, with the exception of lack of incorporation of 'experiment' as a variable in their ANOVA or adjustment for multiple comparisons. The basis for randomization of the rats was unstated, and it is unclear whether there was a habituation period. Several details of the housing arrangements during the study were missing from the study report, including whether the animals were individually-housed during the study and the type of feed or water bottle used. The endpoints measured during the study are interesting from a mechanistic point of view but do not correspond to validated endpoints for addressing the issues of effects on insulin resistance or body weight homeostasis.

Due to the lack of validated endpoints measuring variables related to insulin resistance or obesity, t**his study has no utility for HI and no utility for RA.**

<u>Lifelong Exposure to Bisphenol A Alters Cardiac Structure/Function, Protein Expression, and DNA Methylation in Adult Mice (Patel *et al.*, 2012)</u>

The goal of this study was to determine if exposure to BPA impacts cardiac structure/function, calcium homeostasis, protein expression, DNA methylation of cardiac genes, body weight, body mass index, body surface area, and adiposity in C57BL/6n mice. C57BL/6n mice (Charles River, St Constant, Que) were fed Harlan Teklad Global 2018 diet and housed in standard cages [cage material not specified] with 1/4″corn cob bedding in a 12-h dark/light schedule throughout life. Pregnant dams (n = 8/group) were given drinking water without (VEH) or with BPA calculated to deliver about 0.5 or 5.0 µg/kg bw/day BPA from GD11.5 until euthanasia. The calculated dose assumed a constant drinking water volume of 5 ml/day; water intake by the dams was not measured. A separate group was treated with BPA calculated to deliver 200 µg/kg bw/day from GD11.5 until weaning as a positive control for effects such as longer anogenital distance (AGD). Once weaned, the progeny of BPA 200 dams were treated with VEH water. AGD, body weight (BW), and body length (BL) measurements were collected at weaning and monthly until euthanasia at 4 months. BL was from the tip of the nose to the base of the tail. At euthanasia, AGD, BW, BL, tibia length (TL), and the wet weights of the heart (HW) and adipose deposits surrounding the kidneys, combined testes, combined ovary, and mesentery were collected. In addition, the weights of the uterus and combined ovary in females and the combined weight of the testes, all prostate lobes, and seminal vesicle including contents in males were collected. BMI and body surface area (BSA) were calculated according to standard formulas. Echocardiography

was performed using anesthetized animals around 16 weeks of age using a VEVO 770 ultrasonograph system, and at 15 weeks of age, tail cuff blood pressure measurements on conscious mice were performed for two days, after training on the apparatus for 3 days. Normality using the Kolmogorov-Smirnov test and equal variance about the group mean were passed prior to ANOVA analyses. Significance for all parameters at a level of $P < 0.05$ was evaluated using two-way ANOVA with Student-Newman-Keuls *post hoc* test. Significance was also assessed by ANCOVA using litter size as a covariate. All groups were compared at weaning. In adult mice, BPA 200 groups were only compared with VEH, not the BPA 0.5 or BPA 5.0 groups. The methods section does not state whether the litter was the statistical unit used in these comparisons. Also, the article did not state how many mice/group were used for the EKG and BP measurements, nor did the article state whether BW, BL, BSA, and BMI were analyzed using repeated-measures ANOVA.

The study reported the following results: 1) There was no impact of treatment on litter size at weaning; however, at weaning, BPA 200 male and female pups had reduced BW compared with pups in all other groups. BPA 5.0 males had greater BW, BMI, and BSA at 3 months than other males. BPA 5.0 females had greater BW and BSA versus BPA 0.5 beginning at 3 months and greater BMI versus VEH and BPA 0.5 at 4 months. BPA 200 males and females were smaller than VEH at 1 month. BPA 200 males were similar to VEH at 2 months. BPA 200 females remained smaller than VEH mice at 3 months; 2) BPA 200 pups had increased AGD, AGD/BW, and AGD/BL than VEH pups, increased AGD and AGD/BL compared with BPA 0.5 pups, and increased AGD/BW compared with BPA 5.0 pups; 3) All BPA-treated male mice had increased relative wall thickness (RWT) and no increase in left ventricle mass (LVM) compared with VEH mice. No structural differences were found in female mice; 4) In males, BPA 5.0 had reduced HW and HW indexed to BW and BSA versus other males and reduced HW/TL versus BPA 0.5 males. BPA 0.5 and BPA 5.0 females were similar to VEH. BPA 200 females had reduced HW versus VEH females. HW/TL and HW/BSA were reduced in BPA 0.5 and VEH females compared with similarly treated males, whereas BPA 5.0 females and males were similar; 5) No differences were found in blood pressure values with BPA exposure in males, but BPA 5.0 males had reduced kidney weight versus all other males. In females, systolic blood pressure was increased in BPA 5.0 versus VEH, but the increase did not reach the threshold of clinical significance (140 mm Hg). Diastolic and mean arterial pressures were significantly higher in all BPA-treated females compared with VEH females; and 6) In males, perirenal fat, total fat, total fat/BW, and total fat/BSA were greater in all BPA versus VEH mice. In addition, BPA 5.0 males had increased mesenteric adiposity. In females, BPA 5.0 females had greater gonadal and mesenteric fat, total fat, total fat/BW, and total fat/BSA than VEH females. Total fat/BW, which was unaffected by sex in VEH mice, was increased in all BPA males compared with BPA females. BPA 5.0 males and females had the greatest fat in all deposits.

Effects on the heart were consistent with concentric remodeling in all BPA-treated males, but the implications for HI and RA of this finding are unclear. Further diastolic blood pressure was increased in all BPA-dosed females. Strengths of the study include adequate sample size, route of exposure, and duration. Weaknesses include lack of control for litter effect, the use of a feed with relatively high (150-200 ppm) phytoestrogen content, and the use of corn cob bedding. **The**

study has no utility for HI or RA.

Bisphenol A attenuates phenylbiguanide-induced cardio-respiratory reflexes in anaesthetized rats (Pant *et al.*, 2012)

The study was undertaken to examine the effects of chronic and acute exposure to BPA on cardio-respiratory reflexes elicited by the vasoactive drug phenylbiguanide (PBG). Acute and chronic experiments were performed on adult female rats. In chronic experiments, the animals (n=6/group) ingested pellets containing BPA (2 μg/kg body weight) dissolved in vegetable oil or control pellets for 30 days. Adult female Charles Foster rats (150-200 g) were housed in a temperature- and humidity-controlled room on a 12-h light/dark cycle, with feed and water provided ad libitum. Details of housing and feed composition were not provided in the study report, so it is unclear if the study accounted for BPA-contamination in feed or housing apparatus. The animals were anaesthetized and prepared for recording blood pressure, ECG, and respiratory excursions. PBG was injected through jugular vein to evoke reflexes in these animals. Acute studies involved injection of BPA in ethanol at doses > 5 mg/kg bw/day (35 mg/kg bw n=4-5/group) and, therefore, are not considered further in this review. Data were analyzed by two-way ANOVA (presumably the second variable was time) followed by Newman-Keul's post-hoc testing, with a significance level set at $P < 0.05$. The study did not appear to use repeated-measures ANOVA, which would be the more appropriate statistical test.

PBG administered alone produced bradycardia, hypotension and tachypnea. In BPA-treated group, the PBG-induced heart rate and respiratory frequency changes were attenuated significantly. No effects of BPA were noted on these parameters when administered in the absence of PBG.

The results of this study may be useful to elucidate the mechanism by which BPA may exert effects on the heart and vascular system, but they do not have direct relevance for the HI or RA of the compound, since the effects of BPA were not observed in the absence of PBG. Moreover, the lack of detail in the study report concerning housing conditions, precautions against BPA-contamination, the use of a single BPA dose, and statistical testing used decrease confidence in the overall quality of the study and validity of the reported results. **The study has no utility for HI or RA.**

Subcutaneous exposure

Neonatal xenoestrogen exposure alters growth hormone-dependent liver proteins and genes in adult female rats (Ramirez *et al.* 2012)

The goal of this study was to test the hypothesis that neonatal BPA may perturb the growth hormone (GH) axis and modify sexually dimorphic GH-dependent liver enzymes in female rats. Sprague-Dawley rats (200–250 g, source unknown) were maintained under a controlled 12-h light/dark cycle and temperature conditions, housed in steel cages, and given free access to commercial laboratory chow (feed not specified) and tap water in glass bottles. Pregnant females were housed individually; on the day of birth [postnatal day (PND) 1], litters were culled to eight pups. From PND 1 to PND 10, pups received a daily subcutaneous injection of 50 μl castor oil

("a phytoestrogen-free diluent for steroids") containing 0, 50, or 500 BPA μg. The highest BPA dose of the study (500 BPA μg/50 μl castor oil) was reported to be equivalent to 25–62.5 mg/kg bw, while the lowest dose (50 BPA μg/50 μl castor oil) was reported to be equivalent to 2.5–6.25 mg/kg bw. BPA was purchased from (Aldrich, Milwaukee, WI, USA) [no additional specifications provided]. To control for possible litter effects, each litter included one vehicle female, one 50 BPA μg/50 μl female, one 500 BPA μg/50 μl female, and one vehicle male. The four remaining pups in each litter were untreated males or females not used for the study. Rats were euthanized at 5 months of age. Serum prolactin, growth hormone (GH), and IGF-1 were quantified by radioimmunoassay, and pituitary GH and liver IGF-1 were quantified by an unspecified method. Major urinary proteins (MUPs) were measured in the urine supernatants collected at 5 months of age. The expression level of genes *Cyp2c12*, *Cyp2c11*, *Hnf6* (hepatic nuclear factor 6), *Adh1* (alcohol dehydrogenase), and *Prlr* (prolactin receptor) was quantified in liver by quantitative real-time RT-PCR. Data were subjected to analysis of variance followed by Newman–Keuls test or Tukey's honestly significant difference test for unequal N. Correlation between serum prolactin and liver *Prlr* mRNA was analyzed with the Spearman's correlation test. A P-value < 0.05 was considered significant.

Body weight, serum and liver IGF-1, and pituitary GH were not affected by BPA treatment when compared to the sex-matched (female) control. There was a sex difference in the levels of liver IGF-1 and pituitary GH in vehicle controls, however, BPA-treated females were not statistically different from the male controls for these endpoints. Both doses of BPA significantly decreased the hepatic expression of the female-predominant genes *Cyp2c12* and *Adh1* when compared to same-sex (female) control, and the levels of *Adh1* in BPA-treated females were not statistically different from the male controls. The high, but not low, dose of BPA significantly decreased the expression the female-predominant gene *Hfn6* when compared to same-sex (female) control. BPA treatment did not modulate the expression of the male-predominant liver genes *Cyp2c11* mRNA or MUPs. Female-predominant liver *Prlr* mRNA and serum prolactin were not affected by BPA treatment.

Strengths of this study include the use of steel cages and glass water bottles and the inclusion of a male vehicle control to confirm expected sex-differences and further characterize the effect of BPA in females. Weaknesses include the lack of methodological details (including diet used, method of dams allocation to dose groups, and housing of the animals after weaning), and the lack of adjustment of the dose volume per unit of body weight. Co-housing of littermates dosed with vehicle and BPA greatly impacts interpretation of the data, due to the high risk of BPA cross-contamination between animals allocated to different dose groups. **The study has no utility for HI or RA.**

<u>Perinatal bisphenol A exposure and adult glucose homeostasis: Identifying critical windows of exposure (Liu *et al.*, 2013)</u>

The goal of this study was to assess the critical windows of susceptibility of mice to the development of dysglycemia. C57BL6 mice (male and female; 8 weeks of age; Experimental Animal Center of Nanjing Medical University, Nanjing, China) were housed under SPF conditions in BPA-free polypropylene cages under controlled illumination (12-h light/dark

cycles), humidity (30-50%), and temperature (18-22°C) for a > one-week acclimation period; water in glass bottles and feed (alfalfa and soymeal-free; feed not specified) were provided ad libitum. Mice were mated at the end of the acclimation period, and the day that a vaginal plug was noted in a mated female was designated GD1. Pregnant mice were individually housed under the same conditions as above and were randomized to 8 treatment groups (n>10/group) of either 0 or 100 µg/kg bw/day BPA in 50 µl of corn oil by sc injection for each of the following exposure periods: 1) injections GD 1-6, 2) injections GD6-parturition (GD6-PND 0), 3) injections from lactation through weaning (PND 0-21), and 4) GD6-PND 21. The authors stated that their previous data [reference not provided] verified that the corn oil vehicle had no effect on glucose metabolism at the dose administered during the study. Pregnant dams were observed throughout pregnancy. Litters were culled to 6 pups at PND 0. Pups were weighed on PNDs 0 and 21 and then weekly until 8 months of age. The following assays were performed in F1 mice: intraperitoneal glucose and insulin tolerance tests (3 months, 6 months, 8 months of age; no positive control specified); ex vivo glucose-stimulated insulin secretion from pancreatic islets (n=20 islets/sample); pancreatic islet morphometric analysis using immunohistochemistry (n=5 animals/group, 3-month and 8-month timepoints); PCNA-stimulated islet cell proliferation ex vivo (n=5 animals/group, 3-month and 8-month timepoints); immunohistochemistry on pancreatic islets for apoptosis markers (n=5 animals/group, 3-month and 8-month timepoints); and Western blots on pancreatic islet tissues. Data were analyzed via unpaired Student's t-test and repeated-measures one-way ANOVA at a significance level of $P < 0.05$. Pregnancy success rates (number of mice bearing litters/group) were analyzed via chi-square test. The study authors did not state whether litter of origin was accounted for in each analysis or how many pups/group were used for each endpoint.

Decreased pregnancy success rates were noted in the GD 1-6 and GD 6-PND 0 groups with BPA treatment, with the latter group being significantly different from control. No effects of BPA on birth weights, sex, or litter size were noted across groups. Significant effects of BPA-treatment in offspring are tabulated below:

Effect	GD 1-6 males	GD 1-6 females	GD 6-PND 0 males	GD 6-PND 0 females	PND 0-21 males	PND 0-21 females	GD6-PND 21 males	GD6-PND 21 females
Body weight gain, 3-35 weeks of age	↑BW at 5-7 wks old, no difference after 9 wks old	No effects	↓BW gain	↓BW gain	↑BW gain	No effects	No effects	No effects

Glucose tolerance, 3 months	No effects	No effects	↑peak & total glucose & ↓insulin ogenic index	↑total glucose response & peak glucose & fasting insulin; ↓glucose clearance & insulinogen ic index	↑peak & total glucose; ↓insulino genic index	No effects	↑peak & total glucose; ↓insulinog enic index	No effects
Glucose tolerance, 6 months	No effects	↓insulin ogenic index	↑peak & total glucose, ↓insulin ogenic index	↑total glucose response &↓glucose clearance. ↑Fasting glucose	↑insulino genic index	No effects	↑insulinog enic index	No effects
Glucose tolerance, 8 months	No effects	No effects	↑peak & total glucose & fasting insulin	No effects	↑fasting insulin	No effects	↑fasting insulin	No effects
Insulin sensitivit y	No effects	No effects	↓respons e to insulin, all time points	↓response to insulin, 3- and 6-m.o.	↓respons e to insulin, 3-m.o.	No effects	↓response to insulin, 3-m.o.	No effects
Islet insulin secretion	No effects	↑secreti on, 3 m.o.	↓secretio n, 3 m.o.	↓secretion, 3 m.o.	↓secretio n, 3 m.o.	No effects	↓secretion , 3 m.o.	No effects
Islet morphom etry	No effects	No effects	↑β-cell mass, 3-m.o. No effects, 8 m.o.	↓islet proliferatio n & apoptotic islets cells at 3 m.o. No effects, 8 m.o.	↑β-cell mass, ↓islet proliferat ion & apoptotic islets cells at 3 m.o. No effects, 8 m.o.	No effects	↑β-cell mass, ↓islet proliferati on & apoptotic islets cells at 3 m.o. No effects, 8 m.o.	↑β-cell mass and islet cell proliferation , ↓islet cell apoptosis, 3 m.o. No effects, 8 m.o.

The strengths of the study were the dosing (direct via the dam), the numbers of pups/endpoint for the *in vivo* measurements, the testing of both sexes, and the *in vivo* endpoints measured (insulin and glucose tolerance, fasting glucose and insulin, pancreatic islet cell morphometry). The use of different exposure regimens and assessment of endpoints at several different time points are also strengths. Another strength is the use of BPA-free cages and water bottles, and the use of a phytoestrogen-free diet for both the P0 and the F1 generations. The basis for randomization of

the dams was unstated, and only a single dose of BPA was used in the study. The statistical analyses did not appear to account for either multiple measurements or litter of origin. It is unclear from the description of the study methods whether more than one pup/sex/litter was used for each endpoint. There was also no attempt to determine *in vivo* dosimetry, which would have clarified how the different exposure regimens affected the dose to which the pups were exposed. Further, the study did not appear to use positive controls for the *in vivo* glucose and insulin tolerance tests. A single dose of BPA was tested. Due to the lack of accounting for litter of origin in the statistical analyses of data from the pups, t**his study has no utility for HI and no utility for RA.**

Short-term treatment with bisphenol A leads to metabolic abnormalities in adult male mice (Batista *et al.*, 2012)

The goal of this study was to study the effects of low doses of BPA in insulin-sensitive peripheral tissues and whole body metabolism in adult mice. Swiss albino mice (3 months old, source unspecified) were individually-housed under 'standard housing conditions' (details of housing unspecified). BPA in tocopherol-stripped corn oil was injected sc twice a day for 8 days at a total daily dose of either 0 (vehicle control) or 100 µg/kg bw/day in a volume of 100 µL per injection. Blood glucose, non-esterified fatty acids (NEFA), and insulin were assessed in both fasted and fed animals (n = 7-9/group). Glucose and insulin tolerance tests were administered to fasted animals (n = 7-8/group). Mice (n = 6/group) were also assessed for whole-body energy homeostasis (respiratory exchange ratio, food intake, ambulatory activity, body temperature) after a 4-day habituation period over a 48-hour period, with data collected over the last 24 hours (treatment day 8). Pancreatic islets (n = 4 islets/group) were isolated, and ex vivo glucose production was measured in response to two different levels of glucose. Western blotting was used to analyze levels of the following proteins in skeletal muscle and liver (n = 4-6/group) after immunoprecipitation using a polyclonal antibody to IR-β with or without *in vivo* stimulation of fasted mice via single i.p. injection of insulin 5 minutes prior to sacrifice: IRS-1, Akt, insulin receptor, p-Akt (Thr308) and (Ser473), and p-ERK1/2. The study report states that data were analyzed via Student's t-test, one-way ANOVA, or two-way ANOVA 'as appropriate' at a significance level of $P < 0.05$.

Significantly increased plasma insulin and decreased plasma glucose (fed-state only) were noted, with no significant effects on NEFA levels. Significantly increased plasma glucose levels at all time points were noted during the insulin tolerance test but not the glucose tolerance test. Significantly decreased feed intake, ambulatory activity, and body temperature were noted during the night-phase of the calorimetry monitoring; during the day-phase, only ambulatory activity was significantly decreased from control values. There was no significant difference in respiratory exchange ratios in either phase. The following significant effects were noted in protein levels in muscle: increased IRS-1; decreased immunoprecipitated IR with insulin but not in the absence of insulin; and decreased Thr308-phosphorylated Akt and p-ERK1/2 in the presence, but not the absence, of insulin. The following significant effects were noted in protein levels in liver: increased IRS-1; and decreased immunoprecipitated IR with insulin but not in the absence of insulin.

The strengths of the study were the numbers of mice/endpoint, the fact that the animals were singly-housed, and the *in vivo* endpoints measured (fasting and fed serum glucose and insulin, insulin and glucose tolerance, metabolic parameters). The statistical analyses appear to be appropriate, although the study authors did not appear to adjust for multiple comparisons. As the animals were exposed as adults, there was not a need to adjust for litter in the statistics. The basis for randomization of the mice was unstated. Several details of the housing arrangements during the study were missing from the study report, including the type of cage used to house the animals and the diet. In addition, the description of the housing conditions did not mention whether mice were housed under BPA-free conditions or whether environmental contamination was taken into account in the study. A single dose of BPA was tested. The number of animals used during the study and the *in vivo* endpoints measured but limitation cited above make this **study of limited utility for HI and no utility for RA.** However, the apparent lack of accounting for environmental contamination by BPA and the unspecified phytoestrogen content of the diet may introduce complications in the interpretation of the study results, particularly at the lower doses used in the study.

Developmental exposure to bisphenol A leads to cardiometabolic dysfunction in adult mouse offspring (Cagampang *et al.*, 2012)

The goal of this study was to determine if administration of BPA to pregnant mice between gestational days 11 and 19 leads to changes in growth trajectory and organ size, impairs glucose homeostasis, alters blood pressure, and vascular endothelial responses in adult offspring. MF-1 mice [source not specified] were maintained under a 12-h light/dark cycle and at constant temperature, with water and food (standard chow diet; RM1, SDS, UK) available ad libitum. No further details on housing were available. Virgin MF-1 mice were mated at 9 weeks of age, then plug-positive females were dosed with 100 µg/kg bw/day BPA in sesame oil subcutaneously from gestational day 11-19 (n=8). Control animals (n=6) received sesame oil, which should be considered for potential estrogenicity. Following birth, the litter size was adjusted to 6 animals (3 males and 3 females, if possible). Pups were weighed at birth and every 2 weeks thereafter. At 3 weeks of age, offspring were weaned and group-housed according to sex. Systolic blood pressure was measured in a subgroup (n=1-2/litter) at 12, 14, and 20 weeks of age by tail-cuff plethesmography. At 14 and 18 weeks of age, all offspring were subjected to locomotor (open field) activity test. At 20 weeks of age, offspring were fasted overnight (12 h) and killed the following morning by cervical dislocation. Trunk blood was collected and fasting glucose level was measured. The heart, kidneys, and fat depots [gonadal, retroperitoneal, interscapular brown adipose tissue (iBAT), inguinal, and peri-renal] were immediately dissected out and weighed. Individual tissue weights from each animal were compared with their body weight and calculated as a percentage of total body weight (%bw). The descending aorta (thoracic and abdominal) was dissected, snap-frozen in liquid nitrogen, and used for gene expression analysis. Vascular function in a subgroup of male offspring (n=3 per group, taken randomly from different litters) was assessed using the aortas collected from the 20-week-old offspring. Ex vivo vascular responsiveness and gene expression analysis were not evaluated in this review since these endpoints do not typically serve as the basis for RA decision making. Statistical analyses were performed using litter as the basis of comparison with one-way analysis of variance. The difference was considered statistically significant at $P<0.05$. The study did not appear to use

repeated measures ANOVA to analyze the BW and BP time series data.

The study reported the following results: In dams, there were no changes in behavior or differences in body weight gain between BPA-treated and control dams. In offspring: 1) newborn pups from BPA dams were lighter in weight versus pups from control dams, 2) Both BPA male and female offspring were heavier compared with control offspring from 6-20 weeks of age. 3) Individual weights of the various fat depots (gonadal, inguinal, iBAT, retroperitoneal, and peri-renal) were significantly higher in the BPA male and female offspring versus controls , 4) Blood glucose levels were significantly elevated in BPA versus control male and female offspring, 5) The weight of the heart was similar in the BPA and control groups for both sexes, while kidney weights were significantly lighter in BPA versus control male and female offspring, 6) With regard to locomotor activity, both BPA male and female offspring displayed significantly reduced distance traveled compared to corresponding controls and velocity was only significantly reduced in BPA females compared to control, 6) Mean systolic blood pressure was significantly elevated in BPA offspring at all time points, compared to controls.

The paper reports several findings that suggest that BPA had a biologically significant effect on offspring of female MF-1 mice treated with BPA. The strengths include an acceptable dose (≤ 5 mg/kg bw/day) and statistical analysis (the litter was the unit of comparison); however, repeated measures ANOVA for the BP and BW measurements was not conducted. Other weaknesses include a lack of a dose response (only one dose used), and the low sample size for blood pressure determination. **The results of the paper have limited utility for HI**, based on changes in body weight, weight of fat depots, and blood glucose levels.

Intraperitoneal exposure

Bisphenol A Impairs Mitochondrial Function in the Liver at Doses below the No Observed Adverse Effect Level (Moon *et al.*, 2012)

The goal of this study was to investigate the effects of doses of BPA below the NOAEL (5 mg BPA/kg bw/day) on the hepatic function, especially focusing on oxidative stress and mitochondrial function, both *in vivo* and *in vitro*. Specific pathogen-free C57BL/6 male mice, 3 weeks of age, were purchased from the Orient Co., Ltd. (Seongnam, Korea). Animals were housed in conventional plastic cages with free access to water and chow diet (diet not specified) at 23°C ± 2°C, 60% ± 10% humidity, and a 12-hr light/12-hr dark photoperiod. After 1 week acclimation, mice were divided into three dose groups, n=5 per dose group. Mice were injected intraperitoneally with normal saline, 0.05 mg BPA/kg body weight (bw)/day, or 1.2 mg BPA/kg bw/day for 5 days. BPA (purity > 99%) was purchased from Sigma (St. Louis, MO, USA) and was mixed with 30% ethanol and diluted with water to the appropriate concentrations for the experiments. Each experiment was repeated at least 3 times from 2009 to 2010. At the end of the study, mice were fasted for 8 hr, anesthetized, and sacrificed. To investigate the time-course of effect of BPA, a separate animal study was conducted in which mice (n = 5 per dose group) were injected intraperitoneally with 1.2 mg BPA/kg bw/day and sacrificed 1, 6, and 24 hr after injection. The experiment was repeated twice. For the *in vitro* study, HepG2 cells were treated with 0, 10, or 100 nM of BPA for 2, 6, 12, or 24 hr. Serum AST and ALT were determined by

Beckman Coulter AU480 automatic biochemistry analysis system, and IL-6 and TNF-α were measured by radioimmunoassay. Liver and pancreas sections were examined by transmission electron microscopy. Mitochondrial function assays included measurement of the mitochondrial oxygen consumption rate, cellular ATP production, and mitochondrial membrane potential (MMP). Mitochondrial complexes I - V, interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), catalase, and glutathione peroxidase 3 (GPx3) were quantified in liver samples by western blot. Oxidative stress was measured using the thiobarbituric acid reactive substance (TBARS) assay. Statistical analysis was performed by non-parametric analysis (Mann-Whitney and Kruskal-Wallis tests). Statistical significance was assumed at P-value < 0.05.

Liver weight and histopathology were not affected by BPA. Five injections of 1.2 mg BPA/kg bw/day, but not 0.05 mg BPA/kg bw/day, led to swollen liver mitochondria and decreased mitochondrial oxygen consumption state 3 rate and decreased the hepatic expression of mitochondrial complexes III and IV. Single injection of 1.2 mg BPA/kg bw/day significantly increased serum AST and ALP 24 hours after injection, however this change was not observed after 5 days of treatment. Hepatic MDA, a product of lipid peroxidation, was statistically increased 6 hours after a single dose of 1.2 mg BPA/kg bw/day, while GPx3, an antioxidant enzyme, decreased at 1 and 6 hours after injection. The increase in MDA and decrease in GPx3 were also observed after 5 days of treatment with 1.2 mg BPA/kg bw/day. A single injection of 1.2 mg BPA/kg bw/day led to an increase of both liver and serum IL-6 (at 1 and 6 hours after BPA injection), increase of serum TNF-α (at 6 hours after BPA injection), and decrease in oxygen consumption rate (at 6 hours after BPA injection). Ten and 100 nM BPA led to a dose-dependent increase on the abnormal mitochondrial architecture and decrease on oxygen consumption rates *in vitro*. Both 10 and 100 nM BPA further decreased the MMP at 6 and 12 hours after BPA treatment, and 100 nM BPA increased the MDA concentrations and DHE activities *in vitro*.

Strengths of the study include the assessment of multiple related endpoints and the different exposure scenarios (one and five daily doses of two BPA levels *in vivo*; three time points of two BPA levels *in vitro*). Weaknesses include the small sample size (n = 5 per dose group), the use of a single sex (male), the incomplete description of the methodology (including final % ethanol in the dosing solutions, diet, cage and water bottles material used, randomization of animals per dose group, and number of animals housed per cage), and the incomplete or inconsistent reporting of the data (often it is unclear whether a BPA effect was statistically significant or not, or the data was reported differently between results text and tables/figures, error bars are missing from several graphs, making the sample size and number of replicates unclear). **This study has no utility for HI and no utility for RA.**

Epidemiology Studies

Summary

Forty eight (48) published epidemiologic studies utilizing largely cross-sectional, or to a limited degree case-control or cohort, designs, tested putative associations between BPA exposure (urine or plasma) and various health outcomes or molecular endpoints in samples of human subjects

from around the world. Most studies did not account for extraneous contamination of BPA samples by the collecting device (Ye *et al.*, 2013). Several studies used blood or cord blood concentrations of BPA as the exposure variable; however, blood has been shown to be of limited reliability, since BPA has a short half-life in plasma and is therefore present at very low levels compared to urine (Calafat *et al.*, 2013). Furthermore, plasma BPA levels are unable to adequately discriminate between true signal and noise (background contamination). In almost all cases, measurements of exposure in the reviewed studies took place at a single point in time only, which provides a highly uncertain assessment of internal exposure.

Assessed outcomes were wide-ranging, and no two studies measured endpoints in an identical fashion. No associations (*i.e.*, negative results) were reported between BPA exposure and the following parameters: implantation failure in a prospective cohort of US women undergoing IVF; undescended testes (cryptorchidism) in a prospective cohort of French boys; endometriosis in a sample of US women; age of menarche in US adolescent girls; male idiopathic infertility or semen quality in a sample of adult Han-Chinese men; CYP19 gene expression levels in granulosa cells collected from a sample of US women undergoing IVF; congenital hypothyroidism in a sample of Korean infants; type 2 diabetes in a sample of Korean adults; cases of precocious puberty or metabolic changes in 84 measured hormones in a sample of Chinese girls; adverse coronary events in a sample of elderly Swedish adults; child or maternal health in a sample of Korean lactating mothers/newborns. In one study, although considerable amounts of BPA were shown to be eluted from various dialyzers, patients with chronic kidney disease who were undergoing hemodialysis with different BPA-eluting dialyzers did not show any notable change in the plasma BPA levels during a single course or over four weeks of treatment (Krieter 2013). Most studies reported some sort of weak or possible association/correlation between BPA exposure and measured endpoints, including increased heart rate, female fertility status, nuclear receptor gene expression in peripheral blood mononuclear cells, spontaneous abortion, fetal health/birth outcomes, thyroid function (both positive and negative associations reported), meningioma, oocyte number, BMI (both positive and negative associations depending on age at exposure), school-aged behavior, pre-diabetes, peripheral arterial disease, hypertension, type 2 diabetes, metabolic syndrome, or obesity measure in various samples.

Critical review of the studies indicated significant limitations in study design that made the claims of association questionable or unsupportable. In some cases, an association was reported for transformed, but not the original, data, which casts further doubt on the legitimacy of the conclusion. The most common limitations in study design included use of plastic sample containers of uncertain BPA leaching; small study sample size; use of a single exposure measure (*e.g.*, single spot urine); use of plasma concentration as measure of exposure; absence of control for possible confounders (*e.g.*, diet, BMI, age, race); lack of control for multiple statistical tests within study (favors false positives); inadequate description of timing of sample collection; lack of clinical significance of measured outcome (*e.g.*, decreased T4 without compensatory increase in TSH); use of highly variable outcome (gestational age); arbitrary/nonstandard definition of clinical outcome (*e.g.*, albuminuria); or inability to separate contribution of BPA from association with other measured compounds in a mixture.

Major limitations in study analysis/interpretation included selective reporting of positive results; absence of dose (concentration)-response relationships; inconsistent within-study or between-study (*e.g.*, diabetes, serum T4/T3/TSH) results; equating a reported association/trend for transformed data (*e.g.*, square root of percentage breast density) or parameter estimates with an association for the original study data; lack of generalizability; possibility of reverse causation (*e.g.*, increased levels of lipophilic BPA may be the effect, not cause, of adiposity/obesity). NHANES data were explored in several reviewed studies, but as pointed out by LaKind *et al.* (2012), the use of NHANES data has given contradictory results in the past and may not be appropriate for drawing conclusions about BPA and chronic complex diseases due to the cross-sectional structure of that dataset. Reported associations often disappeared with addition of covariates/possible confounders.

Our review indicated that no single study was able to make a definitive contribution to HI or RA. No study demonstrated, nor was able to demonstrate based on its design, either a causal or a temporal relationship between exposure and measured outcome, since exposures to BPA were uncontrolled. And statistical techniques (*e.g.*, correlation analysis, linear regression), which attempt to quantify *mathematical* relationships between BPA exposure and outcome, are unable to test causal, *scientific* hypotheses. Therefore, no single study was able to definitively identify a hazard or exposure level that could be applied to the current BPA RA. Moreover, it would be inappropriate to selectively choose an isolated result, either positive or negative, out of its context. While we have concluded that the studies evaluated as part of this literature review cycle do not meet the necessary standard for regulatory HI, nor can they provide a quantitative point of departure for RA, they are, nevertheless, meaningful contributions to the growing body of hypothesis generating, epidemiological literature for BPA. The data "explorations" undertaken and reported in these studies might form the basis for more robust, well-designed prospective studies examining any range of chemicals in the future.

Individual Study Reviews

Associations of bisphenol A exposure with heart rate variability and blood pressure (Bae *et al.*, 2012)

This cross-sectional study examined the relationship of BPA concentration in urine with heart rate variability and blood pressure in a sample of 521 non-institutionalized elderly Koreans (mean 71 years) who were participants in the Korean Elderly Environmental Panel Study from 2008 to 2010. Each of the 138 men and 383 women had data from between 1 and 5 physical examinations over those 3 years. BPA was measured in fasted spot urine samples collected in plastic conical tubes[3] on the day of examination using HPLC-MS/MS (no LOD information given)and then adjusted for creatinine level (mean 1.2 µg/g of creatinine). Heart rate variability (HRV) was measured based on ECG for ≥ 5 minutes as mean heart rate (MHR), standard deviation of normal-to-normal intervals (SDNN) and root mean square of successive differences (RMSSD); blood pressure was analyzed as systolic and diastolic blood pressure (SBP and DBP)

3 According to the manufacturer's website, the polypropylene/polystyrene conical tubes were not BPA-free (http://spllabware.en.ec21.com/Centrifuge_Tube--3300647_3302518.html)

and hypertension (HTN; SBP ≥ 140 mm Hg or DBP ≥ 90 mm Hg). Linear statistical models with compound symmetry variance-covariance matrices were used to examine relationships between log transformed BPA and heart rate variability, DBP and SBP. Prevalence odds for hypertension measures were compared across quartiles of BPA (0.37, 0.73 and 1.33 µg/g of creatinine). Regressions were adjusted for (1) age, sex, height, weight, date of examination, (2) previous plus mean fasting blood glucose, smoking status, current alcohol consumption and both were stratified by previous history of hypertension.

Higher BPA was associated with higher MHR, but not SDNN, overall and in women (73.5% of the sample) for both crude and adjusted analyses with a larger effect in those without a history of HTN (the authors commented that those with HTN might be on HTN medication). Higher BPA was associated with lower RMSSD overall but not in subanalyses. The authors noted that RMSSD has been reported to be inversely associated with BP. In unadjusted models, higher urinary BPA was associated with higher SBP and DBP overall and in women and with SBP in men, but in the final adjusted model, urinary BPA was associated only with DBP (P=0.04) in the overall study sample. When urinary BPA was analyzed as quartiles, prevalence odds ratios did not differ for the overall study population. For those without a history of hypertension, both overall and for women only, the odds for HTN and SBP, but not DBP, were significantly higher in the second and fourth quartiles, but not the third, when compared to the lowest quartile. Determination of ORs for DBP was limited by small sample sizes.

Limitations noted by the authors were an elderly population, residual confounding, no dietary information, small size, and lack of recognition of mechanisms. Of these, most would seem to make it less likely to find an association, with the exception of diet where the authors suggest possible positive confounding between BPA containing food and water containers and salty foods. In addition, because urine samples were collected in containers that were not BPA-free, the reported urinary BPA levels should be considered to be unreliable. Reported associations often became less significant or disappeared altogether with inclusion of additional model covariates and lack of dose response across quartiles also argues against the validity of reported associations. In summary, the authors analyzed a short-term measure of BPA exposure and found that log transformed urinary BPA levels were significantly associated in the final adjusted models with increased heart rate, but not blood pressure, in elderly women. Results could represent 'immediate', short-term associations of night-time BPA levels with heart rate and blood pressure measured the next day, but cannot assess longer term temporal relationships nor demonstrate causal relationships. The study provides some useful information on HI and exposure assessment that is compatible with results from previous studies. **This study has no utility for HI and no utility for RA.**

<u>Urinary Bisphenol A and Obesity in US Children (Bhandari *et al.*, 2012)</u>
The objective of this cross-sectional study was to evaluate the association between urinary bisphenol A (urinary BPA) and obesity in children aged 6-18 years from the National Health and Nutrition Examination Survey (NHANES, 2003-2008). A random one-third subset of NHANES participants was selected for participation. Measures of BPA concentration included BPA parent compound and conjugated metabolites. Urinary BPA was measured by using solid-phase

extraction coupled on-line to high-performance liquid chromatography and tandem mass spectrometry. While it is noted that rigorous methodology and quality control were employed in sample collection, specific details are not provided and laboratory manual procedures are not easily obtained. Recent publications report on the increased risk of BPA contamination from environmental exposures including containers used for sample collection, indoor air environment of collection and/or laboratory sites and analyst handling techniques (see review for Ye *et al.*, 2013).

Obesity was defined as a body mass index (BMI) level $\geq 95^{th}$ percentile for age and gender. Urinary BPA was categorized into quartiles (<1.5, 1.5-2.7, 2.8-5.4, > 5.4 ng/mL) and also analyzed as a continuous variable by log transformation due to skewed distribution. The odds ratio with 95% confidence interval of obesity for BPA was calculated using the lowest quartile as the referent using an: 1) age and sex adjusted model, and 2) a multivariable-adjusted model, additionally adjusting for race/ethnicity, parent/guardian education, urinary creatinine, serum cotinine, and moderate physical activity. Subgroup analysis was done by gender and race/ethnicity and tested for statistical interaction.

Among 2200 enrolled children, 48.5% were girls, 62.4% non-Hispanic whites, 55.4% of the parents/guardians had an education above high school, 36.9% reported moderate activity and 34.4% were overweight or obese. A positive association was seen between increasing levels of urinary BPA and BMI/obesity in both statistical models, and the models evaluating linear trend in this association were statistically significant. In the multivariate analysis, children in the highest quartile of BPA (>5.4 ng/mL) had 2.55 odds ratio (95% CI: 1.65, 3.95) for obesity compared to children in the lowest quartile of BPA (<1.5 ng/mL). Although the odds ratio for developing obesity was statistically significant in all quartiles of BPA, it did not monotonically increase with increasing quartiles of urinary BPA exposure.

Within subgroups, a statistically significant association between urinary BPA and obesity was only seen in boys but not in girls ($P_{interaction}$=0.07), and in non-Hispanic whites but not in nonwhites ($P_{interaction}$=0.05). Additional analyses to rule out biases due to higher order polynomial terms for age, sex differences in maturation, selection, and use of gender/race/ethnicity specific BPA quartiles did not have an effect on the results. A stratified age analysis showed that the BPA-obesity association was similar in prepubertal (6-11 years) and postpubertal (12-18 years) children.

The cross-sectional design of this study limits its utility for causality assessment. The strength of this study is its relatively large sample size of US children. However, limitations include the unknown effect of unmeasured confounding factors that may also contribute to development of obesity in children (*e.g.*, maternal gestational diabetes, birthweight, and pre-term delivery). In addition, BPA exposure was characterized and grouped into quartiles based on one urinary BPA level, and the dose-effect relationship is not well established. Similarly, confirmation of proper biomonitoring practices is necessary to validate this study's results. **These data support a plausible relationship between urinary BPA levels and obesity; however, they have limited utility for HI and no utility for RA. Well-designed prospective studies are needed to**

confirm the relationship of urinary BPA levels and obesity in children.

Bisphenol A and phthalates and endometriosis: the Endometriosis: Natural History, Diagnosis and Outcomes Study (Buck *et al.*, 2013)

The objective of this matched cohort design (operative and population cohort) study was to explore the relation between bisphenol A and 14 phthalate metabolites and endometriosis. An operative cohort of 495 women were selected (eligibility: currently menstruating, aged 18–44 years, not breastfeeding ≥6 months, no injectable hormonal treatment in 2 years, and no cancer history save or nonmelanoma skin cancer) after screening cases scheduled for laparoscopy or laparotomy at one of 14 participating clinical centers in the Salt Lake City, Utah and San Francisco, California geographic areas in 2007–2009. The population cohort comprised 131 women matched on age and residence (<50-mile radius using Utah Population Database or a telephone white pages directory for Utah and California sites) of the operative cohort and screened to ensure they were at risk for endometriosis and being diagnosed (*i.e.*, currently menstruating and residing in geographic catchment areas, respectively). Surgically visualized disease was used to define endometriosis in the operative cohort and pelvic magnetic resonance imaging (Siemens Avanto or Espree 1.5 Tesla scanner) visualized endometriosis for the population cohort. A matched exposure (to surgery) cohort design (The ENDO Study), was used to achieve authors' primary objective of estimating the scope and magnitude of endometriosis at the clinical and population level by diagnostic method and choice of comparison group (Buck *et al.*, 2011, Fertil Steril). The operative cohort (n=473) comprised 190 endometriosis confirmed and 283 without endometriosis cases. The population cohort (n=127) comprised 14 endometriosis confirmed and 113 without endometriosis cases. Total BPA concentrations quantified using high-performance liquid chromatography coupled with an API 2000 electrospray triple-quadrupole mass spectrometer. Ongoing quality assurance and control procedures included in each batch of 25 samples a method blank, a spiked blank, and a pair of matrix-spiked sample/duplicates. Detectable limit of BPA, determined from lowest point of calibration standard and sample volume of 0.5 mL was 0.1ng/mL.

Statistical significance was evaluated using the Student t test or Wilcoxon nonparametric test for continuous data. Logistic regression was used to estimate the odds ratio (OR) for an endometriosis diagnosis for BPA (log (x+1) transformed and standardized by their SDs) by cohort. Age (years), body mass index (BMI, Kg/m^2), and urinary creatinine (ng/mL) were defined as potential confounders.

The authors did not observe any significant correlations for BPA and endometriosis in the operative or population cohort in the unadjusted or adjusted (age, BMI and creatinine) models. In the population cohort, only a pattern of higher creatinine-adjusted geometric mean BPA concentrations for women with than without endometriosis was observed. BPA was not associated with endometriosis in multivariate logistic regression, except when parity was included in final phthalate adjusted model (AOR 1.97, [95% CI 1.04–3.72]). **This study has no utility for HI and no utility for RA** as the finding was only observed in the population cohort in which the sample size for endometriosis compared to the cases without endometriosis is small (n=14 vs. n=113). Also, endometriosis was diagnosed from pelvic MRIs and not surgically

visualized, thereby leading to uncertainty in the diagnosis. Authors also discuss the possibility of over adjustment bias resulting from parity adjustment, if endometriosis and parity share a common origin. The other phthalate metabolites and family history for fertility related disorders may also be confounding factors in the analysis. Also, the study needs a longitudinal (multiple urine sample collection over time) study approach to improve the strength of the findings. Buck *et al.* (2011) have also discussed the limitations in diagnosis of endometriosis in women not seeking clinical care.

Exposures to endocrine-disrupting chemicals and age of menarche in adolescent girls in NHANES (2003-2008) (Buttke *et al.*, 2012)

This cross-sectional study aimed to evaluate associations between exposures to previously identified endocrine-disrupting compounds (EDCs) and age of menarche, defined by the Centers for Disease Control and Prevention's National Health and Nutrition Examination Survey (NHANES) as age of first menstruation, taking into account potential confounders specified in the literature, including body mass index (BMI), family income-to-poverty ratio, race/ethnicity, maternal smoking during pregnancy, mother's age at individual's birth, and individual's birth weight.

The study used data from 2003-2008 NHANES 12-to-16-year-old participants who had completed physical examinations with biospecimens and reproductive health questionnaires, with "self"-reported responses provided through proxies of parents or guardians, including the age at menarche outcome. For 2003-2004, NHANES sampled urinary phenols and phthalates using two separate one-third subsets; for 2005-2008, NHANES sampled the urinary phenols and phthalates, as well as parabens, using a single one-third subset. Each subset consisted of a representative sampling of NHANES participants ≥6 years of age from each 2-year study cycle. Solid phase extraction (SPE) coupled on-line to high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/TMS) was used to quantify urinary phenols. SPE-HPLC and atmospheric pressure chemical ionization-HPLC-isotope dilution tandem mass spectrometry (APCI-MS/MS) were used to quantify phenol metabolites. Urine samples with creatinine levels >300 mg/dL or < 30 mg/dL were excluded because they were considered too concentrated or too diluted for accurate analysis (n=11). Non-missing values for urine concentrations below the limit of detection (LOD) were replaced with the value of the LOD divided by the square root of 2. After excluding outliers, the phenol, paraben, and phthalate analytical samples consisted of 440, 287, and 437 participants, respectively. Based on the creatinine-corrected natural log urine concentrations, 18 EDCs found above the LOD in at least 75% of the study participants were assessed as single compounds [benzophenone-3, triclosan, bisphenol A (BPA), 2,4-dichlorophenol (2,4-DCP), and 2-5-DCP] or as summed urinary analyte concentrations in a compound class (parabens, phthalates, and phenols), using molar weights with microgram per gram of creatinine for modeling. Family income-to-poverty ratio, mother's age at participant's birth, and maternal smoking during pregnancy were found not to statistically significantly predict menarche so were excluded from modeling. Cox proportional hazards analysis in SAS 9.2 estimated associations accounting for censored data among participants who had not reached menarche and adjusting for body mass index (BMI) and race/ethnicity.

Statistically significant inverse associations were observed, after adjusting for BMI and race/ethnicity between 2,5-dichlorophenol (2,5-DC) alone and age at menarche [hazard ratio (HR): 1.10; 95% confidence interval (CI): 1.01, 1.19], as well as between the summed phenols 2,5-DCP and 2,4 DCP and the outcome (HR: 1.09; 95% CI: 1.01, 1.19). Associations between the other exposures and age at menarche were not statistically significant. There could be a methodological problem with the adjustment in this study, since race/ethnicity could affect both BMI and age at menarche. BPA was reported as not significantly associated with age of menarche.

Misclassification of the outcome cannot be ruled out because age at menarche was based on self-report through the parent or guardian. Exposures were measured at one point in time (after menarche onset for all except 43 of the participants who had not yet reached menarche) without accounting for compound half-life or interaction between metabolic pathways; also, the time of measurement may or may not bear any relation to the critical time of exposure or represent individual variation based on environmental or genetic/metabolic differences. **Based on these limitations, this study has no utility for HI and no utility for RA.** However, the results suggesting a possibility of ethnic associations should be kept in mind to see if this trend is shown in other BPA studies.

<u>The influence of endocrine disruptors in a selected population of infertile women (Caserta *et al.*, 2013a)</u>
This case-control study examined the relationship between BPA serum concentrations and female fertility status, as part of "PREVIENI", a study funded by the Italian Environment Ministry to monitor the effects of Endocrine Disrupting Chemicals (EDC) on reproductive health. The case group consisted of 48 females aged 18-40 living in Rome and affected by infertility (couples with only a male infertility factor were excluded); the control group consisted of 13 females aged 18-40 living in Rome with a spontaneous pregnancy in the last year. Women who reported smoking, vegetarianism, malabsorption syndromes, or inflammatory or infectious diseases were excluded.

A 20-mL sample of venous blood was collected in glass vials from fasting participants. BPA was measured in serum samples, extracted using a liquid-liquid separation procedures, and measured using HPLC with electrospray ionization tandem mass spectrometry. The limit of detection (LOD) for BPA was 0.5 ng/mL. The authors compared BPA levels in cases and controls using a Chi Square test. The percentage of participants with detectable BPA concentrations was significantly higher in the cases (n=35/48 or 72.9%) than in the controls (3/13 or 23.1%), and this difference was statistically significant ($p<.01$). Among the cases, 6/8 or 75% of infertile women with endometriosis vs. 29/40 or 72.5% of infertile women without endometriosis had detectable BPA concentrations (no p value given).

Limitations of this study include the observational design, small sample size, and insufficient statistical power thereby limiting statistical analyses to the Chi Square test, which cannot adjust for potential confounders such as age. Increasing age is associated with female infertility and also potentially with increased exposure to substances such as BPA. The mean age of the control

group was notably lower (32.6 +/- 5.2 yrs) than the cases (36.0 +/- 4.7 yrs). In addition, the recruitment process for the controls was not well described, and there is potential for selection bias. Finally, there may be potential for residual confounding and reverse causation in this relationship, if unmeasured factors related to fertility status are also related to BPA metabolism. **This study has no utility for HI or RA.**

Correlation of Endocrine Disrupting Chemicals Serum Levels and White Blood Cells Gene Expression of Nuclear Receptors in a Population of Infertile Women (Caserta *et al.*, 2013b)
This cross-sectional study examined the hypothesis that BPA may influence nuclear receptors (NR) involved in hormone response / steroid biosynthesis possibly altering uterine events like implantation. This Italian study compared two groups of women both 18-44 years old. One group was composed of women diagnosed with primary infertility (n =111) at three IVF clinics. The control group was composed of naturally impregnated mothers who had ceased breastfeeding >6 months prior to entry as a control subject (n =44). Before infertile women were given hormone treatment, each participant provided a 20 mL blood sample. Serum and whole blood were separated and frozen. Samples were chemically analyzed HPLC and ESI tandem mass spectroscopy for the presence of bisphenol A (BPA), perfluorooctane sulphonate (PFOS), perflourooctanoic acid (PFOA), phthalate (DEHP), and monoethylhexyl (MEHP). Additionally, nuclear receptor gene expression levels were queried with RT-PCR using GAPDH as a reference gene. Levels of BPA, but not the other chemicals, were significantly more concentrated in infertile subjects than controls (OR 2.79, 1.25-6.19, $P<0.01$). The peripheral blood mononuclear cells of the infertile women also had several NRs that were expressed at a significantly higher rate than in the control group: ERα, ERβ, AR, and PXR. BPA concentration was positively correlated with ERα, ERβ, AR in both groups.

Study strengths include the coupling of genetic and environmental investigations in an attempt to discover the mechanism behind a purported connection between BPA and infertility. However, the study is hampered by the limited population size (n=155) and cross-sectional design. Although the authors sought to eliminate potential confounds in lifestyle across the two groups, inevitably there are differences between mothers and non-mothers that likely confound the results such as exercise, metabolism, sleep habits, and time spent outside of the home. Overall, this study is well designed with reasonable statistical methodology. The validity of serum measurements of BPA are controversial, since BPA metabolizes quickly (Calafat *et al.*, 2013). **For these reasons, this study has limited utility in HI and no utility in RA.**

Association of exposure to phenols and idiopathic male infertility (Chen *et al.*, 2013)
This case-control study investigated the relationship between several urinary phenol concentrations (including BPA) and idiopathic male infertility in samples of adult Han-Chinese volunteers (cases, n=877; controls, n=713) recruited from hospitals affiliated with Nanjing Medical University. Individual total and glucuronide/sulfate-conjugated urinary phenol (BPA, BP-3, PCP, TCS, 4-t-OP, 4-n-OP, 4-o-OP) concentrations, were extracted from urine samples (collected in *glass bottles* on the day of study) and measured using HPLC (BPA LOD 0.36 ng/ml). Volume, sperm concentration, and sperm number per ejaculate were analyzed from individual semen samples collected in metal-free glass containers also on the day of study.

Differences in certain sample population characteristics (*e.g.*, age, BMI, etc.) between cases and controls were tested using simple statistical tests. The relationship between urinary levels of BPA (or other phenols) classified as low (<LOD; 290 cases, 241 controls), median (below median of detectable values; 286 cases, 243 controls), and high (above median of detectable values; 301 cases, 229 controls) and idiopathic male infertility was explored using multivariable logistic regression. Models were adjusted to account for urinary creatinine levels and the potential covariates, BMI, age, smoking and drinking status. WHO reference values were utilized as cutoffs for semen quality.

Small statistically significant differences between cases and control were reported for average age (~1.3 years) and BMI (1/2 unit), but the clinical significance of the difference was not discussed and is not considered to affect study outcome. No differences between the groups were observed for smoking or drinking status. Detection of any phenol in urine ranged from 12.2-62.4% of samples; many were below the LOD. Urinary BPA concentrations in cases or controls were almost identical and ranged from ~0.5 (50^{th}%ile) to 9 ng/ml (95^{th}%ile) with a geometric mean of 0.6 ng/ml. Statistically significant associations (crude or adjusted ORs) were not observed between idiopathic male infertility and any urinary BPA exposure level. Associations were also not observed between urinary BPA levels and cases of male idiopathic infertility when cases were stratified into three subgroups based on WHO cutoffs for semen volume, sperm concentration, or total sperm per ejaculate. A statistical association was observed in this study only between certain individual alkylphenols (4-t-OP, 4-n-OP, 4-o-OP, or their sum) detected in urine and idiopathic male infertility but not with other phenols. Sensitivity analyses confirmed the results of the crude and adjusted mathematical models.

As pointed out by the authors, evidence for an association between male infertility and exposure to BPA was not found in this study. The authors also noted that their study results were consistent with negative results reported in a previous cross-sectional study (Mendiola *et al.*, 2010), which also failed to demonstrate an association between BPA exposure and decreased semen quality in a sample population in the US. **This study has no utility for HI and no utility for RA.**

<u>Parental phenols exposure and spontaneous abortion in Chinese population residing in the middle and lower reaches of the Yangtze River (Chen *et al.*, 2013)</u>

A case-control study was conducted to evaluate the association between parental exposure to various environmental phenols, as measured by parental levels of urinary phenolic compounds, and spontaneous abortion in a Chinese population living in the middle and lower reaches of the Yangtze River (volunteers from affiliated hospitals of Nanjing Medical University). The study included 70 case couples with medically unexplained spontaneous abortion, and 180 control couples without a history of spontaneous abortion and with at least one living child. Urine samples were collected from each subject in the morning and frozen for phenols analysis. Parental urinary phenols were measured by ultra-high performance liquid chromatography-tandem mass spectrometry and included bisphenol A (BPA), benzophenone-3 (BP-3), 2,3,4-trichlorophenol (2,3,4-TCP), pentachlorophenol (PCP), 4-n-octylphenol (4-n-OP), and 4-n-nonylphenol (4-n-NP). All samples were divided into two-level exposure groups, where samples

with concentration less than the limit of detection (LOD) were assigned to the low exposure group, and the remaining samples with detectable concentrations were assigned to the high exposure group.

The smoking history was significantly higher in the paternal case group compared to that of the paternal control group (63.4% versus 44.2%, respectively). Also, the drinking history was significantly higher in the maternal case compared to control group (15.7% versus 7.3%, respectively). The BMI was significantly lower in the maternal case compared to control group (21.5 versus 24.87 kg/m^2, respectively). Because of these baseline differences, the logistic regression model adjusted for paternal smoking, maternal alcohol use, and maternal BMI; it also adjusted for urinary creatinine as a continuous variable to account for urinary dilution. The results indicated statistically significant associations between high paternal urinary PCP levels (OR=2.09; 95% CI, 1.05-4.14), maternal urinary 4-n-OP levels (OR=2.21; 95% CI, 1.02-4.80), maternal urinary alkylphenol(s) levels (defined as exposure to at least one of the two alkylphenols, such as 4-n-OP and 4-n-NP) (OR=2.81; 95% CI,1.39-5.65), and increased odds of spontaneous abortion. Neither high paternal nor maternal levels of BPA were observed to be statistically significantly associated with increased odds of spontaneous abortion (OR 1.28 [95% CI, 0.7-2.35] and 1.38 [95% CI, 0.69-2.75], respectively).

This study has no utility for HI and no utility for RA. This is a small sample size study, and the case-control study design limits the ability to make a causal conclusion based on its results. Also, the study looked at only one urinary BPA sample, defining low and high exposure based on LOD, and it is unclear when the urinary samples were obtained in relation to the time of spontaneous abortion. The study was conducted in a population from a limited area of China, and study subjects were obtained from hospitalized patients because urine samples were required. Thus, it is unlikely that the reported findings are generalizable.

Biomonitoring of bisphenol A concentrations in maternal and umbilical cord blood in regard to birth outcomes and adipokine expression: a birth cohort study in Taiwan (Chou *et al.*, 2011)
This study was conducted to assess BPA concentration in pregnant women and umbilical cord blood, and to investigate whether maternal BPA exposure affected fetal outcomes including lower birth weight (LBW), smaller size for gestational age (SGA), and high leptin (HLP) and low adiponectin (LAD) secretion. The study recruited 157 healthy pregnant women between January 2006 and August 2007 at an obstetrics and gynecology clinic in Hsinchu County, Taiwan. Of all women consented and completed questionnaire, 97 mother-newborn pairs enrolled in the study and 37 dropped out before delivery. Maternal blood corresponding to umbilical cord blood samples were respectively collected in glass heparin tubes at full-term delivery.

The BPA level was determined by a reverse-phase high performance liquid chromatography (HPLC). The BPA concentrations in plasma were determined using HPLC chromatography connected to a UV detector. A multivariable logistic regression was used to model the odds of adverse outcome variables (LBW, SGA, HLP and LAD). The analysis accounted for important confounders such as maternal age, BMI, serum BPA concentration, smoking and socioeconomic status. In addition, maternal metabolic parameters (*i.e.*, HDL, TC and TRG) were selected as the

independent variables for newborn LBW and SGA outcomes.

The geometric means of BPA concentration in maternal blood and fetal cord blood were 2.5 ng/ml and 0.5 ng/ml, respectively. In male neonates, increased odds of LBW, SGA HLP, and LAD were reported when the highest quartile of maternal BPA exposure was compared to the first quartile. In female neonates, the highest quartile of maternal BPA level was associated with statistically significant increased odds of SGA and HLP, but not LBW or LAD. The odds for adverse effects on fetal outcomes did not monotonically increase with each increasing quartile of maternal BPA levels.

Health outcomes (LBW, SGA, HLP and LAD) are clearly defined and measurement methods have been validated previously. One limitation of the exposure assessment was that the maternal BPA level was measured at a single point in time rather than multiple measurements in the time periods prior to delivery (especially considering the variability of BPA exposure over time and its short half-life). As the study pointed out, parity, a potential confounding factor for birth weight, was not controlled for. Also, information was not provided to assess the comparability between the women enrolled and the women who dropped out. The statistical power in this study from Taiwan was limited by its relative small sample size. It is not clear if the reported findings are generalizable since all patients came from a single site in Taiwan. **This study has no utility for HI and no utility for RA.** Most importantly, a uniform dose-response relationship was not observed across the 4 quartiles of maternal BPA levels (non-linearity). The problematic nature of blood measurements have been described in the PK section of this document. Also, the sample size is relatively small, which limits the study's power to reliably assess the risk posed by BPA.

Maternal Urinary Bisphenol A during Pregnancy and Maternal and Neonatal Thyroid Function in the CHAMACOS Study (Harley *et al.*, 2013)

Data from the Center for the Health Assessment of Mothers and Children of Salinas (CHAMACOS) longitudinal birth cohort study between 1999 and 2000 was used to examine the association between maternal urinary BPA levels and maternal and neonatal thyroid function. Serum collected from 169 mothers was insufficient for assay for reasons not reported by the authors. The final sample consisted of 335 mothers and 364 neonates. Free maternal Thyroxine (T4) and Thyroid Stimulating Hormone (TSH) was measured from maternal serum, and neonatal TSH was obtained from dried blood spots. BPA (free + conjugated) was measured via spot urinary samples collected in BPA-free cups was measured using online solid-phase extraction coupled with isotope dilution–HPLC–TMS during the first and second halves of pregnancy. The two concentration adjusted samples measured were averaged for measure exposure. The primary statistical methods were ANOVA and Pearson's correlations. The correlation between the two BPA measurements was only 0.16 which shows a high variability. The association between BPA and T4 was significant only when the closer BPA measure was used (β= -0.13 μg/dL per $\log_2$ unit: 95% CI: -0.25, 0.00), and in that case it was only borderline significant since 0.00 is included in the confidence interval. The average maternal BPA measure was associated with reduced TSH in boys (-9.9% per log2 unit; 95% CI: -15.9%, -3.5%), but not girls. This association was stronger with BPA measures from the third trimester.

Study strengths include a large sample size, observational cohort design, and two BPA measurements. Limitations include that while a majority of statistical tests did not find a statistical significance, authors generalize from the minimal positive results to say that there is therefore a relationship between maternal BPA and thyroid hormones without explaining the negative results. **This study has no utility for HI and no utility for RA.**

The relationship between urinary bisphenol A levels and meningioma in Chinese adults (Duan *et al.*, 2012)

The objective of this case-control study was to investigate whether an association exists between the diagnosis of meningioma and BPA exposure level in Chinese population (>35 years). A total of 418 patients with neuroradiology-confirmed meningioma (documented lesion consistent with meningioma on MRI or histological diagnosis), without concurrent or previous cancers, were registered (May 15, 2009-2010) at Union Hospital in Wuhan, China. A total of 247 patients (93 men, 154 women) with meningioma were finally enrolled of which 146 were newly diagnosed cases (59 %) and 101 patients (41 %) received their diagnosis and treatment before their Union Hospital visit. A total of 258 healthy, nonneoplastic controls (90 men, 168 women) were selected among individuals participating in health examinations at the same hospital who were frequency matched based on gender, age (35–55, 56–65, 66–75, ≥76 years), and self-reported race (Chinese or non-Chinese Han). Data were collected via interview for HRT use, medical history, and family history for cancer. Height and weight were measured and BMI was calculated and categorized based on WHO standard ranges. Total urinary concentrations of BPA were measured and analyzed using solid-phase extraction coupled with high-performance liquid chromatography–mass spectrometry (HPLC–MS). A comprehensive quality control system, including reagent blanks, was used to ensure that samples were not contaminated during handling, storage, and analysis.

The distribution of categorical variables was compared between patients and controls using the Pearson χ^2 test. Odd ratios (ORs) and 95% CI of meningioma in association with urinary BPA levels (quartiles: <0.53, 0.54–0.91, 0.92–1.69, and >1.69 ng/ml; lowest quartile as reference group) were estimated using unconditional logistic regression with adjustment for gender, age, race, BMI, past or current hormone replacement therapy (HRT) with use of estrogen or with progestin, and family history of cancer restricted to first degree relatives. All statistical analyses were performed using SPSS version 15.0 (SPSS, Cary, NC, USA) and SAS software, version 9.2 (SAS Institute, Cary, NC, USA) with two-sided tests ($p<0.05$ as significant). Female gender, HRT use, and BMI $\geq$25 kg/m^2 were significantly associated with increased risk of meningioma (p's$\leq$.002).

The authors report a positive association between increasing urine BPA levels and meningioma in the multivariable-adjusted model (OR's 1.40-1.57, p=0.003). Also, this observation was reported to be independent of BMI, HRT use and smoking status. The result was based on one-time analysis of urine BPA, rather than several observations over a period of time. Also, the study did not report the blinding protocols, time of sample collection and urine analysis or the percentage of population in which BPA was detected in the urine. Grade of meningioma, duration of treatment for meningioma, urinary creatinine adjustment, dietary exposure,

medication, and other health conditions (diabetes, coronary heart diseases) was not considered in the analysis. Also, the WHO BMI cutoffs may not be appropriate for the Chinese population. Thus, due to these limitations**, this study has no utility for HI and no utility for RA.**

Urinary Bisphenol A Concentrations and Implantation Failure among Women Undergoing *in vitro* Fertilization (Ehrlich *et al.*, 2012a)

This prospective study was conducted at a single center in 18-45 (mean 35.8) year old female *in vitro* fertilization (IVF) patients (n =137) recruited between November 2004 and April 2010, MA, US. The study aimed to determine if there was a correlation between egg implantation failure following IVF treatment. The urine (two spot–urine samples collected day 3-day 9 of treatment cycle and day of egg-retrieval procedure) was divided into aliquots, frozen, stored at –80°C and shipped on dry ice overnight to the CDC where they were stored at ≤ –40°C until analysis (limit of detection for BPA was 0.4 μg/L). The spot urine levels of BPA were analyzed using online solid phase extraction–high performance liquid chromatography–isotope dilution tandem mass spectrometry. Implantation failure was defined as a serum β-hCG level < 6 mIU/mL typically measured 17 days after egg retrieval. Multivariate generalized estimating equation models (SAS version 9.2; SAS Institute Inc., Cary, NC) for repeated measures were used to evaluate the association between implementation failure, specific gravity (handheld refractometer) adjusted cycle specific urinary BPA concentrations, and other potential risk factors like age, day of embryo transfer and IVF protocol. The odds of implantation failure increased linearly with increasing quartiles of urinary BPA concentrations. However, it became statistically insignificant after adjusting for age, day of embryo transfer and IVF protocol (p-trend=0.06). This relationship was within the bounds of the 95% CI in all three quartiles.

Overall, the findings of this paper fail to provide a convincing statistical correlation between urinary BPA concentration and the failure of implantation in women undergoing IVF. Also, the infertility factors (male, female, family history for infertility), health status of the patients and partners (stress associated with the procedure, diabetes mellitus, timeliness of IVF medication), and status of previous IVF failure may be confounding factors in the analysis. Finally, the embryos were selected by an embryologist. The authors do not state if this person was blinded which in the case of this particular project is somewhat problematic. In light of the inherent challenges and bias, this study is not compelling. If anything, the lack of a statistically meaningful relationship does nothing to bolster the underlying hypothesis. **This study has no utility for HI and no utility for RA.**

Urinary Bisphenol A Concentrations and Cytochrome P450 19A1 (Cyp19) Gene Expression in Ovarian Granulosa Cells: An *in vivo* Human Study (Ehrlich *et al.*, 2013)

This molecular epidemiology study was designed to investigate a possible relationship between urinary BPA concentrations and CYP19A1 gene expression in granulosa cells collected from a subsample of women enrolled in a larger prospective cohort study studying environmental chemical-fertility/pregnancy relationships. Follicular fluid samples were taken from 61 women who underwent an IVF oocyte retrieval procedure as part of fertility treatment at Mass General Hospital Fertility Center between 2009 and 2011. Study participants were predominantly Caucasian (88%) with a mean age of 35. Granulosa cells were separated from oocytes and

resuspended in follicular fluid, frozen in liquid nitrogen, and shipped for further analysis (RNA) by collaborators in two batches (Apr 2011 and Jan 2012). Total RNA was extracted from follicular samples and reverse-transcribed to form cDNA using commercially available kits. Quantitative (q) PCR assays, conducted in duplicate, were used to measure CYP19 gene expression levels relative to β-actin in each sample. Serum FSH levels were measured on cycle day 3 and peak serum E2 was measured 1.5 days prior to oocyte retrieval in each study participant. One to two spot urine samples were provided per woman per IVF cycle; analyses used urine BPA concentrations from the morning of the day of oocyte retrieval due to the close proximity in time to collection of granulosa cells whose gene expression would be expected to change within hours if there was a response to BPA exposure. Urine samples were collected in polypropylene specimen cups, frozen at -80C, and shipped to collaborators for BPA measurement. Storage container and lab equipment materials were not specified as BPA-free. Following enzymatic hydrolysis (of conjugated BPA), total BPA levels in 100-µl samples were measured using HPLC-tandem MS and adjusted for the specific gravity of the urine sample. Characteristics of study participants were summarized using simple statistics, while more complex, mixed-effect statistical models were used to test for associations between urinary BPA concentrations on day of oocyte retrieval and CYP19A1 gene expression levels.

BPA was detectable in 91% of spot urine samples, and it was stated that urinary BPA concentrations were similar to those of the larger IVF study as well as the general US population. The median CYP19 gene expression level was 0.43x that of β-actin. No association was observed between urinary BPA levels (quartiles) and CYP19 gene expression levels (log transformed), either with or without adjustment for BMI or batch date. The only statistically significant association reported was that between batch date and CYP19 expression level ($P \leq 0.003$). The authors stated that both the adjusted and unadjusted models "suggested" a non-monotonic dose response between urinary BPA and parameter estimates for log CYP19 gene expression. However, the data did not support that statement, since none of the parameter estimates in either model was statistically significant, nor was any test for trend. This was verified by visual inspection of Fig 1 and the significant overlap in 95%CI for each quartile. No dose response association, therefore, was observed between urinary SG-adjusted BPA and Cyp19 granulosa gene expression. The authors also reported (in text) a statistically significant negative association between adjusted urinary BPA (25^{th}-75^{th}%ile) and peak serum E2, as found in previous studies. However, the validity of the comparison is uncertain since the cutoffs for each of the stated urinary quartiles did not match those reported in Table 2 for the CYP19 models, and since the results from other possible comparisons (*e.g.*, 25^{th}-100^{th}%ile, 0^{th}-75^{th}%ile, etc.) were not reported and therefore are unknown. **This study has no utility for HI and no utility for RA.** Had the study not been negative, the study utility was still further constrained by lack of generalizability because of its small n and the all-Caucasian study sample.

<u>Urinary bisphenol A concentrations and early reproductive health outcomes among women undergoing IVF, Human Reproduction (Ehrlich *et al.*, 2012b)</u>
This observational study was designed to evaluate the association between urinary bisphenol A (BPA) concentrations and ovarian response (peak serum estradiol) and early-stage reproductive outcomes (oocyte maturation, fertilization, embryo quality, and cleavage rate). A cohort of 174

women (18-45 years old) undergoing *in vitro* fertilization (IVF) were prospectively followed until they had a live birth or discontinued fertility treatment (25% of the women, n =44, were assayed for >2 IVF cycles). Two samples were collected during each cycle, yielding 429 samples across 237 cycles. IVF-cycle-specific urinary concentrations of the sum of free and conjugated BPA (total BPA) were measured by solid-phase extraction (SPE) coupled with isotope dilution-high-performance liquid chromatography (HPLC)-tandem mass spectrometry (MS/MS). Eighty-eight percent of samples had BPA >LLOD, whereas some samples were <LLOD (n =51). Trend-test analysis showed that higher measured BPA (highest quartile ~470 pg/ml estradiol) was associated with decreased estradiol compared to lowest BPA quartile ($p<0.001$). Secondly, at the time of retrieval, mean decreases in numbers of normally fertilizing oocytes (1, 6, and 24%) at the highest three BPA quartiles were identified, compared with the lowest BPA quartile. Finally, the incidence of poor-quality embryos (as rated by a standardized internal protocol) in BPA quartiles 2, 3, and 4 compared with quartile 1 (the lowest) were 1.43 (95% confidence interval 1.01, 2.01), 0.89 (0.60, 1.32), and 1.05 (0.73, 1.51), respectively. After adjustment for age and other potential confounders such as smoking, there was a significant inverse relationship between urinary BPA concentrations and the number of oocytes, number of fertilized oocytes, and estradiol concentrations. Interpretation of the data is limited by the variability in BPA measurements and the small study sample size. Additionally, data from women undergoing IVF treatment may not be generalizable to fertile, normally cycling women, and the assessment of reproductive outcomes does not consider potential contributions from the male partner. **This study has no utility for HI and no utility for RA.**

Unconjugated bisphenol A cord blood levels in boys with descended or undescended testes (Fénichel *et al.*, 2012)

This case-control study evaluated the relationship between fetal exposure to BPA and cryptorchidism (undescended testes) by comparing the levels of unconjugated BPA (uBPA) in cord blood samples of boys with or without undescended testes. Newborn males who were included in a prospective study (Brucker *et al.*, 2008) on cryptorchidism in Nice and Grasse, France, were participants; all boys born alive at ≥ 34 weeks of gestational age were eligible. Over the 3-year period, 102 were diagnosed with cryptorchidism, of which 95 parents wanted to participate in the study. Two control boys born on the same ward around same time, with examination by the same pediatrician, were recruited as a control for each case; they were matched for gestational age, birth weight, and parental geographical origin if possible. Of 95 cryptorchid boys and 188 matched controls, BPA samples were available for 46 cryptorchid and 106 control newborns. The BPA levels were measured in cord blood using a radioimmunoassay validated by gas chromatography-mass spectrometry, with special care to avoid BPA contamination from polycarbonate equipment. The limit of detection for BPA was 0.08 ng/mL.

The maternal or newborn characteristics were similar between case and control groups. The uBPA levels were detected in all cord blood samples, and the levels were similar between male newborns with or without cryptorchidism. The median level of uBPA was 0.92 ng/mL in cryptorchid boys (range 0.14-4.75 ng/mL) compared to 0.86 ng/mL in the control group (range 0.14-4.76 ng/mL). The mean level of uBPA (±SD) was also not significantly different between the two groups: 1.26 ng/mL (±1.13) in cryptorchid boys and 1.12 ng/mL (±0.86) in the control

group. The lack of correlation between BPA and presence of undescended testes was confirmed by a non-parametric Kruskal –Wallis test (p=0.05).

This study has no utility for HI and no utility for RA. This is a small sample size study measuring BPA exposure through cord blood in a small group of newborns in a geographically focused area in France. As the authors noted, the cord blood levels of BPA do not necessarily reflect fetal exposure during the period of physiological testicular descent.

Prenatal and postnatal bisphenol A exposure and body mass index in childhood in the CHAMACOS cohort (Harley *et al.*, 2013a)

This study uses data from the Center for the Health Assessment of Mothers and Children of Salinas (CHAMACOS) longitudinal cohort study of environmental factors and child growth and development. The study sample included women over 18 years old who qualified for low income health insurance in the Salinas Valley in California and their children. The final sample included 311 mother-child pairs. Maternal BPA levels were measured by spot urine samples collected from mothers during the first trimester and second trimester of pregnancy; the two measures were averaged to approximate exposure during pregnancy. Child BPA levels were measured at ages 5 and 9 years. The LOD was 0.4 ng/mL. Child fat percentage was measured by bio-impedance. Child height, weight, and waist circumference was measured by trained study staff; body mass index and z scores were then calculated. Children with BMI between 85th and 95th percentile were classified as overweight, and children above the 95th percentile were classified as obese, using sex-specific percentile data issued by CDC in 2000.

Authors reported that BPA concentrations during pregnancy were higher in mothers who had lived longer in the U.S., and BPA concentrations at 9 years of age were higher in children who were obese and who drank more soda (p's<.05). Prenatal BPA concentrations were not correlated with child BPA concentrations at 5 and 9 years, birth weight, or timing of puberty. Prenatal and 5 year old BPA concentrations were not associated with 9 year old body size measures for boys or for the combined sample; in girls, there was a negative association between the highest vs. lowest tertile of prenatal BPA concentration and 9 year old BMI z scores (-0.47, 95% CI: -0.87, -0.07; p=0.05 for gender*BPA interaction term). This association was stronger for pre-pubertal girls than girls who had begun puberty; this finding was unexpected. Interactions of prenatal BPA by gender were not significant for other body measurements.

BPA concentrations at age 9 were cross-sectionally associated with increased BMI z score, waist circumference, and body fat, and increased odds of obesity/overweight at age 9, after controlling for maternal pre-pregnancy BMI, household income, maternal education level, maternal years of residence in the U.S., child environmental tobacco smoke exposure, soda intake, fast food intake, and sweet consumption at age 9. Compared to children with no detectable level of BPA, those with measurable concentrations below the median were 3.08 (95% CI: 1.18, 8.02) times more likely and those with concentrations above the median were 4.20 (95% CI: 1.60, 11.02) times more likely to be overweight or obese. There were no significant cross sectional associations between BPA levels and body measurements at age 5.

Though this study utilized a large sample size and a cohort design, it did not find a relationship between BPA in pregnancy and BMI at 5 and 9 years of age. The authors note that the cross sectional associations do not provide information about temporality. BPA concentrations may be associated with other lifestyle or diet characteristics that are predictive of obesity, especially consumption of canned food and packaged, processed food. In addition, increased BPA concentrations may be a result of simply consuming more calories, or atypical metabolic processes in the overweight. Thus no conclusions can be drawn about a causal relationship between BPA and overweight.

An additional limitation is the transient nature of BPA measurements of spot urine samples. The study sample consisted of low-income Latina women living in the Salinas Valley in California, so external validity is limited. Finally, confidence intervals around effect estimates were large (*e.g.*, 1.60-11.02), making interpretation of effect estimates more difficult. **This study has no utility for HI and no utility for RA.**

Prenatal and early childhood bisphenol A concentrations and behavior in school-aged children (Harley *et al.*, 2013b)

This study examined the relationship of BPA in urine of pregnant women and in urine of their offspring at 5 years with behavioral assessments of offspring at ages 7 and 9 years. Total BPA was measured in spot urine samples from pregnant women (average of 13.6 week and 26.4 week measures) using online solid phase extraction with HPLC-MS/MS (LOD 0.4 µg/L) and then adjusted for specific gravity for mothers and creatinine level for children. Behavior at 7 and 9 years of age was assessed using age-standardized T-scores, transformed to log base 2, of teacher and mother responses to the Behavior Assessment System for Children 2 (BASC-2) and Conner's ADHD/DSN-IV Scales (CADS). The Connors' Continuous Performance Test (CPT) was also used to directly assess ADHD at 9 years. Maternal and child BPA levels were included in all multivariable linear models, with β coefficients interpreted separately. Analyses of teachers' and mothers' reports were adjusted for mother's country of birth, maternal education, marital status, child's age, home environment, household income, number of siblings, and maternal dialkyl phosphate metabolite levels during pregnancy. Models of mothers' reports also included maternal language of interview and maternal depression at 7 years.

BPA levels in the children at age 5 years were approximately twice the levels in their mothers during pregnancy but all were lower than the average for the general U.S population. Prenatal urinary BPA was positively associated with internalizing problems in boys only based on both maternal and teacher reports. Depression and aggression were also positively associated in teacher reports. Childhood BPA was associated with internalizing problems, anxiety and inattention in boys based only on teacher reports. In girls, childhood BPA was associated with internalizing problems, conduct problems and inattention in mothers' reports and internalizing problems, depression and hyperactivity in teacher reports. There were no significant associations with CPT test results. The authors also reviewed 4 other studies of BPA and childhood behavior and results from laboratory animal behavior studies, noting that inconsistent results have been found across studies.

Spot urine BPA levels measure short-term exposure. Measures in the study population were lower than the general US population. The authors reported that the 2 prenatal measurements that they averaged, made 13 weeks apart, were only weakly correlated. Therefore, the BPA measures used in this study cannot accurately reflect the true long-term exposure of BPA. Yet, children's behavior development is a long-term result of many factors. Further, BPA was measured at 5 years for children and the outcomes were at 7 and 9 years, there were no behavioral measures at 5 years to show that the exposures preceded the outcome measures. Differences in exposures could have resulted from behavioral problems or, more likely, both BPA and behavioral measures could be related to other family lifestyle differences – that could potentially increase exposure to food and beverages in containers with BPA. Additionally, mothers were known to have multiple occupational exposures as agricultural workers which could have had effects on the children and were not considered. The lack of consistency and the lack of association between BPA and the CPT direct assessment results are also of concern, particularly with the multiplicity of statistical comparisons. More than two dozen outcomes were evaluated against the same set of covariates. **This study should be considered as part of the growing body of work assessing relationships between BPA exposure and behavior, however it has no utility for HI and no utility for RA.**

<u>The association between some endocrine disruptors in human plasma and the occurrence of congenital hypothyroidism (Jung *et al.*, 2013)</u>
This is a cross-sectional study to look at the associations between endocrine disruptors and the occurrence of congenital hypothyroidism and passage of target compounds from the mother, using plasma samples of 59 mother-infant pairs collected at Soonchunhyung Hospital, Seoul, South Korea. Ages of infant donors ranged 1-54 months, weight ranged 2.50-18kg.

Measurements and statistical methods: The levels of phthalates (DEHP, MEHP, DBP, MBP and PA), alkylphenols (n-NP and t-OP), bisphenol, and isoflavones (equol, daidzein and genistein) were determined by gas chromatography–mass spectrometry (GC–MS) in infants. Values below the limit of detection (LOD) were valued as zero for calculating the total concentrations of chemicals. P- values less than 0.05 were considered statistically significant. Correlation (r) between the levels of investigated compounds in mother and infant was tested using Pearson's coefficient of determination. (3) Results: t-OP and PA concentrations in the patient group were significantly higher than in normal infants. Genistein concentrations in normal infants were significantly higher than in patients. BPA concentration was not significantly different in patients vs. controls.

The study's sample size is very small, no confounders or modifiers were assessed. No new information regarding BPA and hypothyroidism were provided. No information was provided regarding patient enrollment and participation/decline rates. **This study has no utility for HI or RA.** There is no dose-response relationship found in this study.

<u>Association between urinary concentrations of bisphenol A and type 2 diabetes in Korean adults: A population-based cross-sectional study (Kim *et al.*, 2012)</u>
Cross sectional data from the 2009 Korean National Human Biomonitoring Survey in Daegu,

South Korea from August to September 2009 was used to examine the association between urinary bisphenol A (BPA) levels and type 2 diabetes. The sample consisted of 1210 participants aged 40-69 years old. Participants were categorized as having type 2 diabetes based on self-report or a doctor's diagnosis. One BPA sample was analyzed using liquid-liquid extraction and gas chromatography coupled with mass spectrometry and the lower limit of detection was 0.05 ng/mL. BPA measurements were categorized into quartiles for analysis: <1.36, 1.36-2.14, 2.15-3.32, and >3.32ng/mL.

Overall, the mean BPA level was higher in diabetic patients (2.40 ng/mL) than non-diabetic patients (2.03 ng/mL) (p=0.05). When adjusted for age and sex, this was no longer significant. Data was stratified by sex, age, body mass index (BMI), education, cigarette smoking status, income and urban or rural residence. The unadjusted mean BPA level was significantly higher in diabetic participants who were 50-59 years old (no diabetes: 1.91 ng/mL, diabetes: 2.66 ng/mL, p=0.025), those who lived in urban environs (no diabetes: 1.95 ng/mL, diabetes: 2.54 ng/mL p=0.013), and female participants (no diabetes 1.98 ng/mL, diabetes: 2.67 ng/mL, p=0.008). Four different multivariate regression models included adjustment for creatinine, age, sex, body mass index, education, cigarette smoking, income, and place of residence. The multivariate logistic regression models showed no clear significant relationship between BPA quartiles and type 2 diabetes.

The largest limitation is the cross sectional design of the study. This does not allow the study to measure BPA exposure over time, or to determine cause and effect. BPA is a variable measure and it was only measured once. Notably, there was no statistically significant difference after adjusting for age and sex. The categorization of BPA and BMI into categorical variables likely led to some residual confounding, but it is unlikely that affected the result. The binary smoking variable may have also caused some residual confounding and it would have been more precise to utilize serum cotinine instead. Study strengths include the large sample size, and the reporting of negative results. **This study has no utility for HI and no utility for RA.**

Bisphenol A in Chronic Kidney Disease (Krieter *et al.*, 2013)

This study was conducted to evaluate plasma BPA levels in patients with chronic kidney disease (CKD), and to determine whether dialyzers with different elutable BPA affect plasma BPA levels in patients on maintenance hemodialysis. BPA was eluted from three different dialyzers *in vitro* on by recirculating water for 180 minutes; all had polycarbonate housings but different dialysis membranes (high-flux polyethersulfone PUREMA H [PUR-H], high-flux polysulfone [HF-PSu], and low-flux polysulfone [LF-PSu]).

A cross-sectional study measured single plasma BPA level (see PK section for issues related ot blood serum measurements) in maintenance dialysis outpatients with different stages of CKD from four different nephrology centers; the control group included 24 healthy volunteers with no history of renal impairment, diabetes, or arterial hypertension. A prospective, randomized, crossover study was done in 18 adult patients receiving maintenance dialysis (CKD Stage 5D), where patients successively received 4 weeks of three-times weekly hemodialysis treatment with each LF-PSu, HF-PSu, and PUR-H. The fractions of protein-bound and free BPA were obtained

in a subset of these dialysis patients. The mass of eluted BPA was statistically significantly lowest in PUR-H compared to HF-PSu or LF-PSu. A statistically significant higher BPA was seen with LF-PSu compared to HF-PSu (140.8+38.7 vs 48.1+7.7 ng; $p<0.01$). In the cross-sectional study, 152 patients with CKD were enrolled and the sample size of different CKD stages varied. The mean plasma BPA levels in patients with CKD stages 1 (n=6) and 2 (n=12) were below the limit of detection (0.2 ng/mL) and similar to controls. For each increasing CKD stage, the BPA levels showed statistically significant elevation compared to controls; the BPA levels were 0.7 ng/mL in stage 3 (n=31) and 4 (n=40), and 1.6+1.8 ng/mL in stage 5 (n=10; $p<0.05$ compared to stages 1-4 and controls). In CKD stage 5D (n=53) in which patients are on maintenance dialysis, BPA levels were statistically significantly elevated compared to any other group (10+6.6 ng/mL; $p<0.001$). No difference was seen between dialysis patients on polysulfone or polyethersulfone dialysis membranes. The plasma BPA levels of controls and CKD patients (except 5D) showed an inversely proportional relationship with eGFR, which was statistically significant. In the prospective study, the change in plasma BPA levels was not significant during any hemodialysis session in any patient, and the plasma BPA levels remained similar after 4 weeks of hemodialysis with any of the three dialyzers. Plasma BPA was highly protein bound in both dialysis and healthy controls; 74% and 70% of BPA were protein-bound in dialysis patients and healthy volunteers respectively.
Although considerable amounts of BPA were shown to be eluted from dialyzers with polysulfone membranes, CKD patients undergoing hemodialysis with these different BPA-eluting dialyzers did not show any notable change in the plasma BPA levels during a single course or over 4 weeks of treatment. **This study has no utility for HI and no utility for RA.**

Use of NHANES data to link chemical exposures to chronic diseases: A cautionary tale (LaKind, *et al.*, 2012)
This article uses four waves of NHANES data to analyze the association between BPA levels and three outcomes: diabetes, Coronary Heart Disease, and heart attack. The authors argue that NHANES is not an appropriate data source to study these associations due to its cross sectional study design and inability to draw conclusions about temporal and causal relationships. The National Health and Nutrition Examination Survey (NHANES), conducted by CDC, is a nationally representative cross sectional survey. Urinary BPA is measured for a subsample of the NHANES population. The LOD was 0.36 ng/ml for the 2003-2004 survey, and 0.4 ng/ml for the other three surveys (2005-2006, 2007-2008, and 2009-2010). The authors report no statistically significant associations between BPA levels and diabetes, CHD, or heart attack in any of the four waves, after adjusting for creatinine, age, gender, race/ethnicity, education, income, smoking, BMI, waist circumference, heavy drinking, family history of diabetes or heart attack/angina, hypertension, sedentary activity, blood cholesterol, and daily energy intake. They also report no significant associations in unadjusted and fully adjusted analyses of pooled data from all four data waves [odds ratios: Diabetes 0.995 (95% CI: 0.982, 1.007), CHD 1.004 (95% CI: 0.998, 1.009), heart attack 1.002 (95% CI: 0.998, 1.007)].

These results differ from those of previous studies using NHANES data which found significant associations, mostly due to differences in inclusion criteria and case definitions. The current authors did not exclude based on age (<18 or >74) as previous authors did. Regarding the

diabetes analyses, they chose to compare individuals with diabetes vs. those with no diabetes or borderline diabetes, whereas previous authors chose to compare individuals with diabetes or borderline diabetes vs. those without diabetes. Finally, the current authors included more covariates in their analyses than previous authors. The long list of covariates presents some concerns about model selection and collinearity. The authors conclude that when using the NHANES dataset it is difficult to draw conclusions about BPA and chronic complex diseases, due to its cross-sectional design. **This study has no utility for HI and no utility for RA.**

Changes in steroid metabolism among girls with precocious puberty may not be associated with urinary levels of bisphenol A (Lee *et al.*, 2013)

This case-control study focused on evaluating steroid metabolism in two subsets of girls with clinical precocious puberty (PP): Central (n =42) and Peripheral (n =40) and age-matched, healthy girls (n =32) who attended the same clinic for regular growth check-ups. In addition to comparing urinary levels of 84 steroids between the three groups of girls, steroid activity and correlations between steroids were compared between girls with higher (>10 μg/g creatine) and lower (<5 μg/g creatine) levels of urine BPA.

Case girls ranged from 7 to 11 years of age. Central PP was diagnosed by GNRH stimulation test as: (i) breast budding <age 8, (ii) bone age >1 year over chronological age, and (iii) LH peak values >5 mIU/mL and peak LH/FSH ratios >1.0 under GnRH stimulation conducted <9 years old. Subjects who met criteria (i) and (ii) but not (iii) were diagnosed with peripheral PP. Control girls had height and BMI between the 25^{th}-85^{th} percentiles of the national reference standard and showed no signs of premature sexual development. First morning urine was collected and assayed for an 84-steroid profile and BPA analysis by GC-MS. No information was presented on the type of collection container. The limit of quantification (LOQ) for BPA was 0.2 ng/ml, with only one girl, in the control group, having a level below the LOQ. The ratio of steroid metabolites to precursors was calculated and normalized to the mean values of controls as an indicator of enzymatic activity. Groups were compared using unpaired t-tests and ANOVAs. Pairwise correlations (Pearson) between 84 measured steroids were calculated and plotted as heat maps for girls (pooled across cases and controls) with high and low levels of urine BPA.

The authors reported significant differences between levels of some androgens, estrogens, progestins, corticoids and sterols and some metabolic ratios between girls with central- and peripheral-PP and/or controls. However, there were no significant differences in levels of BPA in either PP subset versus controls and no significant correlations between differences in steroid metabolism and BPA concentration. Significantly higher levels of urinary testosterone, and pregnenolone were present in girls with high versus low levels of urinary BPA within each subset while 17β-estradiol levels differed only for girls with central-PP. The heat maps showed higher levels of correlation among steroids for girls with high BPA than low BPA, but only visual comparisons were made.

This was a small case-control study with measurement of urinary BPA and steroids in a single urinary sample from each girl. The cross-sectional nature of the urine measurements makes it impossible to infer any causal relationships. Without information to show that urine collection

tubes were BPA-free, the reported urinary BPA levels should be considered to be unreliable. No associations were found between BPA levels and either type of PP or with metabolic changes in 84 measured hormones, which was the primary BPA-related question in the study. The high number of statistical comparisons, based on all steroids and their metabolic indices and the subsets of girls, might be expected to produce some spurious findings of significance. Other than age-matching, no other genetic, epigenetic, or environmental co-factors were considered that may provoke manifestation of PP. **This study has no utility for HI and no utility for RA.**

Exposure to bisphenol A is associated with low-grade albuminuria in Chinese adults (Li *et al.*, 2012)

This cross-sectional study investigated the potential relationship between spot urinary BPA concentration and low-grade albuminuria in a subsample of 3455 Chinese adults enrolled in a larger study of BPA and chronic disease. Current study participants were randomly selected from Jun–Aug 2009 from the larger study population of 10,185 adults aged 40 years or older and represented individuals with diabetes (27%), impaired glucose regulation (33%), or normal glucose regulation (40%). Participants (mean age>60) with either macroalbuminuria (urinary albumin-to-creatinine ratio [UACR]>300 mg/g; n=67) or microalbuminuria (UACR between 30 and 300 mg/g; n=333) were included in the study but analyzed separately from the 3055 participants with UACRs <30 mg/g. Demographic and clinical information was collected by interview. Fasting and 2-hr oral glucose tolerance test (OGTT)-blood samples were collected and assayed for a number of clinical chemistry endpoints. Single-void first morning urine samples were collected in two separate BPA-free containers. The first container was analyzed for albumin and creatinine within 1 hr of collection and UACR calculated, while the second was frozen at -70C within 4 hrs of collection and later assayed for total BPA concentrations. "Low-grade albuminuria" was defined by the study authors as UACR in the highest quartile (9-71-29.9 mg/g) of UACR levels <30, while UACR levels <9.71 were referred to as "normal". However, it is important to point out that in the published literature, all UACR levels <30 (which would include the authors' "low-grade albuminuria" group) are considered "normal" (Xu *et al.*, 2007).[4] The authors' classification, therefore, appears arbitrary. Urinary BPA concentrations were log-transformed. Demographic and clinical characteristics of study participants were summarized using simple statistics, while more complex statistical regression models were developed using SAS 8.1 to evaluate possible associations between spot urinary BPA concentrations and, primarily, low-grade albuminuria.

Among participants with UACR<30 mg/g, the median BPA level in the low-grade albuminuria group (0.95 ng/ml) was higher (P<0.0001) than that in the (rest of the) normal group (0.79 ng/ml). When the low-grade and (other) normal groups were combined, the BPA level in quartile 4 (Q4) was significantly higher than that in quartile 1 (P<0.0001). However, Pearson's correlation analyses indicated that many clinical and clinical chemistry endpoints other than urinary BPA levels were associated with UACR in the participants. When adjusted for those possible confounders, an association was observed between UACR and urinary BPA in Q4 or

4 http://www.mayoclinic.com/health/microalbumin/MY00143/DSECTION=results http://www.aacc.org/members/nacb/LMPG/OnlineGuide/PublishedGuidelines/diabetes_update/Documents/Chapter%2012.pdf

Q3, relative to Q1, with ORs of 1.2 and 1.23, respectively. Importantly, however, the reported median BPA concentration in the combined micro/macroalbuminuria group (0.77 ng/ml) was *lower* than that in the low-grade albuminuria group and almost identical to that in the normal group, and there was no significant association observed between UACR and increasing BPA quartile in the combined micro/macroalbuminuria group. This absence of a dose-response relationship does not support the authors' reported (very weak) association between urinary BPA and low-grade albuminuria. **This study has no utility for HI and no utility for RA.**

Urine Bisphenol-A Level in Relation to Obesity and Overweight in School-Age Children (Li *et al.*, 2013)

The study was ancillary to a larger national study of pubertal development and health of adolescents in which anthropometric measures and information on pubertal development had already been collected. Parents of all students were sent a consent form describing the nature of the study and details of the urine collection. Parents were asked to inform the student's teacher if their child was not to participate. However, due to infeasibility, signed consent forms were not required. Assent forms are not mentioned. While students were informed by their teachers of the study purpose, process and voluntary nature of the evaluation, the authors report that participants were not informed of the hypothesis of the study, as it was an ancillary study.

Spot urine samples were collected anytime between 9am to 4pm. All urine kits were made of BPA free materials. Methods to avoid BPA contamination from external sources were employed. The limit of detection for BPA was 0.31 µg/L. Weight, not BMI, was used as a measure of overweight/obesity. Overweight was defined as greater than the 90^{th} percentile for age and gender as that for the 85^{th} percentile was not available. Logistic regression with 95% confidence intervals was used to estimate odds ratio of obesity measurements associated with urine BPA levels. 1,451 students were eligible, 18 refused to participate, 17 samples were damaged, and 72 female students were menstruating at the time of collection, leaving 1,344 available samples. However, the authors report only 1,326. Seven-hundred-forty-eight (748) students (57.4% female) had urine BPA levels < 2 µg/L and 578 students (42.6% female) had levels ≥ 2 µg/L. The analysis was adjusted for the following potential confounders: school grade, parental education, parental overweight, unbalanced diet, depression scores, and activity levels. In a separate analysis, these variables did not show any association with urine BPA levels. However, those who spent more versus less time playing video games had higher BPA levels ($P = 0.03$). High urine BPA levels were associated with overweight among female students entering the pubertal stage (9-12 y) of development (crude odds ratio (OR) 2.08 [95% CI 1.2, 3.7]; adjusted OR 2.38 [1.2, 4.7]). In this group, girls with BPA levels $> 90^{th}$ percentile had a five times increased risk of overweight (adjusted OR 5.2 [1.7, 16]). An association between high urine BPA levels and overweight/obesity was not seen in older girls or boys. Urine BPA levels were associated with a hip circumference $> 90^{th}$ percentile (adjusted OR 2.9 [1.1, 7.5], but, not associated with higher measures of waist circumference, waist-height ratio, skinfold thickness, or BMI.

Limitations of this study include the unusual consent form process and the discrepancy in the number of subjects enrolled versus number of available urine results. Also, the timeline between

the collection of anthropometric measures and urine BPA levels is not clear. Other possible confounders not accounted for in this study include maternal presence of gestational diabetes, birthweight and pre-term delivery. **This study supports clinical research implicating BPA as a potential obesogen, especially in young females. However, the level of evidence from this study is questionable and has no utility for HI and no utility for RA.**

Urinary bisphenol A concentration and angiography-defined coronary artery stenosis (Melzer *et al.*, 2012a)

This cross-sectional study examined the relationship between urinary BPA and angiographically graded coronary atherosclerosis (CAD) in a sample of 591 patients who participated in the UK Metabonomics and Genomics in Coronary Artery Disease (MaGiCAD) study. Patients were classified as having normal vessels (120; 20%), severe disease (385; 65%) or intermediate disease (86; 15%). Total BPA was measured in spot urine samples collected on the day of angiography using online solid phase extraction with SPE-HPLC-MS/MS (LOD 0.5 µg/L). The 117 (17%) with values below the LOD were assigned a value of 0.28 µg/L. Multivariable logistic regression was used to examine the relationship between standardized Z-score BPA values and CAD (severe versus normal or intermediate versus normal) adjusted for age, sex, occupational social class, diabetes, and BMI (categorized as underweight, recommended, overweight, obese I and obese II). Sensitivity analyses were used to examine effects of serum creatinine, older age, diabetes, and alcohol intake (and potentially smoking which was listed in the methods but not the results or discussion).

The initial proposed analysis, comparing BPA levels between those with one or more severe stenosis to other patients, did not find significant differences. When patients with severe disease were separately compared to those with normal coronary arteries, the odds were elevated in models adjusted for just age and sex (1.51; 1.07-2.14) and in the fully adjusted models (1.43; 1.03-1.98). For those with intermediate disease, the results were close to significance (P=0.06) with slightly larger odds ratios (1.79; 0.98-3.27 and 1.69; 0.98-2.94, respectively) but a small sample size. The factors examined in the sensitivity analyses did not change the results.
Spot urine BPA levels measure short-term exposure. Therefore, there is no way to establish temporality, *i.e.* that differences in BPA levels predated CAD changes. Both BPA and CAD could be associated with other lifestyle differences – potentially related to diet and to consumption of food and beverages in containers containing BPA. Also, BPA has been reported to be associated with obesity, a risk factor for CAD, raising concern that categorization of BMI may have allowed positive associations due to residual confounding. Additional limitations include the study's small sample size, and the fact that odds ratios and confidence intervals were either not statistically significant or close to the null. This study does examine a potential intermediate in the development of cardiovascular disease; however, **this study has no utility for HI and no utility for RA.**

Urinary bisphenol A concentration and risk of future coronary artery disease in apparently healthy men and women (Melzer *et al.*, 2012b)

This nested case-control study used participant data from the European Prospective Investigation of Cancer (EPIC) and Nutrition-Norfolk cohort to assess the relationship between spot urinary

BPA (uBPA) and coronary artery disease (CAD) by comparing concentrations from baseline clinical examination stored samples of cases who developed CAD with those of controls who remained free of CAD during follow-up.

The current study's analytical sample of 758 incident CAD cases and 861 controls followed for 10.8 y was based on a previously selected EPIC-Norfolk CAD case-control set (Boekholdt *et al.* 2004a), derived from those original recruits, that matched 2 controls to each case by sex, age within 5 y, and date of clinic visit within 3 months; however, because only the CAD-, diabetes mellitus (DM)-, and stroke-free 40-to-74-y-olds with an available urine sample and valid uBPA measure were included, the original Boekholdt *et al.* 2004a matching did not always hold. Demographic and covariate data were collected by questionnaires. The case definition was hospital diagnosis or death from coronary heart disease during follow-up, which occurred until first CAD onset or December 2003.

Nonfasting blood samples taken at baseline were processed "soon after," using a chemistry analyzer, or stored at -80°C. Total (free and conjugated) uBPA concentration measurement used NHANES/CDC methods; the Good Laboratory Practice-compliant quality control system included reagent blanks to confirm that the EPIC stored samples contained "almost exclusively" metabolized compound and minimal leaching. The limit of detection (LOD) was <0.050 ng/mL uBPA; the limit of quantification was 0.50 ng/mL uBPA. For analysis, the value 0.28 ng/mL was assigned to uBPA assays below the LOD [n=190 out of 861 (22.1%)] and cases [n=140 of 758 (18.5%)]; outlier uBPA concentrations exceeding 80.1 ng/mL were excluded. Some of the original EPIC age-, sex, and clinic date-matched case-control sets were incomplete because of urine sample availability (and other reasons): 217 of 861 controls (25.2%) had no matched case, and 251 of 758 cases (33.1%) had no matched control. Mean uBPA in the study was 3.65 ng/mL; median uBPA was 1.3 ng/mL. Controls without, compared to those with, matched cases had significantly higher uBPA [odds ratio (OR): 1.41, 95% confidence interval (CI): 1.17-1.70; p<0.0001] and were less likely to be obese (OR: 0.04, 95%CI, 0.21-0.75, p=0.005). Significantly fewer women than men were observed among the cases without, compared to those with, matched controls (OR: 0.58, 95% CI: 0.37-0.90), p=0.014). Given these significant differences among the matched compared to the unmatched cases and controls, the main analysis assessed the case-control groups without matching, using unconditional logistic regression models; a subanalysis assessed the matched sets using conditional logistic regression models. The models adjusted for potential confounders, such as socioeconomic markers, urinary creatinine, age, sex, education, occupation, body mass index (BMI), smoking, systolic blood pressure (mmHg), total cholesterol, HDL, LDL, triglycerides, and physical activity. Generalized additive models with penalized cubic regression splines were used to explore the relationship between uBPA-CAD diagnosis and identify departures from linearity.

In unconditional logistic regression modeling (cases and controls not matched, n=1619), after adjusting for age, sex, education, occupation, and urinary creatinine, the per standard deviation (SD) of 4.56 ng/mL uBPA linear increases in uBPA concentration were significantly associated with incident CAD (n=1579; adjusted OR (AOR): 1.14; 95% CI: 1.03-1.26, p=0.021), but with further adjustment for CAD risk factors (BMI, smoking blood pressure, total cholesterol LDL,

HDL, triglycerides, physical activity), the association weakened (n=1477 [note: abstract wrongly shows n=1744]; AOR=1.11, 95% CI: 1.00-1.23, p=0.058) and was not statistically significant. The generalized additive model showed a trend for a linear relationship between SD increases in uBPA and incident CAD (for smoothed term, p=0.068) but was not statistically significant. Results of sensitivity analyses using variations of the CAD risk factor/fully adjusted unconditional logistic regression model (*e.g.* adjusting for medical conditions associated with CAD such as obesity and inflammation) supported the positive association between uBPA and incident CAD: Excluding the earliest 3 years of follow-up to remove those closer to CAD onset at uBPA sample collection (AOR: 1.12, 95% CI: 1.00-1.26, p=0.050). The conditional logistic regression model of data from the case and control pairs matched on age, sex, and clinic visit date indicated a significant association between SD increases in uBPA and incident CAD (OR: 1.34, 95% CI: 1.12-1.62, p=0.0015), but the authors did not specify covariates taken into account.

The timing of the single spot urine samples was not specified, and variation was not addressed. Although the authors could confirm that "the EPIC stored samples contained almost exclusively metabolized compound, showing minimal leaching of BPA from collection or storage vessels," they did not address post-storage cross-contamination exposures or safeguards. The authors point out that low exposure reduces power to detect true associations, observing the relatively low median concentration of uBPA in this analytical sample compared to NHANES 2003-2004 (2.7 ng/mL). In addition, the relatively high percentage of controls with uBPA concentrations below the LOD is problematic because of the indeterminable potential effect on the estimated association between uBPA and incident CAD. Important potential confounders were measured at only one point in time; furthermore, changes in case behaviors after diagnoses were not explored. Nevertheless, this study is unique in its use of prospective data, with some results indicating a positive association between uBPA and incident CAD although without dose-response effect, *i.e.*, demonstrated increases in uBPA associated with CAD; **the findings from the main models and sensitivity analyses merit further examination of this possible association and may have limited utility in HI and no utility for RA.**

<u>Associations between circulating levels of bisphenol A and phthalate metabolites and coronary risk in the elderly (Olsén *et al.*, 2012)</u>

The objective of this cross-sectional study was to investigate the association of circulating levels of BPA, four phthalate metabolites [monoisobutyl phthalate (MiBP), monomethyl phthalate (MMP), monoethyl phthalate (MEP), and mono-2-ethylhexyl phthalate (MEHP)] or both to (i) coronary risk estimated by the Framingham Risk Score (FRS) and (ii) the cardiovascular risk factors included in FRS considered separately. FRS is a scoring system that uses age, gender, smoking history, blood pressure, HDL-cholesterol, LDL-cholesterol, and blood glucose levels to estimate coronary event risk over the course of ten years among individuals without previously diagnosed coronary heart disease.

The study analyzed data on cardiovascular risk factors (BMI, gender, smoking history, blood pressure, HDL-cholesterol, LDL-cholesterol, and blood glucose levels), circulating levels of BPA and phthalate metabolites from 1016 senior (70year-old) residents of Uppsala Community,

Sweden (PIVUS study). All samples and measurements were taken after an overnight fast. Blood serum BPA and phthalate metabolites samples from brachial artery (arterial cannula) were analyzed using isotope liquid chromatograph/tandem mass spectrometer (API4000LC-MS/MS), following the Centers for Disease Control and Prevention general procedures. The assay used blank and spiked calf serum as negative and positive control. Composition of storage containers and lab equipment was not addressed. Blood pressure was measured by a calibrated mercury sphygmomanometer in the non-cannulated arm. Lipid variables and fasting blood glucose were measured by standard laboratory techniques described by Carlsson *et al.* (2010).

Variables of the compounds were log-transformed to achieve normal distributions. The statistical program STATA 11 (College Station, TX, USA) was used to evaluate relationships by linear multiple regression, logistic regression, and multivariable fractional polynomial models. Gender, BMI, serum cholesterol and triglycerides, hypertension, smoking, and diabetes mellitus were used as confounders in the analysis, except for the confounding variable being analyzed as the outcome.

BPA, MEHP, and MMP were associated ($p<0.05$) with LDL-cholesterol, and BPA and MEHP were associated with HDL-cholesterol. However, after Bonferroni correction ($p<0.0012$), the authors concluded there were no significant relationships between circulatory BPA and coronary risk, assessed by the FRS. The authors also did not observe any significant associations between BPA and any of the coronary risk factors assessed separately in this elderly population.

The study did not find any associations between circulatory BPA and coronary risk estimated using FRS or cardiovascular risk factors in an elderly Swedish population. Furthermore, the very narrow population of this study, which enrolled only 70-year-old Uppsala residents, constrains the generalizability of its findings. Other important limitations include its cross-sectional design, with exposure measurement at one point in time, which may or may not relate to the critical window of exposure for the coronary outcome, as well as measurement of BPA from serum, which is problematic (Calafat *et al.*, 2013) because of the possibility that contamination may mask actual exposure, and unspecified composition of lab equipment and storage vessels, which may have introduced another source of contamination. **This study has no utility for HI and no utility for RA.**

<u>Relationship between urinary bisphenol A levels and prediabetes among subjects free of diabetes (Sabanayagam *et al.*, 2013)</u>

This study uses data from the 2003–2008 National Health and Nutrition Examination Survey (NHANES), a cross-sectional, multistage, stratified, cluster-sampling population-based survey, to examine the association between urinary BPA levels and prediabetes in 3,516 subjects. The study sample consisted of (N = 4,792) participants aged ≥20 years with urinary BPA measurements available. They further excluded subjects with diabetes (n = 471), self-reported cardiovascular disease (n = 499) and subjects with missing data on covariates included in the multivariable model (n = 306), leaving 3,516 participants for the current analysis of whom 1,108 had prediabetes.

Measures of environmental phenols were derivatized to alkyl or acyl derivatives before GC-mass spectrometry (GC/MS) analysis and were measured using solid phase extraction coupled to high-performance liquid chromatography - isotope dilution tandem mass spectrometry (HPLC-MS) with peak focusing. The lower limit of detection for BPA concentrations was 0.36 ng/ml in 2003–2004 and 0.4 ng/ml in 2005–2006 and 2007–2008. A value of 0.28 ng/ml for BPA concentration was assigned to the samples with measurement below the level of detection. Prediabetes was defined as fasting plasma glucose concentration of 100–125 mg/dL (5.6–6.9 mmol/L) or 2-h glucose concentration of 140–199 mg/dL (7.8–11.0 mmol/L) or an A1C value of 5.7–6.4 % among those without diabetes based on the recent American Diabetes Association recommendations. They calculated the odds ratio [(OR) (95 % confidence interval (CI)] of prediabetes associated with tertiles of BPA by taking the lowest tertile (tertile 1) as the referent, in two multivariable logistic regression models: the age and sex-adjusted model, the multivariable model, additionally adjusting for race/ethnicity (non-Hispanic whites, non-Hispanic blacks, Mexican Americans, others), education categories (below high school, high school, above high school), smoking (never smoker, former smoker, current smoker), alcohol intake (non-drinker, moderate drinker, heavy drinker), physical inactivity (absent, present), BMI (normal, overweight, obese), mean arterial blood pressure (mm of Hg), total serum cholesterol to high-density lipoprotein (HDL) cholesterol ratio, and serum C-reactive protein (mg/dL). BPA was modeled as an ordinal variable. To examine the consistency of the association between BPA and prediabetes, they performed subgroup analyses stratified by gender and BMI. Interactions were formally evaluated. Sample weights that account for the unequal probabilities of selection, oversampling and non-response were applied for all analyses. All analyses were done using SAS (version 9.2; SAS Institute, Cary, NC) and SUDAAN software.

Overall, a positive association was observed between higher levels of urinary BPA and prediabetes, independent of potential confounders including body mass index, alcohol intake, blood pressure, and serum cholesterol levels. Compared to tertile 1 (referent), the multivariate adjusted odds ratio (95 % confidence interval) of prediabetes associated with tertile 3 of BPA was 1.34 (1.03–1.73), p-trend = 0.02. In subgroup analysis, this association was statistically significant among female and obese subjects.

This is a large multiethnic, nationally representative sample of US adults. The measurement methods of BPA were validated. Care has been taken to avoid BPA contamination in measurement procedures. The covariates measurements were validated, and outcome was defined within clinical context. The modifying effects, however, were not clearly identified. The study results were derived from single urine samples that may not represent the exposure that is relevant to development of prediabetes. Creatinine adjustment for urine BPA measurements was not performed, and information on sampling such as sampling day food intake or time of sampling was not reported.

No clear overall dose-response relationship is found (non-linearity). Instead, a similar effect is shown for both second and third tertiles, when compared to the lowest tertile. However, the association between BPA and prediabetes was statistically significant among female and obese subjects. Given the limitations in the study, the results are interesting and merit further examination to see if the finding can be repeated (*e.g.*, whether it is statistically significant and identified in other

subgroups. **This study has limited utility for HI and no utility for RA.**

Bisphenol A and Peripheral Arterial Disease: Results from the NHANES (Shankar *et al.*, 2012a)
Data from the 2003-2004 wave of the National Health and Nutrition Examination Survey (NHANES) were used to examine the association between urinary BPA levels and peripheral arterial disease (PAD). The sample consisted of 753 men and women over 40 years of age. PAD was defined as having an ankle-brachial index (ABI) of less than 0.9, in accordance with American Diabetes Association guidelines. Individuals with ABI of more than 1.5 were excluded from analyses due to severe arterial rigidity (n=3). BPA was measured in a urinary spot sample using gas chromatography-mass spectrometry analysis, and the lower LOD was 0.36 ng/mL. The number of individuals with BPA values below the LOD was not given. Urinary BPA was categorized into tertiles: lowest (<1.4 ng/mL), middle (1.4-3.5 ng/mL), and highest (>3.5 ng/mL). BPA exposure tertile was positively associated with PAD for comparisons of the highest to lowest tertile but not for the middle compared to the lowest tertile. Compared with the lowest tertile, those in the highest tertile of BPA exposure had 3.73 (95% CI: 2.03, 6.86) times higher odds of PAD in analyses controlling for age and sex. After controlling for age, sex, race/ethnicity, education, household income, smoking status, pack-years of smoking, alcohol intake, BMI, hypertension, diabetes, urinary creatinine, glomerular filtration rate, and total cholesterol, the odds ratio for PAD for the highest compared to the lowest tertile of BPA exposure was 2.69 (95% CI: 1.02, 7.09). The association persisted when BPA was treated as a continuous variable. In supplementary analyses, the authors also report that those in the highest BPA tertile had 1.83 (95% CI: 1.01, 3.31) times higher odds of self-reported cardiovascular disease than those in the lowest tertile.

This study has a number of strengths, including the use of NHANES, a nationally representative dataset. The main limitation is the cross sectional study design, which limits the ability to draw conclusions about temporality or causality. Diet, particularly, might differ due to knowledge about current atherosclerotic vascular disease and lead to changes in BPA levels. Residual confounding was also a major concern and could have accounted for the observed differences. The authors showed that a number of factors associated with PAD were unequally distributed across BPA tertiles. When adjusted for covariates, even with relatively broad categories, the odds ratios for the highest tertile decreased so that the lower bound of the 95% CI was very close to 1.0. Also urinary BPA was measured in a single spot sample rather than multiple samples, which is not ideal due to the variability involved in urinary BPA levels. Finally, the confidence intervals of several of the odds ratios are very close to including the null value (1.0) so it is difficult to draw conclusions from these results. **This study has no utility for HI and no utility for RA.**

Urinary Bisphenol A and Hypertension in a Multiethnic Sample of US Adults (Shankar *et al.*, 2012b)
This cross-sectional study investigated the association between urinary BPA levels and hypertension. The authors included 1380 subjects from the National Health and Nutritional Examination Survey 2003-2004 who had urinary BPA levels and information on covariates included in the multivariate model, 580 of whom had hypertension. Subjects were considered to have hypertension if they reported concurrent blood pressure reducing medication use and/or

blood pressures >140/90mmof Hg. Patients were grouped into tertiles based on urinary BPA level. The authors found a positive association between increasing levels of urinary BPA and hypertension in the multivariate logistic regression model adjusting for age, gender, race/ethnicity, educational categories, smoking, alcohol intake, body mass index (BMI), hypertension, diabetes mellitus and total serum cholesterol levels. Compared to BPA Tertile 1 (referent; <1.5 ng/mL), the multivariate adjusted odds ratio (95% confidence interval) of hypertension was 1.50 with Tertile 3 (>4.0 ng/mL) (95% CI; 1.12−2.00; P-trend = 0.007); however, a statistical significance was not seen when Tertile 2 (1.5-4 ng/ml) was compared to Tertile 1. Although not statistically significant, the positive magnitude of association between increasing BPA levels and hypertension was seen in various stratified subgroup analyses by race/ethnicity, smoking status, BMI, and diabetes status.

The strength of this study is that the study population is a large multiethnic and representative sample of US adults from the National Health and Nutritional Examination Survey 2003-2004. However, this is a cross-sectional study looking at the relationship between one urinary BPA level and hypertension, which limits the ability to make a causal conclusion. The BPA exposure was characterized based on one urinary BPA level, which may not be reflective of chronic exposure related to the possible development of hypertension. Also, there is a possibility that patients who took anti-hypertension drugs were not actually hypertensive but treating other diseases. The study only showed statistically significant association between hypertension and the highest tertile urinary BPA level. Therefore, the dose-effect relationship is absent. **Based on the stated limitations, this study has limited utility for HI and no utility for RA.**

Urinary bisphenol A and type-2 diabetes in U.S. adults: data from NHANES 2003-2008 (Silver *et al.*, 2011)

This cross-sectional study investigated a possible statistical relationship between spot urinary BPA concentration and HbA1c or Type II diabetes mellitus (T2DM) in previously reported National Health and Nutrition Examination Survey (NHANES) data acquired from 4,389 adults from the 2003/4, 2005/6, and 2007/8 cycles. Study participants (mean age: 46.5) had been selected from a larger population of 4,792 NHANES adults aged 20 or older based on completeness of data. Spot urine samples had been provided by a random subset of 1/3 of NHANES participants; total BPA (free + conjugated) was measured using HPLC/MS following extraction. HbA1c levels had been measured in single blood samples from NHANES participants using HPLC. T2DM was defined as HbA1c≥6.5% or self-reported use of diabetes medication. Demographic information in NHANES had been collected by self-report, interview, or physical exam. Generalized linear models were constructed to assess possible relationships between urinary BPA and HbA1c/T2DM data. Model covariates included age, gender, BMI, urinary creatinine, race/ethnicity, education, income, waist circumference, and smoking status.

Mean creatinine (Cr)-corrected BPA levels were 2.4, 1.8, and 2.0 ng/mg in NHANES 03/4, 05/06, and 07/08 samples, respectively (p<.001). Percentage of participants with T2DM was 7.8, 9.0, and 10.7 across the three samples, respectively (p=.06). Visual inspection of Table 1 indicated that Cr-corrected urinary BPA values did not track with mean HbA1c levels (5.4%, 5.4%, 5.6%), proportion of subjects utilizing diabetes medication (6%, 6.9%, 8.1%), or

proportion of subjects with diabetes (values above) for the respective 03/04, 05/06, or 07/08 samples. Rather, urinary BPA levels were higher in males, current smokers, adults aged 20-39, and those with household income <$20k, but not in those with diabetes (Table 2). Logistic regression models of the data produced a final adjusted OR for T2DM of 1.08 (95% CI: 1.02, 1.16) and a value of 0.017 (95% CI=0.001-0.032) for the final adjusted linear regression coefficient for HbA1c(%), relative to a doubling in urinary BPA concentration. We agree with the authors that the reported values for the pooled data were driven by the slightly positive association reported for the 2003/4 cycle. CIs for the ORs or coefficients for the 2005/6 and 2007/8 cycles overlapped with unity (OR) or were negative (β), respectively, indicating a lack of a positive association between T2DM/HbA1c and urinary BPA in more recent NHANES cycles. The lack of a positive association in 2 out of 3 NHANES survey cycles argues against the presence of a true association in the overall population, which is also reflected in the very weak signal (OR=1.08) reported for the pooled data. The cross-sectional nature of the study, other limitations in study design (single spot urine, single blood draw per subject, and lack of controlling for potential confounding by dietary factors such as consumption of canned soda), and the weakness and inconsistency of the reported association preclude a conclusion of causal relationship. **This study has no utility for HI and no utility for RA.**

Fetal Growth and Prenatal Exposure to Bisphenol A: The Generation X Study (Snijder *et al.*, 2013)

This cohort study evaluated the effect of prenatal exposure to BPA, as measured by urinary BPA level, on fetal growth. The study used a Dutch population-based prospective cohort study; 9778 pregnant women with expected delivery between April 2002 and January 2006 participated in the study (61% response). In 2006, random samples of 100 women with one urine sample were analyzed. In 2010, random samples of 120 women with multiple urine samples were used; 80 women had three samples and 40 women had two samples. After excluding one twin pregnancy, 219 women with 419 urine samples were available, 26% from first trimester, 28% from the second trimester, and 46% from the third trimester of pregnancy. All urine samples were collected between February 2004 and November 2005, between 0800 and 2000 hours in 100 mL polypropylene urine collection containers. The urine specimens were analyzed for BPA using tandem mass spectrometry. The specimens from 2006 and 2010 were analyzed at different institutes in Germany; the limit of detection was 0.26 µg/L for 100 specimens analyzed in 2006 and 0.05 µg/L for 120 specimens analyzed in 2010. The fetal growth rates were estimated using the second- and third-trimester fetal ultrasound measurements, combined with measurements of fetal size at birth. Two measures of fetal growth were available for 99% women (n=217), and three measures of fetal growth were available for 72% of women (n=152).

Potential confounders of fetal growth were included as covariates in models for estimating associations: maternal age, pre-pregnancy weight, height, educational level, ethnicity, parity, smoking, alcohol use, and folic acid use. Creatinine-based urinary BPA levels were log-transformed ($lnBPA_{CB}$) to obtain normal distributions. Linear regression models with repeated-measures were used to estimate associations for $lnBPA_{CB}$ in urine samples from the first, second, and third trimester with fetal growth measurements from the second trimester, third trimester, and at birth, respectively. BPA_{CB} was also analyzed as a categorical variable (quartiles).

The univariate and multivariate repeated linear regression analyses using all available measurements (419 samples from 219 women) did not show statistically significant associations for BPA_{CB} as a categorical or continuous predictor of fetal growth rate (*i.e.*, fetal weight or head circumference). Among 80 women with three BPA measurements, women with the highest BPA_{CB} quartile (> 4.22 µg/g creatinine) had statistically significant lower growth rates for fetal head circumference than did women with the lowest BPA_{CB} quartile (< 1.54 µg/g creatinine), with estimated differences in mean values at birth of –3.9 cm (11.5% of mean); however, a statistically significant lower growth rate was not seen for fetal weight in women with the highest BPA_{CB} quartile when compared to the lowest BPA_{CB} quartile. A linear regression analyses for repeated measures limited to these 80 women showed a statistical significance between continuous $lnBPA_{CB}$ and fetal growth rate, where decreases in beta coefficient (representing the average decrease in SD) for fetal weight (-0.017) and head circumference (-0.018) were observed per unit increase in BPA; however, the association did not show a relationship in categorical analysis, as the fetal growth rate did not monotonically decrease with increasing quartiles of BPA_{CB} exposure. Compared to the study population as a whole, 80 women with three samples were more likely to be highly educated and of Dutch origin.

Although the results of this study suggest that increasing the number of urinary BPA measurements per subject during pregnancy may result in better exposure-response estimates, the study is not conclusive in demonstrating that higher BPA levels in prenatal urine may result in lower fetal growth rate, since the findings were only positive in a subgroup of 80 women with all three urinary BPA measurements. Other limitations of the study include a relatively small sample size and the generalizability of study results. **The findings on the BPA-associated lower fetal growth rate in a subgroup of 80 women with triplicate measurement needs to be further confirmed in a well-designed prospective study. This study has limited utility for HI and no utility for RA.**

Circulating serum xenoestrogens and mammographic breast density (Sprague *et al.*, 2013)
This cross-sectional study examined the relationship between serum BPA and breast density in 231 postmenopausal women, aged 55-70 years who had screening mammograms between in 2008 and 2009 as part of the Wisconsin Breast Density Study. The study also analyzed serum phthalates, parabens and other phenols. Total breast area and dense breast area were classified from digital images of the left breast and percentage of dense to total area determined. BPA was measured in blood collected immediately following mammography using solid phase extraction with HPLC-MS/MS with APCI negative ionization (LOD 0.24 µg/L). BPA was categorized as below LOD (193; 84%), below median of detectable values (median=0.55 µg/L; 35, 15%) and above median (34, 11%). Information on potential confounders was collected using a questionnaire at the same time. Multivariable linear regression was used to examine the relationship between ordinal BPA categories and square root of breast density adjusted for age, BMI, parity, family history of breast cancer, physical activity, and smoking, estradiol levels and BMI as potential effect modifiers. Square root of dense area was also analyzed as a separate outcome.

BPA was associated with square root of percentage breast density (p_{trend}=0.01) but was modified by BMI, with an increased trend in 166 women who were not obese (graphical plots showed percent breast density to be about 15%, 16% and 23% across categories) but a flat trend in 96 women who were obese (about 8%, 9% and 8%). The relationship of BPA with square root of dense area approached significance (p_{trend}=0.08) and there was no significant effect modification with obesity.

Serum BPA levels measure short-term exposure. Therefore, there is no way to establish constancy of exposure or temporality, *i.e.* that differences in BPA levels predated changes in breast density. Epithelial and stromal tissues contribute to radiodensity while fat tissue is more radiolucent. The authors cite studies that suggest that BPA may be present and released as a source of continuing exposure from fat tissue. This would seem to suggest that higher serum BPA should be associated with lower breast density. Also, higher BPA has been reported to be associated with obesity, the group with the no trend between breast density and BPA. Diet was not examined - both BPA and breast density could be associated with other lifestyle differences – potentially related to diet and to consumption of food and beverages in containers containing BPA. The authors also expressed concern about limitations and study interpretation. **This study has no utility for HI and no utility for RA.**

Association between bisphenol A and abnormal free thyroxine level in men (Sriphrapradang *et al.*, 2013)

This cross-sectional study used data from the nationally representative Thai National Health Examination Survey (NHES) IV 2009, to assess the relationship between BPA exposure and thyroid function. The participants from this study derived from the NHES IV 2009's multistage, stratified sampling of the population, which consisted of a final sample of 20,450 individuals. Face-to-face interviews with standard questionnaires were used to collect demographic, medical history, and medications data, followed by physical examination and blood sample collection. Pregnant women and individuals with thyroid disorder or thyroid function-altering medications (such as lithium and amiodarone) use histories, as well as those with recent iodinated contrast exposures, were excluded. Body mass index (BMI) was calculated from measurements taken during physical examination. A subset of study participants was randomly selected by software program according to age group (15-29, 30-44, 45-59, 60-69, 70-79, and ≥80 years), sex, and region (urban, rural) for serum analysis of BPA, TSH, free thryroxine (FT_4), thyroid peroxidase antibody (TPOAb), and thyroglobulin antibody levels (TgAb). Of the 2700 randomly selected, 2586 serum samples had available serum samples. Serum samples for BPA, TSH, FT_4 , TPOAb, and TgAb analyses were collected at the same time. BPA was measured by competitive enzyme-linked immunosorbent assay with intra-assay and inter-assay precision of 7.0% and 13.6%, respectively. Electrochemiluminescence immunoassays with respective intra-assay precision of 3.6%, 1.31%, 9.2%, and 6.1% were used to measure serum TSH, FT_4, TPOAb, and TgAb. Of the data from 2586 participants who were 15 years of age or older and had complete serum data available, 107 were excluded because of outlier values for TSH or FT_4 . Those aged 15-17 years old were analyzed separately because of significant differences between adults and adolescents in thyroid hormone levels indicated by previous studies and also observed among the participants of this study. Those with TPOAB (n=368) and TgAb (n=280) autoantibodies were also analyzed

separately because of the possibility of abnormal thyroid function. Analyses included means, standard deviations, interquartile ranges, and comparisons of differences between means and medians using the *t* and Mann-Whitney *U* tests. Multivariable linear regression in SPSS was used to assess the relationship between thyroid function (TSH or FT_4 level) and BPA level, adjusting for covariates in men and women.

For the study sample of 2340 Thai adults, BPA was detected in 52.8% of the serum samples (median: 0.33 ng/mL, range: 0.00-66.91 ng/mL). Among men but not women, a significant inverse relationship was detected between serum BPA and FT_4 levels ($r=-0.14$, $p<0.001$). No significant associations were observed between serum BPA and TSH among the men or the women.

While the study includes a large nationwide sample, it has significant methodological limitations. BPA exposure was measured in serum, which current evidence indicates is problematic (Calafat *et al.*, 2013) because of the inability to distinguish between sample contamination and true exposure; also, unspecified containers and equipment were used. In addition, the limitations of cross-sectional study design apply here, including exposures measured at one point in time that may or may not bear any relation to the critical time of exposure for observed thyroid function levels and that may represent individual variation based on environmental or genetic/metabolic differences or interactions with other exposures. **Based on the problematic use of serum BPA measurement of exposure and the limitations of cross-sectional design, this study has no utility for HI and no utility for RA.**

Associations of prenatal exposure to phenols with birth outcomes (Tang *et al.*, 2013)
A study of 567 pregnant women was conducted to investigate the relationship between prenatal phenol exposure and birth outcomes, including birth weight, length, and gestational age. The participants were recruited from hospitals affiliated with Nanjing Medical University in China between September 2010 and April 2012. Eligible women with singleton pregnancy were ≥18 years old and reported no assisted reproduction and medical complications (*e.g.*, gestational or preexisting diabetes, hypertension, HIV infection or AIDS). Newborn infants with severe neonatal illness, *e.g.*, very premature births (delivery at <32 completed gestational weeks or birth weight <1500 g), genetic abnormalities, or malformations, were excluded. A total of 592 women met the eligibility criteria.

Urine samples were collected from each subject during hospital admission for delivery, and were frozen at -20^{o} C until analysis. No information about time of day of urine collection was provided. No information addressed whether collection, storage, pipetting and other utensils in contact with urine were BPA-free. Concentrations of bisphenol A (BPA), benzophenone-3 (BP3), 4-n-octylphenol (4-n-OP) and 4-n-nonylphenol (4-n-NP) were measured in maternal urine using ultra high performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS). Infant sex, birth date, parity, weight, and crown-heel length were obtained from hospital delivery logs and medical records. A questionnaire was conducted with each participant by face-to-face interview, collecting further demographic and health information. A three-level ordinal variable was formed: all samples with concentrations <LOD were assigned to the lowest group, and two

equally sized groups were formed among the samples with detectable concentrations to form the middle- and high-exposure groups. Multivariate linear regression modeling was used to examine the relationship of prenatal exposure to phenols and birth outcomes.

No statistically significant association between gestational duration was found with highest compared to lowest urinary BPA. Between middle and low exposure groups, BPA was negatively associated with gestational duration (beta coefficient adjusted = -0.48 week; 95% confidence interval = -0.91, -0.05). After stratification by gender, consistent results were found in infant boys (compared to those in all infants), but no significant association was observed for girls.

The study sample is a convenience sample recruited at NMU-affiliated hospitals in a metropolitan city in China with questionable generalizability to other populations. Of the confounders taken into account, most were self-reported and not otherwise verified, except for BMI for which measurements were taken at delivery. Exposure measurement methods were validated, but BPA concentration was measured from urine samples taken at only one time point (at admission for delivery, introducing the inconsistency of different times of day), and it is unknown how that measurement relates to exposure during the rest of the pregnancy. Also, it is not clear how storage time affects measured BPA concentrations. **This study has limited utility for HI and no utility for RA.** Non-linearity in effect was observed. There is no clear dose-response relationship found in this study for BPA and decreased time of gestation in the population of pregnant women from Nanjing, China.

Bisphenol A in polycystic ovary syndrome and its association with liver-spleen axis (Tarantino *et al.*, 2013)

This cross-sectional study examined associations of serum bisphenol A (BPA) levels with markers of low-grade chronic inflammation, hepatic steatosis, insulin resistance, and hyperandrogenism in women with polycystic ovary syndrome (PCOS). Forty premenopausal women with PCOS were consecutively enrolled from 2009 to 2011 at the Federico II University Hospital in Naples, Italy. Diagnosis of PCOS was based on the Rott-PCOS criteria. Twenty healthy (*e.g.* regular menstrual cycles and no hyperandrogenemia, hirsutism, or acne), age-matched control women were also selected from among hospital employees. Subjects with concurrent conditions or use of medications that could affect sex hormone, inflammation/metabolic status or body weight, *i.e.* hypothyroidism, use of oral contraceptives, insulin sensitizing agents, glucocorticoids, anti-androgens, ovulation inducing agents, or anti-obesity drugs were excluded.

Ovulatory state and hirsutism were assessed using the Ferriman-Gallwey score. Homeostasis Model Assessment of Insulin Resistance (HoMA-IR), laboratory liver tests, Testosterone, Sex Hormone-Binding Globulin (SHBG), Free Androgen Index (FAI), C-Reactive Protein (CRP), Interleukin (IL)-6were determined, and hepatic steatosis (HS) and spleen longitudinal diameter (SLD) quantified through ultrasound. Serum BPA concentrations were determined using an anti-rabbit IgG antibody solid-phase competitive ELISA kit (IBL Co., Ltd., Gunma, Japan). Although the kit measurement range was reported to be 0.3–100 ng/ml BPA, reported results ranged from 0.1-6 ng/ml. The assay was reported to have 100% cross-reactivity with BPA, 85% with BPA-

glucuronide, 68% with BPA-Na-sulfate and 8.5% with bisphenol.

Although all measured variables were compared between case and control women using unpaired t-tests or Mann-Whitney U- tests, much of the study actually focused on comparisons only among women with PCOS. The controls were used to define the cut-off for high BPA (0.45 ng/ml as their 95^{th} percentile, *i.e.* 1/20 controls above this level). Among women with PCOS, the authors found mean values of HoMA-IR, FAI, HS, CRP and SLD, but not age, BMI, or IL-6 differed among women dichotomized by BPA level and that mean serum BPA differed between women when dichotomized by HoMA-IR, FAI, HS, and SLD. There were no differences in BPA levels between lean (18-24.9 kg/m^2) and overweight/ obese women (25-40 kg/m^2) with PCOS. The authors found a linear correlation of serum BPA with liver enzymes and BMI.

Data from all 60 women were included, ignoring case or control status, in a stepwise, multivariable, linear regression, using serum BPA level as the outcome. BMI, HoMA-IR, HS severity, FAI, and SLD were included in the model. SLD and FAI were reported as predictors of BPA level (β coefficients 0.379, p=0.007 and 0.343, p=0.014, respectively).

The study's cross-sectional design, using a single measurement of serum BPA (see PK section for issues related to serum measurements) after PCOS diagnosis, is inadequate to support a causal role of BPA in the hypothesized pathway of PCOS pathogenesis. Further, although variables were dichotomized many different ways for the analyses, sizes of comparison groups were never reported. Results from different analyses of the same variables were not always consistent. Combining PCOS cases and hospital employee controls in a single linear model with serum BPA as the outcome rather than as a predictor variable is not an appropriate analysis nor does this, or the correlation analyses, test a hypothesis of a causal pathway involving BPA. **This study has no utility for HI and no utility for RA.**

Bisphenol A and Metabolic Syndrome: Results from NHANES (Teppala, *et al.*, 2012)
Data from the National Health and Nutrition Examination Survey (NHANES), 2003-2008, was used to examine the association between urinary BPA levels and Metabolic Syndrome (MetS). The sample consisted of 2,104 men and women over 18 years of age. MetS was defined according to the revised Adult Treatment Panel III guidelines, using the following five characteristics: abdominal obesity, hypertension, elevated serum triglycerides, glucose intolerance, and reduced HDL. Subjects who met 3 or more of the 5 criteria were classified as having MetS (n=741). BPA was measured in a urinary spot sample using gas chromatography-mass spectrometry analysis, and the lower LOD was 0.36 ng/mL. Urinary BPA was categorized into tertiles: lowest (<1.4 ng/mL), middle (1.4-3.4 ng/mL), and highest (>3.4 ng/mL).

Descriptive analyses showed that participants with higher levels of BPA were more likely to be non-White, currently smoking, have higher levels of alcohol intake, higher triglycerides, lower HDL, and higher urinary creatinine levels. BPA exposure tertile was positively associated with MetS in both age and sex-adjusted models and after adjusting for age, gender, race/ethnicity, household income, smoking, alcohol intake, moderate physical inactivity, and urinary creatinine. Compared with the lowest tertile of BPA exposure, those in the highest tertile had 1.53 (95%CI:

1.09, 2.15) times the odds of MetS in the age and sex-adjusted model, and 1.51 (95%CI: 1.07, 2.12) times the odds of MetS in the fully adjusted model. Those in the middle tertile had 1.26 (95%CI: 0.95, 1.68) times the odds of MetS in the age and sex-adjusted model, and 1.24 (95%CI: 0.93, 1.65) times the odds of MetS in the fully adjusted model.

This study is cross sectional in nature, meaning that no conclusions can be drawn about temporality or causality. In addition, it is important to account for the potential confounding role of diet. Consumption of canned soda and packaged, processed food is associated with BPA exposure as well as increased overweight/obesity and MetS. The authors did not control for any dietary factors in their analyses. Confidence intervals were close to the null (1.0) or included the null, meaning that strength of the associations was low. Also urinary BPA was measured in a single spot sample rather than multiple samples, which is not ideal due to the variability involved in urinary BPA levels. Strengths of the study include the use of four waves of NHANES, a nationally representative dataset. **This study has no utility for HI and no utility for RA.**

Association between urinary bisphenol A concentration and obesity prevalence in children and adolescents (Trasande *et al.*, 2012); Editorial: Brent RL. JAMA. 2013 Jan 9;309(2):134; Editorial: Trasande *et al.*, JAMA. 2013 Jan 9;309(2):134-5

A cross-sectional sample from the 2003-2008 National Health and Nutrition Examination Survey (NHANES) was used to examine the association between bisphenol A (BPA) levels and obesity in children and adolescents. The sample consists of 2,838 participants between the ages of 6 and 19 years who were randomly selected from 9270 NHANES children and adolescents for the measurement of urinary BPA. Body mass index (BMI) was assessed by trained health technicians, converted to sex- and age-standardized z scores, and used to classify subjects as overweight [Z-score $\geq$ 1.036 (85th percentile for age and sex)] or obese [Z-score $\geq$ 1.64 (95th percentile for age and sex)]. One spot urine sample from each participant was analyzed with liquid chromatography and tandem mass spectroscopy to determine BPA level. A BPA value of 0.3ng/mL was used when urinary BPA concentration was below the limit of detection. Urine was also analyzed for Benzophenone-3,4-tertoctylphenone and triclosan to test for specificity of association with BPA. BPA levels were categorized into quartiles-- quartile 1: urinary bisphenol A concentrations <1.5 ng/mL; quartile 2: 1.5-2.7 ng/mL; quartile 3: 2.8-5.5 ng/mL; quartile 4, ≥5.6 ng/mL. Information was obtained on potential confounders including age, sex, race/ethnicity, caregiver education, poverty-income ratio, 24-hour caloric intake, physical activity levels, daily hours of television watching, serum cotinine levels (to assess smoking).

Quartile 1 was defined as the reference group. In a logistic regression analysis adjusted for urinary creatinine concentration, the odds for obesity were increased in all three quartiles above reference [quartile 2: OR 2.22(95% confidence interval (CI) 1.53-3.23), quartile 3: OR 2.09 (CI 1.48-2.95), quartile 4: OR 2.53 (CI 1.72-3.74), all p<0.001]. When stratified by race/ethnicity, this finding was significant only in non-Hispanic white participants [quartile 2: OR 4.32 (CI 2.08-8.99), quartile 3: OR 4.21 (CI 2.01-8.77), quartile 4: OR 6.03 (CI 2.88-12.62)]. In multivariable regression analysis, controlling for the potential confounders noted above, results were similar with BPA quartiles 2, 3, and 4 significantly associated with being obese, [quartile 2: OR 2.24 (CI 1.54 to 3.24), quartile 3: OR 2.08 (CI 1.46 to 2.96) , quartile 4: OR 2.57 (CI 1.72-

3.83)], but not with being overweight. Analysis using log-transformed BPA concentrations (for non-normality of the data) showed a similar significant positive correlation between BPA concentration and obesity. In general other phenols were not associated with overweight or obese in bivariate analyses. Urinary benzophenone levels were associated with obesity, but this association was believed to have arisen by chance. Detailed discussion of this association is not provided.

In a letter to the editor, Robert L. Brent asserted that there may be higher levels of BPA in obese children is because BPA is a fat soluble molecule, making higher BPA levels the result of obesity and not the cause. Further, only one urine sample was taken for BPA analysis even though BPA urine levels can vary dramatically.

The cross sectional design of this study limits its utility for assessment of causality. Though the study collects dietary intake data for caloric estimates, these data cannot determine dietary sources of BPA. **This study has no utility for HI and no utility for RA.**

Bisphenol A exposure is associated with low-grade urinary albumin excretion in children of the United States (Trasande *et al.*, 2013)

This cross-sectional study evaluated whether spot urinary BPA is associated with low grade albuminuria, using NHANES data in US children aged 6-19 years. The study examined data from 710 of 2596 children in the 2009-2010 NHANES who had urinary BPA measurements and first morning urine samples with creatinine measurements. BPA was measured in one spot urine sample from each child, and was done on a different urine sample than the first morning urine sample for albumin:creatinine ratio (ACR) calculation. The ACR was log-transformed because of skewed distribution, and children with macro- (≥300 mg/g, n=5) or microalbuminuria (30-300 mg/g, n=38) were excluded in the analysis of ACR as a continuous variable. The probability of having either macro- or microalbuminuria was modeled using categorical analysis. BPA was analyzed using high-performance liquid chromatography and tandem mass spectroscopy. For BPA levels below the level of detection (50 or 7.1%), 0.3 ng/mL was substituted. Urinary BPA was log-transformed, and unweighted sample quartiles for urinary BPA were created to allow for non-linearity. BPA levels were adjusted for urinary creatinine level. Analyses were adjusted for the following confounders: hypertension, serum cotinine, race/ethnicity, caregiver education, poverty-income ratio, age, body mass index, cholesterol, and calculated HOMA-IR (insulin resistance calculated from fasting insulin and glucose). Using Stat 12.0, weighted univariable, bivariable, and multivariable analyses were conducted applying sample weights according to the National Center for Health Statistics guidelines. The relationship between log-transformed urinary BPA and ACR was modeled 1) using quartiles of ACR in the sample and 2) log-transformed ACR. Univariate regression analyses were done between ACR and confounders, and between urinary BPA and confounders, adjusting only for dilution. Multivariate regression analysis was done for ACR adjusting for urinary creatinine and confounders. Sensitivity analyses were done in unweighted modeling, and multiple imputations for missing data.

Adjusting for urinary dilution and confounders, the multivariate logistic regression analysis showed that children with the highest quartile of urinary BPA (≥ 4.3 ng/mL) had statistically

significant higher ACR (0.91 mg/g) compared to the lowest quartile (<1.1 ng/mL). Also, multivariate linear regression analysis showed a statistically significant increase in ACR of 0.28 mg/g for each log unit increase in urinary BPA. Statistically significant relationship was not found for odds of micro- or macroalbuminuria with urinary BPA.

This is a cross-sectional study looking at the relationship between one spot urine BPA measurement and one measure of ACR, which limits the ability to make a causal conclusion. ACR is subject to substantial day-to-day variability, and a single spot urine sample of BPA may not reflect actual long term BPA exposure. Both BPA level and albuminuria can be related to lifestyle factors, and the investigator did not have information on diet and exercise which are potential confounders. Authors define "low grade albuminuria" as ACR <30 mg/g, but in the published literature, ACR <30 mg/g are considered normal and albuminuria is defined as ACR >30 mg/g.[5] The study showed a statistically significant 0.28mg/g ACR increase seen for each log unit increase in urinary BPA, indicating a possible dose-response relationship**.** In addition to limitations cited, it is not clear that such small increases in albuminuria would be associated with a clinically adverse effect. **The results of this study would need to be further confirmed in a well-designed prospective study, but this possible dose-response information could have limited utility for HI but no utility for RA.**

Urinary bisphenol A (BPA) concentration associates with obesity and insulin resistance (Wang *et al.*, 2012c)

This cross-sectional study examined potential relationships of urine BPA with overweight, obesity and insulin resistance in 3390 adults, aged 40 years or older in Songnan Community, Baoshan District, Shanghai, China. Weight, height, waist circumference, and blood pressure were measured. An oral glucose tolerance test was conducted and plasma glucose measured at 0 and 2 hours. Overweight was defined as having a BMI of 24 to less than 28 kg/m2, and obesity was defined as having a BMI of 28 kg/m2 or higher according to Chinese criteria. Insulin resistance was defined as homeostasis model assessment of insulin resistance (HOMA-IR) higher than 2.50. BPA was measured in morning spot urine samples using solid phase extraction with HPLC-MS/MS (LOD 0.30 µg/L). BPA was categorized into quartiles at 0.47 µg/L, 0.81 µg/L and 1.43 µg/L. Urinary creatinine was also determined. Information on age, education level, smoking and alcohol consumption was collected using a questionnaire at the same time. Multivariable linear regression, adjusted for age, sex and urinary creatinine was used to test for trend across the BPA quartiles. Multivariable logistic regression was used to examine the relationship between higher BPA quartiles and overweight, obesity and insulin resistance adjusted for age, sex, urinary creatinine concentration, smoking, alcohol drinking, education levels, systolic blood pressure, high and low density cholesterol (HDL-C and LDL-C), triglycerides (TG), serum alanine aminotransferase (ALT) and glutamyltransferase (GGT), high-sensitivity C-reactive protein (hs-CRP), fasting plasma glucose, and fasting serum insulin. A *P* value 0.05 (two sided) indicated statistical significance. Insulin resistance was further stratified for BMI at 24 kg/m^2.

5 http://www.aacc.org/members/nacb/LMPG/OnlineGuide/PublishedGuidelines/diabetes_update/Documents/Chapter%2012.pdf
http://www.mayoclinic.com/health/microalbumin/MY00143/DSECTION=results

Higher BPA was associated with a trend of increased BMI, waist circumference, fasting glucose, and fasting insulin and decreased HDL-C, although mean values did not consistently follow the trend across categories. BPA was associated with higher odds of abdominal obesity for quartiles 2 through 4, compared to 1, however without a dose-response pattern. There was a significant difference for quartile 4 for generalized obesity and also for insulin resistance, with the later shown to be only present in the thinner participants.

In this cross-sectional study, there is no way to determine whether measures of obesity and insulin resistance might be leading to differences in BPA levels or vice versa. The authors noted that diet was likely to differ between those who were obese and thinner and that this might have changed exposure to BPA. Diet and other lifestyle exposures were not examined. No methods were described to assure that urine collection containers did not introduce BPA contamination. Also, urine BPA measures very short-term exposure at the time of the examination, while the outcomes of interest were long term processes. The lack of dose-response increases across quartiles also weakened the evidence for a causal relationship. **This study has no utility for HI and no utility for RA.**

High urinary bisphenol A concentrations in workers and possible laboratory abnormalities **(**Wang *et al.*, 2012a)

This cross-sectional study was conducted using subjects representing relatively equally two small private semiautomatic epoxy resin factories in China. Workers who may have been exposed to the potential liver toxicant, chloropropane, at the facilities were excluded from the study. A total of 28 day-shift workers (21 male and 7 female; mean age: 39) who were exposed to BPA via the dermal and possibly the inhalational route (feeding operator, office workers and crushing/packing workers) participated in the study. Demographic and health information was collected by questionnaire filled out by the worker. Although the smoking and alcohol status of the workers was presented, it was not used for any further analysis. A single urine sample was collected for each worker at the end of his/her shift on a Friday afternoon. The material construction of the collection container was not reported. Total (free+conjugated) BPA was measured using Ultra-high performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS). The levels of liver enzymes (GPT, GOT, GGT, ALP, LDH), markers of glucose homeostasis (Insulin, Glucose) and thyroid function (TSH, TT4, TT3, FT4, FT3), and CRP were measured in single, fasted venous blood samples in the morning; additionally, albumin was assessed in urine. One-way ANOVA, least significant difference for multiple comparisons, and Pearson correlation were the major methods of statistical analysis. Urinary BPA, but not blood analyte, levels were log-transformed before statistical analysis without explanation.

The mean (geometric) creatinine-adjusted urinary concentration of BPA was highest in feeding operators (192.45 μg/g; $P<0.01$) followed by crushing and packing workers (17.08 μg/g) and office workers (11.6 μg/g). However, there was no statistically significant dose-dependent correlation between BPA levels in those groups and any clinical chemistry endpoint (Table 3). In the absence of any association, the authors then pooled all workers on study and divided them into three groups based on (non-log-transformed) urinary BPA percentile (undefined) and

reported a linear trend for FT3 (free T3) and, after excluding two outliers, a moderate positive correlation between log-transformed BPA and FT3 (r=0.57). However, the association disappeared when both office workers and crushing/packing workers were excluded from the analysis. Importantly, only five of the feeding operators and three of the crushing/packing workers had levels of FT3 that exceeded the upper limit of normal. All other subjects on study had FT3 in the normal range (n=20).

As the authors stated, the results of this study should be interpreted with caution because of the small sample size and cross-sectional nature of the study. The study also had several other limitations which make interpretation of the results difficult. These include failure to include possible confounding factors (smoking and alcohol status) in the analysis. Secondly, urine samples were collected in an uncontrolled environment and whether a plastic or glass container was used for collection was not mentioned. Third, urinary BPA was assessed at one time only, which may not indicate long-term exposure in the study population. **Given these limitations, the absence of a reproducible and consistent association, and the fact that most study participants had FT3 levels within the clinically normal range to begin with, this study has no utility for HI and no utility for RA.**

Urinary bisphenol a concentration and thyroid function in Chinese adults (Wang *et al.*, 2013b)
This cross-sectional investigation aimed to assess the relationship between urinary bisphenol A (BPA) and thyroid function among 3394 participants ≥40 years of age from a June-August 2009, population-based Shanghai study. Trained staff captured sociodemographic, medical and family history, and lifestyle data, including age, education, smoking status, occupation, and alcohol consumption, by interview. Glucose and lipid profiles were measured within 2 hours of obtaining fasting blood samples. First morning spot urine samples were collected and stored at -80°C, but specimen container material was not addressed. Autoanalyzer measurements of urinary creatinine and lipid profiles were used. Body mass index (BMI) was computed from weight and height measured according to protocol by experienced nurses. Based on fasting plasma glucose levels, participants were classified as normal (<5.6 mmol, no diabetes history), impaired (5.6 to <7.0 mmol, no diabetes history), and diabetes (≥7.0 mmol, diabetes history). Because those with lower compared to higher glucose levels were expected to have lower participation rates, participants were randomly selected from the 3 groups for further investigation by survey, with sampling ratios of 1.0 (diabetes): 1.2 (impaired): 1.44 (normal). After exclusions for thyroidectomy, thyroid conditions, use of thyroid hormones or medications affecting thyroid function, or insufficient serum, 3394 participants remained and were found not to differ from nonparticipants significantly by age or sex. Exposures [total (free and conjugated) urinary BPA concentration] were quantified from single spot first morning urine samples after enzymatic hydrolysis by HPLC-MS. For analysis, levels below the 0.30 ng/mL limit of detection were assigned a value of 0.15 ng/mL. Outcome thyroid function measurements by chemiluminescent microparticle immunoassay included serum free triiodothyronine, free thyroxine, and TSH, as well as serum thyroid peroxidase and thyroglobulin antibodies. Multivariable-adjusted linear regression models assessed the relationship between urinary BPA and thyroid function; after participant classification by serum free thyroid hormone and TSH concentrations into 5 categories, including overt and subclinical hyperthyroidism, euthyroid, and subclinical and overt hypothyroidism,

logistic regression was used to further evaluate the relationship between urinary BPA and thyroid function group. Both the multivariable linear and the logistic models adjusted for age, sex, urinary creatinine, BMI, education, occupation, smoking, alcohol intake, total cholesterol, triglycerides, HDL-C, LDL-C, and thyroid peroxidase and thyroglobulin antibodies.

Median BPA concentration was 0.81 ng/mL (interquartile range: 0.47-1.43 ng/mL). Geometric means for thyroid measures showed little variation across BPA quartiles and bounds of 95% CIs were well within reference ranges. In the multivariable-adjusted model, each one-quartile increase in BPA was observed to relate to an increase of 0.068 pmol/l [95% confidence interval (CI): 0.065, 0.071] in free triiodothyronine and a 0.084 μIU/ml decrease (95% CI: -0.099, -0.069) in thyroid-stimulating hormone (TSH) among the men, and to a 0.10 pmol/l (95% CI: 0.09, 0.11) increase in free triiodothyronine and a 0.13 μIU/ml decrease (95% CI: -0.14, -0.11) in TSH among the women. In the adjusted logistic regression model, the highest compared to the lowest quartile of urinary BPA was associated with increased thyroid function [Adjusted odds ratio (AOR): 1.71, 95% CI: 1.26, 2.32].

Important limitations include the method used to measure free triiodothyronine and TSH, as well as the failure to account for dietary intake, socioeconomic status, and other potentially relevant confounders. Contamination-related exposure mismeasurement cannot be ruled out because of unspecified lab containers. The usual limits of the cross-sectional design also apply: exposure was measured at one point in time, which may or may not bear any relation to the critical time of exposure; does not take individual variation into account; allows potential for reverse causality if thyroid function affects urinary BPA excretion; and may conflate measured exposures with individual variation based on environmental or genetic/metabolic differences in a Chinese population that may or may not be representative of the US population. **Because of these limitations, this study has no utility for HI and no utility for RA.**

Association between bisphenol a exposure and body mass index in Chinese school children: a cross-sectional study (Wang *et al.*, 2012b)

The objective of this cross-sectional study was to investigate the association between BPA exposure and body mass index (BMI) in school children of the Changning district of Shanghai City, China. A total of 360 students were randomly selected (20 obese, 10 overweight, and 30 normal weight students aged 8-15 years from 3 (out of 26) primary schools (N=518, 448, and 573) and 3 (out of 30) middle schools (N=467, 541, and 374) on the basis of the most recent physical examination conducted in October 2011. A total of 259 subjects (24-normal, 53 overweight, and 82 obese, [according to the Working Group on Obesity in China (WGOC) cutoffs shown in a supplement to this article (Additional_file_1)] were included; those with liver, kidney, or endocrine diseases were excluded. Body weight (kg) and height (cm) were measured using the standard apparatus and procedures (Cameron1978).

Instructed to avoid contact of urine with plastic products, the participants self-collected urine samples (January 3-6, 2012) in glass centrifuge tubes (rinsed by acetone and baked at 350°C for 2 hours), which were transported “as soon as possible” (range of time before transport not given) to a lab and stored in dark at -20°C until analysis. Solid-phase extraction coupled with ultra-

performance liquid chromatography isotope dilution tandem mass spectrometry was used to analyze (limit of detection-0.07 ng/mL) total urine BPA (free and conjugated). All urine analyses were carried out in a random order and completed in March 2012 to reduce potential systematic drift. Specific gravity (SG) of urine (measured by handheld refractometer, Atago PAL 10-S; Tokyo, Japan) was used to correct for urinary dilution (Mahalingaiah *et al.*, 2008). The correction formula was $SG_{corrected}$ BPA concentration (ng/mL) = experimental BPA concentration (ng/mL) × [(1.024–1)/(SG–1).

Multiple linear regression analyses to study the associations of naturally log-transformed urinary BPA concentrations (and daily BPA intake estimated, but not measured) with BMI and analyses of variance for mean differences in BMI (95% CIs) between quartiles (2, 3 or 4 and 1 as reference, ranges not specified) of urine BPA concentration were carried out in all subjects and stratified by age or sex, before and after SG correction. Data analyses (two-sided, $p < 0.05$ significant) were performed using SPSS (version 17; SPSS, Inc., Chicago, IL, USA).

BPA was detected in 84.9% of urine samples with a geometric mean of 0.45 ng/mL (95% CI:0.37-0.55), but handling of samples with undetectable levels was not addressed. Multiple linear regression analyses showed urine BPA concentration were significantly associated with increasing BMI as a continuous variable in all subjects (Crude analysis) and in models with adjustment for age and sex, before and after urine BPA concentrations were corrected by SG. In age and sex stratification analysis, 8-11 year and female group showed significant positive association of SG uncorrected BPA concentration with BMI (not significant after SG correction). The urine BPA concentrations uncorrected by SG were similar in boys and girls (stratified by gender) but were significantly higher in older than younger ones (stratified by age) and in obese compared with the normal group. In this study, a significant positive association of urine BPA concentrations with BMI in Chinese school children is reported. **The results of this study are of potential interest. However, because the results were based on a one time analysis of urine BPA measured three months after physical assessment for BMI, the results have serious limitations and provide no utility for HI and no utility for RA. Any future studies for consideration should take into account important confounders, including total dietary intake, calorie composition, pubertal status, family income level, family history for obesity, or degree of correlation of other phthalate metabolites.**

<u>Effects of maternal exposure to phthalates and bisphenol A during pregnancy on gestational age (Weinberger B, *et al.*, 2014)</u>

This study evaluated the relationship of maternal urinary levels of phthalate and bisphenol-A (BPA) with gestational age. Urinary phthalate and BPA metabolites were measured in 72 pregnant women with single gestations from a high risk obstetrics clinic at Robert Wood Johnson University Hospital had urinary phthalates and urinary BPA metabolites measured. Gestational age was based on the best obstetric estimate from the medical record (from either sonographic dating or date of implantation). Estimates were consistent with physical examination findings of the infant.

Several different phthalate and BPA metabolites[6] were measured from urine samples collected at the last prenatal visit before delivery. Values were normalized to urine specific gravity. Linear regression models unadjusted and adjusted for parity and maternal race (and other predictors of gestational age [*e.g.*, maternal education, race, gravidity, age, employment, paternal employment, fast food consumption, birth country] found to be significant in univariate models) were used to estimate the change in gestational age associated with each interquartile range increase in phthalate or BPA metabolite concentration. The same modeling was performed after stratifying by infant gender. Adjusting for parity and maternal race, each increase in interquartile range for MEHHP (21.9 ng/mL) was associated with a change of -4.2 days of gestation, and each increase in interquartile range for the combination of free BPA and BPA glucuronide (180 ng/mL) was associated with a change of -1.1 days of gestation (p=0.03 for both). Evaluating the changes by gender showed that these changes are primarily due to changes in the days of gestation in male infants (MEHHP: -5.1 days, p=0.03; BPA (free+glucuronide): -1.1 days, p=0.03). No statistically significant relationship was seen in the female infants, or between the other phthalate/BPA metabolites and gestational age. Results from models that included adjustments for other important risk factors for preterm delivery, such as maternal age, were not reported.

This study has no utility for HI and no utility for RA. The findings of this study are consistent with some previous publications (Latini *et al.*, 2003; Meeker *et al.*, 2009) which found a relationship between higher concentrations of certain phthalate/BPA metabolites and shorter gestation. However, it should be noted that the metabolite(s) associated with shorter gestation was not the same across studies. Other studies have reported opposite results (Adibi *et al.*, 2009), showing women with concentrations of DEHP metabolites at the 75th percentile had a longer gestation than women with concentrations at the 25th percentile.

Limitations of this study include the small number of patients, and the fact that patients came from a single site. Thus, it is not clear if the reported findings are generalizable. Testing of multiple different metabolites introduces the problem of multiplicity. Also, the study only looked at one urinary sample and it is unclear whether this accurately reflects maternal exposure during pregnancy. The observed changes in gestation are small, and it is unclear whether this is truly a result of exposure or if it is normal variation within the error of gestational age estimation. Depending on the timing and method of gestational age estimation, the accuracy can vary by ± 3 or more days.[7] While shorter gestation could be associated with greater neonatal complications, it is unclear whether the small change reported here poses a real clinical risk. Outcomes of the deliveries are not reported, which limits the value of this study in determining the clinical significance of these findings.

Association between bisphenol A and waist-to-height ratio among children: National Health and

6 Includes mono-2-ehylhexyl phthalate (MEHP), monomethyl phthalate (MMP), monoethyl phthalate (MEP), monobutyl phthalate (MBP), monocyclohexyl phthalate (MCHP), mono-(2-ethyl-5-hydroxylhexyl) phthalate (MEHHP), mono-3-methyl-7-phthalate (isodecyl, MDP), mono-(2-ethyl-5-oxohexyl) phthalate (MEOHP), mono-n-octyl phthalate (MOP), mono-3-methyl-5-dimethylhexyl phthalate (iso-nonyl, MNP), free BPA, BPA sulfate, and BPA glucuronide

7 ACOG Practice Bulletin No. 98: Ultrasonography in pregnancy. *Obstet Gynecol*. 2008, 112: 951.

Nutrition Examination Survey, 2003-2010 (Wells *et al.*, 2014)
This study uses the National Health and Nutrition Examination Survey (NHANES) data from 2003-2010 to examine the cross-sectional association between urinary levels of BPA and waist-to-height ratio (WHR) among children. The sample consisted of 2,836 children aged 6-18 years old. Waist and height measurements were collected by trained study staff. Urinary BPA was measured in a single spot urine sample using gas chromatography/mass spectrometry. The authors reported that BPA levels were classified into quartiles: Q1 <1.4 ng/mL, Q2 1.2-2.6 ng/mL, Q3 2.6-5.1 ng/mL, and Q4 >5.1 ng/mL. There is overlap between the reported ranges for Quartiles 1 and 2, which may be a typographical error. Caloric intake was measured using 24 hour dietary recall (authors do not state whether respondent is parent or child).

The authors reported that BPA levels were significantly associated with survey year, race/ethnicity, and serum cotinine (a biomarker for nicotine exposure) in bivariate analyses, but exact results were not presented. The authors reported a positive association between urinary BPA levels and WHR. In models adjusted for urinary creatinine, age, sex, race/ethnicity, education, smoking status, and caloric intake, children in the second, third, and fourth quartiles of BPA had 0.011 (95% CI 0.001, 0.020); 0.010 (95% CI 0.001, 0.019), and 0.016 (95% CI 0.007, 0.026) units increase in WHR, respectively, compared with children in the first quartile. Unadjusted results were not presented. In stratified multivariate analyses, the association between BPA and WHR was statistically significant for males but not females, and for non-Hispanic white and non-Hispanic black subjects but not for subjects of other races/ethnicities.

When looking at the association between BPA levels and body fat measures such as WHR, the potential confounding role of diet always needs to be considered. Consumption of canned soda and packaged, processed food is associated with increased overweight/obesity as well as BPA exposure. The authors adjusted for caloric intake, but there remains a potential for residual confounding by dietary choices (*e.g.*, consumption of canned soda and processed food). Other limitations include the fact that this study is cross-sectional in nature, meaning that no conclusions can be drawn about temporality or causality, and the possibility of reverse causality cannot be ruled out. Confidence intervals for some effect estimates were close to the null (0.0). Also urinary BPA was measured in a single spot sample rather than multiple samples, which is not ideal due to the variability involved in urinary BPA levels. Strengths of the study include the use of NHANES, a nationally representative dataset. **This study has no utility for HI and no utility for RA.**

Association between Endocrine Disrupting Phenols in Colostrums and Maternal and Infant Health (Yi *et al.*, 2013)
This study evaluated BPA and 4-tertiary-octylphenol (OP) and 4-nonylphenol (NP) of alkylphenols (APs) in colostrum to assess the risk of exposure to these endocrine-disrupting chemicals in neonates. The study was conducted in 325 lactating mothers staying at postpartum care centers in Seoul, South Korea, who donated 10 mL of their colostrum. Questionnaires were administered to obtain physical characteristics, lifestyle patterns including dietary habits, and health and pregnancy-related factors from mothers. To avoid phenol contamination, glasswares were used to analyze the colostrum, and BPA, NP, and OP levels were quantified with

LS/MS/MS analyses. The investigators also performed analyses of HPLC/FLD to confirm LC/MS/MS results in samples with low phenol levels and got high reproducibility between the two analyses. The total forms (conjugated and free phenols) and free forms of BPA and APs for each colostrum sample were quantified with/without enzyme hydrolysis, and conjugated phenols were calculated by subtracting the amount of free form of phenols from total phenols. The limit of detection and limit of quantification were calculated with signal to noise ratio 10 and 30 respectively.

The study subjects were reflective of the middle class in South Korea based on educational level, occupation, and monthly household income. About 11% (n=35) of mothers had clinical diseases including toxemia (n=22), thyroid disorders (n=6) and gastritis (n=1). A similar percentage of infants (n=34) as mothers were sick at birth, most recovering within 2 weeks except for two infants with congenital malformation. There was no association of disease presence between the mothers and infants. As a result, the investigators separately analyzed the effects of phenol exposure on mothers and infants and found that the concentrations of total NP, but not BPA or OP, were higher in both mothers and infants with diseases compared to healthy mothers and infants, which reached statistical significance only in mothers. Two infants with congenital malformation of uvulas showed high concentrations of total NP and OP (medians 21.5 and 18.1 ng/mL respectively).

The distribution of phenols in colostrum were skewed to the left and not normally distributed. The OP and NP concentrations were not detectable in most colostrum samples. The total BPA concentrations\s were detectable in 71% of 325 colostrum samples with a median concentration of 7.8 ng/mL. Based on this, the authors estimate 1.2 µg/kg of daily BPA exposure in infants, which is determined to be 10-fold higher than estimated exposure in adults based on Korean adult urine samples. Consumption of dairy products and use of “detergents for food” in mothers showed statistically significant positive correlation with total BPA concentrations based on Spearman’s Rho pair-wise correlation analyses.

Although this study showed that a large proportion of colostrum samples had detectable BPA levels, BPA levels were not correlated with diseases in infants or mothers. The study only measured one colostrum sample. BPA levels were left skewed towards zero and the authors did not log transform the data before analysis. This study has limited generalizability since it included mostly middle-class mothers from one city in South Korea, Seoul, which is the most urbanized city in the country and would be expected to have different environmental exposures to phenols (*e.g.*, air pollution) compared to the rest of the country. Although the investigators correlated total BPA levels with a couple of dietary factors, more detailed information related to dietary or environmental factors are needed (*e.g.*, canned goods, use of plastic wrap, etc.). **This study has no utility for HI and no utility for RA.**

Association between precocious puberty and some endocrine disruptors in human plasma (Yum *et al.*, 2013)

This is a case-control study to look at whether there is an association between precocious puberty and some endocrine disrupting chemicals (EDCs) in human plasma. Plasma samples were

obtained from female patients (150 precocious puberty patients and 90 control subjects) admitted to the department of pediatrics, Kyung Hee University Medical Center, Seoul, Korea in 2009. The subjects in the study were selected among the children, living in Seoul and Kyoung-gi area who visited the pediatric endocrine clinic for the evaluation of precocious pubertal development. The precocious puberty patients were from 6 to 12 years of age (8.91 ± 1.40) and showed secondary sex characteristics under the age of 8 or menarche that had occurred before 9.5 years. A parallel control group (8.50 ± 1.68) included ninety healthy children, who did not exhibit any evidence of endocrine disease or pubertal signs and had visited the clinic during the same time period.

The levels of 7 EDCs and 3 isoflavones that exhibit estrogen-like actions were measured in the plasma of precocious puberty patients and compared to control subjects to determine if there is an association between the onset of precocious puberty and the levels of EDCs in the plasma. EDCs examined in this study were bisphenol-A (BPA), di(2-ethylhexyl) phthalate (DEHP), dibutyl phthalate (DBP), mono(2-ethylhexyl) phthalate (MEHP), monobutyl phthalate (MBP), n-nonyl phenol (n-NP), and t-octylphenol (t-OP), and whereas the isoflavones were equol, genistein, and daidzein. AGC/MS instrument consisting of an Agilent 6890GC interfaced with an Agilent 5975 mass selective detector was used to detect the target compounds. Two sample t-tests were conducted.

BPA plasma levels (3.53 ng/mL) in precocious puberty patients were lower than those (7.56 ng/mL) of the control group; the trend, however, was non-significant (see PK section for issues related to blood serum measurements). The study did not control for any potential confounders or effect modifiers. The selection process of control is not clearly stated in order to gauge the quality of control selection. **This study has limited utility for HI and no utility for RA.** There is no overall clear dose-response relationship found in this study for BPA and precocious puberty.

<u>The effects of bisphenol A (BPA) exposure on fat mass and serum leptin concentrations have no impact on bone mineral densities in non-obese premenopausal women (Zhao *et al.*, 2012)</u>
Cross sectional data from a study of 246 premenopausal women ≥20 years old in Shanghai, China was used to examine the relationships between urinary BPA, body composition (from body mass index (BMI) and waist-hip ratio, each calculated from measurements), serum estradiol, serum leptin, serum osteocalcin, urinary bone resorption marker N-terminal telopeptides of type 1 collagen (NTx) exposures, and the outcome of bone mineral density (BMD).

BMI, fat mass, fat-free mass, and bone mineral densities were measured with dual-energy X absorptiometry (DXA). Estradiol, leptin and osteocalcin were measured from fasting blood samples collected during the follicular phase of the menstrual cycle (ascertainment of follicular phase not described). Second morning urine samples were collected from each participant and stored at -80°C until analysis; composition of materials used to collect and vessels used to store the urine was not addressed. Of the samples from 251 individuals available for analysis, 246 had BPA above the 0.3 ng/mL limit of detection (LOD). As noted by the authors, the urinary BPA

levels were measured at the Shanghai Institute of Materia Medica, determined by enzymatic hydrolysis using a sensitive and selective liquid chromatography-tandem mass spectrometry method, and expressed relative to creatine excretion, with the intra- and inter-assay coefficients of variation at 5-19% and 3-5%, respectively. As also noted by the authors, the analytical sample for the study consisted of those 246 women with BPA detected above the LOD. Analyses included partial correlation with adjustment for age, BMI, fat mass, or fat-free mass and multivariate linear stepwise regression modeling to identify important exposure variables accounting for changes in lumbar spine and femoral neck BMD, as well as leptin. Results were expressed as mean ± standard error (SE).

After adjustment for age, BPA was found significantly positively correlated with fat mass ($r=0.350$, $p<0.001$), fat-free mass ($r=0.186$, $p=0.009$), leptin ($r=0.362$, $p<0.001$), body weight ($r=0.240$, $p=0.001$), BMI ($r=0.298$, $p<0.001$), waist circumference ($r=0.296$, $p<0.001$), hip circumference ($r =0.270$, $p<0.001$), and WHR ($r=0.149$, $p=0.035$). After adjustment for both age and BMI, BPA was still significantly correlated with fat mass ($r=0.193$, $p=0.006$) and leptin ($r=0.236$, $p=0.001$), but not with fat-free mass. The significant positive correlation remained after adjustment for both BMI and fat-free mass ($r=0.249$, $p<0.001$), but was attenuated after adjustment for age, BMI, and fat mass ($r=0.175$, $p=0.014$). In all cases the effect size of the observed significant correlation was small. No significant correlations were observed between BPA and estradiol, NTx, or osteocalcin. In three separate multivariate regression models respectively using lumbar BMD, femoral neck BMD, and leptin as outcomes, no statistically significant associations between urinary BPA and any of the outcomes were observed.

Study strengths included a relatively large sample size. Limitations included the cross-sectional study design, which does not allow for the determination of cause and effect; BPA exposure was measured from a single urine sample taken at one point in time bearing an undeterminable temporal relationship to the BMD outcome. **This study has no utility for HI and no utility for RA.**

NCTR GLP / NTP Technical Report (E2176.01): Evaluation of the toxicity of Bisphenol A (BPA) in male and female Sprague-Dawley rats exposed orally from gestation day 6 through postnatal day 90 (subsequently published as Delclos *et al.*, 2014)

The following review is abbreviated from Attachment 1, Memorandum by Y. Gu and R. Mitkus dated August 2, 2013.

INTRODUCTION

Study Design

A specialized subchronic toxicity study with *in utero* exposure and direct-dosing to pups was conducted in NCTR Sprague-Dawley (CD) rats by administering BPA to characterize the dose-response, evaluate the adverse effects in rats, particularly at low levels of exposure potentially attainable in humans, and to serve as a dose-range finding study for a chronic toxicity study in rats. BPA was administered orally by gavage at doses 2.5, 8, 25, 80, 260, 840, 2,700, 100,000,

and 300,000 µg/kg bw/day to pregnant dams from gestational day (GD) 6 through parturition and then directly to pups from postnatal (PND) 1 (day of birth = PND 0) to PND 90. The BPA exposure levels at 2.5 to 2,700 µg/kg bw/day were considered as Low dose BPA; whereas, 100,000 and 300,000 µg/kg bw/day as High dose BPA. Two ethinyl estradiol (EE2) groups (0.5 and 5.0 µg/kg bw/day) were included as reference controls. A group administered with 0.3% carboxymethylcellulose and another group without any treatment served as vehicle and naïve controls, respectively.

In this study, the litter was utilized as the unit for statistical analysis and the target litter number was 20 per dose group. The animals in the study were divided into three different subset groups:

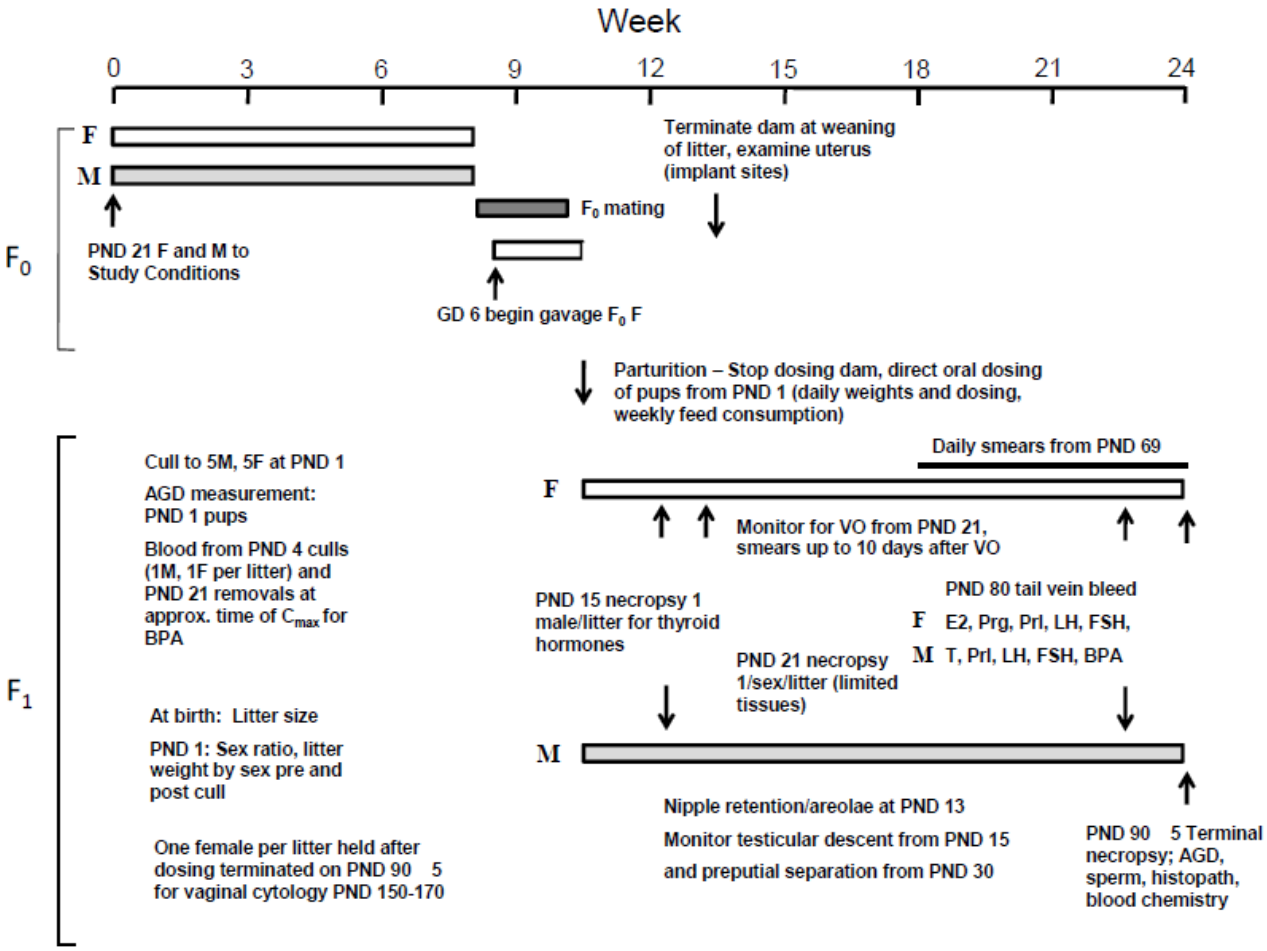
1) Histopathology arm (F1): for histopathology and standard measurements on PND 90 (18-23/sex/group);
2) Frozen tissue arm (FZ): for providing tissues to non-GLP studies; and
3) Delayed vaginal cytology arm (VC): for vaginal cytology on PND 150-170 (females only).

In addition, a group of animals (both sex) was designated for histopathology examinations of mammary glands on PND 21.

The statistical analyses were, in most cases, performed by using Low or High dose BPA groups in each subsets compared with the concurrent vehicle controls.

Because of the specified study purpose, a number of additional observations/end-points was examined compared to that in a standard subchronic oral toxicity study for regulatory purposes.

The study design summary and treatments are depicted at below. (Adopted from Figure 1 on page TR000160 of the report)



Study Objectives

The purpose of this study is designed to characterize the dose-response for orally administered BPA in the NCTR Sprague-Dawley (CD) rat, to evaluate the adverse effects in rats near levels of exposure potentially attainable in humans, and to serve as a dose-range finding study for a chronic toxicity study. The dose groups were as follows:

1) Low dose BPA: to detect and characterize the dose-response of potential toxic effects of BPA;
2) High dose BPA: to characterize and evaluate the toxic effects of BPA;
3) EE2: to characterize estrogenic effects of EE2 and confirm the sensitivity of the NCTR CD rat model; and
4) Naïve: to assess effects of gavage procedure (vehicle control) on endpoint measurements.

Guidelines

The study was reportedly conducted in compliance with FDA Good Laboratory Practice Regulation (21 CFR, Part 58). There is no currently validated study protocol or guidelines for a study with *in utero* exposure plus pup direct dosing available. A quality Assurance statement was dated and signed on March 1, 2013 by NCTR Quality Assurance (QA) unit.

Testing Facility
National Center for Toxicological Research, Food and Drug Administration,
NCTR Road, Jefferson, Arkansas

Study Protocol:	March 10, 2010
Initiation Date:	May 3, 2010
Experimental Start Date:	May 20, 2010
Experiment Completion Date:	November 5, 2010
Audit of Draft Final Report:	February 21, 2013
Final Report:	March 4, 2013

Test Substance
Bisphenol A: CAS RN 80-05-7, aka 4,4'-(1-Methylethylidene)bisphenol, TCI America, Portland, OR; Lot: AOHOK; purity >99% by Mass Spectrometric analyses, Nuclear Magnetic Resonance Spectrometry and High-Performance Liquid Chromatography-Photodiode Array Analyses in study performing laboratory
Ethinyl estradiol (EE2): CAS RN 57-63-6; purity >98%, Sigma-Aldrich Corporation, St. Louis, MO; Lot 028K1411
0.3% Carboxymethylcellulose sodium salt: from Sigma-Aldrich Corporation, St. Louis, MO: Lot #048K0023
Stability: 35 days for lowest BPA and EE2; 47 days for 100 mg/ml BPA
Homogeneity: performed with the highest dose of BPA, 300,000 µg/kg and homogeneity was acceptable
Concentrations: For BPA, the mean of accuracy of dosing was 104±15% (S.D.) ranging from 80-139%; for EE2, the mean accuracy of dosing was 92±16% (S.D.) ranging from 70-154%.
Storage: No problems reported

Animals
Animals: Sprague-Dawley/CD23 NCTR BR males and females fed with 5K96 rodent chow (Catalogue No. 1810069, verified casein diet 10 IF round pellets, irradiated, Purina Mills, Richmond, IN) was provided to animals *ad libitum*.

Feed: Phytoestrogen contents of daidzein and genistein in the six diet lots were 0.249±0.064 (S.D.) and 0.374±0.118 (S.D.) ppm, respectively. The mean level of BPA in the diet was 2.6±0.8 ppb.

Water: Millipore-filtered water (Jefferson, municipal supply) via glass water bottle with food-grade silicone stoppers was provided *ad libitum* over the course of study. Contaminants in the water were reportedly screened.

Rooms and cages: temperature 23^{o}± 3^{o}C; humidity 50%±20%; light cycle 12/12 hour dark/light; ventilation--10 air changes/hour. Polysulfone cages with Microisolator tops (Ancare Corporation, Bellmore, NY) were changed weekly and wire-bottomed twice weekly during mating. Bedding with hardwood chips (P.J. Murphy, Montville, NJ) were changed weekly.

Parameters measured

The following parameters were measured in dams (GD 6 –parturition) and F1 pups (PND-1 to 90): Body Weight, body weight gains and feed intake and feed (aka metabolic) efficiency, mortality (twice daily), and clinical observations (daily)

The following parameters were measured in F1 pups only (PND-1 to 90):

Clinical chemistry:
PND 15: T_3 T_4 and TSH (males only);
PND 80: LH, FSH, E_2, and prolactin (females were based on estrus by vaginal cytology); and
PND 90: general parameters.

Reproductive parameters including:
The numbers of implants, resorptions, total live pups, male and female pups, unsexed pups, dead pups were counted. Total litter weights, male and female pup weight, sex ratio, anogenital distance, prewean survivals, vaginal opening (age), time to first estrus, testicular descent (age) and preputial separation (age), estrous cycle (estrus, diestrus, abnormal, PND 69-90 and 150-170), vaginal cytology, and sperm parameters (sperm morphology, % motility, caudal sperm count, testicular spermatid count)

Necropsy:
Terminal - PND 90 organs/tissues weights (absolute and relative) including: adrenal gland, brain, fat pad (ovarian parametrial, retroperitoneal, and epididymal), heart, kidney, liver, ovary, pituitary gland, spleen, thymus, thyroid gland, uterus, epididymis, prostate (dorsolateral & ventral), seminal vesicles (with coagulating gland), and testis were measured.

Histopathology:

PND 21- mammary glands only from both sexes

PND 90 - Pancreas, liver, aorta (thoracic), heart, mandibular lymph nodes, bone marrow (femur), spleen, thymus, kidneys, right testes, right epididymis, seminal vesicle with coagulating glands, prostate (dorsolateral and ventral), ovaries, uterus, oviduct, vagina, brain, eyes, pituitary glands, 5^{th} left (inguinal) mammary glands, thyroid gland, adrenals, and retroperitoneal fat pads

Statistical analysis

The table below lists the methods used in statistical analyses of different end-points/measurements.

METHODS OF STATISTICAL ANALYSIS	PARAMETERS TESTED
Analysis of variance (ANOVA) or analysis of covariance (ANOCOVA)	Gestation length, gestational body weight gain, and gestational metabolic efficiency; litter parameters, anogenital distance, preweaning body weights, clinical chemistry, body weight food consumption and metabolic efficiency, organ weights, sperm analysis

Dunnett's test	Puberty markers and comparisons
Cox regression	Preweaning pup survival
Cochran-Armitage trend	Estrous cycle analyses
Poly-k test with average lesion severity reported Jonckheere-Terpstra/Shirley-Williams (JT/SW) Relative treatment effect (RTE) test	Histopathology data

As summarized above, non-neoplastic histopathological findings were analyzed using three separate statistical tests: 1) the Poly-k test (k=3); 2) the Jonckheere-Terpstra/Shirley-Williams (JT/SW) test which integrates lesion severity scores in its analysis and is designed to assess monotonic dose response; and 3) the relative treatment effect (RTE) test which is designed to assess nonmonotonicity in dose response and can also account for the severity score of histopathological observations. However, it should be noted that the study authors reported only the results of the Poly-3 test in the final report, apparently based on traditional NTP practice. Yet, the JT/SW and RTE tests were often more sensitive, and no explanation was given as to why the results from those two tests were not integrated into the final study results and discussion.

RESULTS

Since the primary purpose of this study was focused on the effects of Low dose BPA, statistical analyses were performed with three separated comparison groups, namely, Low dose BPA to vehicle control, High dose BPA to vehicle control, and EE_2 to vehicle control in each subsets of animal groups. The summarized results presented here will follow these three treatment groups for convenience but combined with all animal subsets when it is available and feasible.

As noted, hematology, urinalysis, neurotoxicity and ophthalmological data are not included in the study report. However, this study was not a standard subchronic toxicity study and was designed primarily to assess effects on reproductive tract development as well as other nonstandard toxicity endpoints.

Low dose BPA

Briefly, no significant test material-related effects were observed for many measured parameters in this study, which include feed consumption, feed efficiency, body and organ weight or weight gain, clinical observation, clinical chemistry, reproductive parameters, or histopathology.

Some statistically significant changes/findings were observed sporadically. But those changes/findings were considered by the study authors or these reviewers to be non-test material related or non-biologically significant since there were no dose-response pattern/trend, single time point/measurement, in single sex, no concomitant of histopathology finding or within a ±10% range. In addition, some of these changes/findings were within referenced range as

compared with normal values reported in the same strain animals[8] or considered as background by the study authors or these reviewers.

On PND 21, a significant trend in mammary gland duct hyperplasia was observed in the low dose BPA groups, and the 2,700 and 100,000 μg BPA/kg bw/day dose groups differed significantly from the vehicle control in females using the Poly-k test. Specifically, the incidence of mammary gland duct hyperplasia of minimal severity was 29% (P=0.023), 35% (P=0.009), and 25% (P=0.057) in the 2,700, 100,000, and 300,000 dose groups, relative to vehicle (0%) controls. It is unclear whether the incidences would have been statistically significant in the treatment groups if compared to the incidence in naïve controls (12%), given that there was no statistically significant difference in incidence between the vehicle and naïve control groups. It also should be noted that fewer animals were assessed in the highest dose group (n=12) compared to the two next lower dose and control groups (n=16-17), and this may account for the slight lack of statistical significance using the Poly-k test in the highest dose group. However, when the incidences of female mammary gland duct hyperplasia were analyzed using the JT/SW or RTE tests, the 2,700, 100,000, and 300,000 μg BPA/kg bw/day dose groups were all significantly different from vehicle control. As mentioned, the increase in ductal hyperplasia at 2,700 μg BPA/kg bw/day in PND 21 females was considered a treatment-related effect by the study authors, but not by the study pathologists.

In a subsequent evaluation of the study report, CFSAN Pathology did not consider the reported mammary gland ductal hyperplasia at 2,700 and 100,000 μg BPA/kg bw/day in PND 21 females to be a treatment-related effect, but rather considered the observation to reflect inherent background and sampling variability when compared with naïve controls (for details, see Attachment 2 Memorandum by S. Mog and S. Francke-Carroll dated July 19, 2013).

On PND 90, the incidence of mammary gland ductal hyperplasia was statistically significantly increased in females in the highest dose BPA group only when the Poly-k test was used. Specifically, the incidence of duct hyperplasia was 55% (P=0.171), 65% (P=0.063), and 74% (P=0.013) at 2,700, 100,000, and 300,000 μg BPA/kg bw/day, respectively, relative to the vehicle (35%) controls. The study authors considered the incidences to be increased at both 100,000 and 300,000, but not 2,700, μg BPA/kg bw/day. The severity of the observations increased from minimal to mild in BPA treated groups, but without clear dose response. When analyzed using either the JT/SW or RTE statistical test, both of which take into consideration incidence along with severity scores, the incidences of ductal hyperplasia were statistically significant at 2,700 μg BPA/kg bw/day and above, with evidence of trend. It is unclear whether the incidence at 2,700 μg BPA/kg bw/day would have been statistically significant if compared to the incidence in naïve controls (45%), given that there was no statistically significant difference in incidence between the vehicle and naïve control groups. The original study pathologists reported "there may be a slight dose response trend in the 2,700 to 300,000 μg/kg bw/day BPA groups" for duct (ductular)

8 Since no historical data were provided in the report, this review checked and compared with the publication entitled "Clinical laboratory parameters for Crl:CD(SD) rats" which is available during this review at http://www.criver.com/SiteCollectionDocuments/rm_rm_r_clinical_parameters_cd_rat_06.pdf

hyperplasia. However, as for their assessment of the PND 21 histopathology data, their assessment for the PND 90 data was apparently conducted in the absence of knowledge of the statistical testing results for these observations.

In a subsequent review of the study report, CFSAN Pathology did not consider the mammary gland ductal hyperplasia at 2,700, 100,000 and 300,000 μg BPA/kg bw/day in PND 90 females to be a treatment-related effect, but rather caused by inherent background and sampling variability when compared with naïve controls.

High dose BPA

A. Body weights and feed efficiency:
The significant body weight gain reduction was observed in dams and F_1 animals at PND 4 and beyond, approximately 6 – 13% with observed lower means in feed (metabolic) efficiency in F_1 animals.

B. Mortality and clinical observations:
No significant difference was reported for mortality. No clinical observation data was found in the report.

C. Clinical Chemistry:
PND 15 (male only) – Increase of serum T3 in both high dose groups; decreased TSH in highest dose BPA.
PND 80 – Increased serum estradiol and decreased progesterone levels in females; no significant changes in males.
PND 90 (overnight fast) – Decreased cholesterol, triglycerides, and leptin and increased TSH levels in females; decreased cholesterol and leptin and increased creatinine levels in males.

D. Reproductive parameters:
   a. Gestational length – no significant changes.
   b. Gestational metabolic efficiency – no significant changes.
   c. Litter Parameters – no significant changes on counts of live pups, sex ratio, body weight of pups, number of implants or resorptions, etc.; an observed lower prewean survival rate in both sexes.
   d. Puberty Markers
   Vaginal opening – no significant changes in age or body weight.
   Onset of first estrus – no significant changes.
   Nipple retention, testicular descending and preputial separation – no retained nipples observed; significant increase in age at testicular descent (highest dose BPA); no significant changes in either age or body weight at preputial separation.
   e. Sperm parameters – no significant changes.
   f. Estrous cyclicity & Vaginal cytology - 63% of the females with asynchronous (highest dose BPA) and significant increase in proportion of females showing abnormal cycles in highest dose BPA at PND 69-90

g. Anogenital Distance -
PND 1- no significant changes in both sexes.
PND 90 – changes in highest dose BPA in males only.

E. Organ weights
Female – reduced weights including spleen, fat pad, ovary; increased including liver (most are confined to highest dose BPA);
Male – reduced brain weight; other organ weight reduction is significant in absolute, but not in relative weight, thus it is considered secondary.

F. Necropsy and Histopathology

PND 21 (mammary gland only): As noted above in the context of the low dose group results, significant increases in incidence of mammary gland and duct hyperplasia in 100,000 and 300,000 μg BPA/kg bw/day only in females. But the severity for the hyperplasia was minimal.

PND 90: No treatment-related neoplastic findings were reported.

Male - significant increase in the incidences of cyst and nephropathy in kidney was observed. Mammary gland lesions were noted, but not significant. Decreased testicular size was noted in highest dose BPA. Degeneration of the seminiferous tubular epithelium was correlated microscopically with decreased testicular size in less than half of the animals.

Female - bilaterally small ovaries and thick vagina wall were noted frequently in highest dose BPA which correlated microscopically with depletion of corporal lutea and antral follicles, and increased incidence of follicular cysts. Increased incidence of cystic endometrial hyperplasia in the uterus was observed in highest dose BPA. As noted above in the context of the low dose group results, the incidence of mammary gland ductal hyperplasia was increased in both high BPA groups. Significant higher incidences of renal cysts with the incidence and severity were also noted and considered treatment-related.

EE2

EE2 was used as reference controls in this study since the reported effects of BPA are considered to be associated with estrogen signaling pathway.

EE2 did not significantly affect feed consumption, gestation length, gestational metabolic efficiency, anogenital distance and T_3, T_4, TSH serum levels. However, EE_2 caused lower means of feed efficiency in both F_1 sexes, but higher body weights in females. It significantly delayed vaginal opening and onset of first estrus in females, and preputial separation and testicular descent in males. EE_2 also produced a 100% of female animals showing abnormal estrous cycles. EE_2 profoundly altered the serum estradiol, progesterone, testosterone levels. In males, EE_2 reduced caudal sperm count, epididymal size and testicular size. It significantly induced smaller

ovary, thick vaginal wall, and increased uterine weight, incidence of follicular cysts and depletion of corpus lutea and antral follicles. EE_2 significantly increased incidence of ductal hyperplasia in mammary gland in males (PND 21 and PND 90) in the study.

EVALUATION AND COMMENTS ON STUDY

This study was effectively designed to evaluate the effects of BPA administered directly by oral gavage to the maternal animal and offspring, particularly at low doses, and to serve as a dose-range finding study for a chronic toxicity study. The study was not designed to choose points of departure for risk assessment (*e.g.*, LOAELSs and NOAELs). The study was well conducted and each of the targeted parameters/end-points was thoroughly investigated, recorded and evaluated. In addition to a broad range for dose exposure (especially the low dose range), the use of the litter as the appropriate unit for data analysis, the strictly controlled micro-environment in the study, and *in utero* exposure of and direct-dosing to pups in this study all contributed to a robust assessment of potential adverse effects of BPA especially at doses that are closer to the low-dose human exposure scenario.

A broad dose range of BPA was tested in this study (2.5 µg/kg bw/day up to 300,000 µg/kg bw/day). There is a huge dose gap in the dose selection, between the Low dose BPA (2.5–2,700 µg/kg bw/day) and High dose BPA (100,000 and 300,000 µg/kg bw/day) ranges. It is noted that this kind of dose selection may not be appropriate for determination of a NOAEL or a full dose-range finding study, given the 37-fold difference between the highest Low dose and the lowest High dose. However, as stated by the study authors, a major goal of this study was to evaluate and characterize the low dose effects of BPA in rodents; to serve as a dose-range finding study for a chronic low dose toxicity study, and the highest doses served as positive controls in the study. Therefore, the dose range and selection in both the Low and High dose BPA groups adequately served that purpose.

It is noted that the effects of Low dose BPA and High dose BPA were independently compared with vehicle controls in three animal subset groups in the study. The statistical analyses were performed in the same way, *i.e.*, Low dose BPA to the vehicle control or High dose BPA to the vehicle control. It is not clear whether such statistical analysis results would be the same or different if the analyses were performed using combined data of both Low and High dose BPA groups as one single data set, particularly given the positive trend test results.

As mentioned, non-neoplastic histopathological findings were analyzed using 3 separate statistical tests: 1) the Poly-k test (k=3); 2) the Jonckheere-Terpstra/Shirley-Williams (JT/SW) test, which integrates lesion severity scores in its analysis and is designed to assess monotonic dose response; and 3) the relative treatment effect (RTE) test, which is designed to assess nonmonotonicity in dose response and can also account for the severity score of histopathological observations. However, it should be noted that the study authors reported only the results of the Poly-3 test in the final study report. The study authors seemed to have ignored the results from the JT/SW and RTE tests, which were often more sensitive and designed specifically to include lesion severity and/or to assess nonmonotonicity in the dose-response

curve. As a result, while the study authors considered the increase in duct hyperplasia at 2,700 µg/kg bw/day and above in PND 21 females to be treatment-related, the study authors appeared to have ignored the statistically significantly increased incidence of mammary gland duct hyperplasia at 2,700 µg/kg bw/day and above in females that were assessed on PND 90.

A few significant differences were observed between the vehicle control and naïve control (noted below). However, it is not clear whether these differences are caused by the effects of vehicle or the stress created by the treatment procedure (gavage or handlings) based on available data in the study report. It is likely that the gavage dosing procedure is unrelated to the differences in these few observations between the vehicle and naïve control groups.

No historical control data were provided in the report for any measured parameters in this study. In the study report, all comparisons and statistical analyses for treated groups were appropriately made against the concurrent controls, namely vehicle control. Values for naïve controls were also provided for reference. The primary function of historical control data is to provide an indication of whether values observed in concurrent control group(s) are reasonable or consistent with previous studies (Haseman, 1995). Thus, the historical control data might provide some insights into any differences observed between the naïve and concurrent vehicle controls, as well as the marginal statistically significant differences in effect in treated groups relative to vehicle control. The historical data should be provided for this study, if possible, and included in the future reports.

A number of deviations were reported in the study (See Technical report Appendix II for details). We consider that those deviations are minor and negligible, and do not affect the final outcomes of this study.

SUMMARY AND CONCLUSIONS ON STUDY E2176.01

A few statistically significant differences between naive and vehicle controls have been noticed in this study, but such differences would be expected for negative controls and are considered incidental. It is unlikely that the gavage dosing procedure is related to any of these differences between the control groups, except the prewean survival rate which might be related to gavage error.

Low dose BPA did not induce significant effects for many measured parameters in this study. Some statistically significant changes and sporadic findings were reported. But those changes/findings were considered by the study director and these reviewers to be non-biologically significant since they either had no dose-response pattern/trend or observed at a single time point/measurement or in a single sex, or without any concomitant histopathology findings or within a ±10% range. In addition, some of these changes/findings were within reference range as compared with normal values reported in the same animal strain or background.

Low dose BPA resulted in a statistically significant increase in the incidence of mammary gland ductal hyperplasia of minimal severity (2,700 µg) in females at PND 21 with a positive trend.

This increase was considered treatment-related by the study authors but not by the original study pathologists nor in subsequent review of the study report by CFSAN Pathology. The incidence(s) of mammary gland duct hyperplasia at the 300,000 µg BPA/kg bw/day dose level in females at the PND 90 examination was statistically significantly increased when analyzed using the Poly-k test. The incidence(s) was also statistically significantly increased at 2,700, 100,000, and 300,000 µg BPA/kg bw/day at PND 90 when the JT/SW or RTE tests, which integrate lesion severity score and/or nonmonotonicity in the dose-response curve, were used. This increase was considered a possible trend by the original study authors, but not treatment-related by CFSAN Pathology in a subsequent review of the study report. Taking the incidences, statistical testing results, and all pathologist and study author opinions together, we conclude that the evidence for duct hyperplasia in the mammary gland of females on either PND 21 or 90 is weak. We agree with the study authors that there were no clearly adverse effects in the low-dose range. We consider it to be an equivocal finding that may be a reflection of normal biological variability and/or a reflection of limits in tissue processing. However, we also believe that this conclusion and putative explanations will be tested by the results of the chronic toxicity study with BPA, for which the current study served primarily as a dose-range finding study.

High dose BPA did not affect feed consumption, gestation length, gestational metabolic efficiency, prewean survival, anogenital distance in both F_1 sexes; vaginal opening and first time estrus in F_1 females; or retained nipples, preputial separation, testicular descent, sperm mortality and count in F_1 males.

High dose BPA lowered the means of feed efficiency and reduced body weight gain, decreased the serum levels of T_3, creatinine, cholesterol, testosterone, and leptin and increased TSH, and decreased epididymal fat, epididymis, prostate, seminal vesicle and testes weights while increasing adrenal, pituitary and spleen weights in males.

In females, high dose BPA significantly increased the incidences of renal tubule cyst in kidney, lowered the means of feed efficiency and reduced body weight gain in both dams and F_1 females, increased the serum levels of T_3, estradiol, creatinine and the proportion of animals showing abnormal estrous cycles; while decreasing TSH, progesterone, cholesterol and leptin levels. High BPA increased incidence of follicular cysts, corpus luteal depletion and antral follicle depletion in ovary and cystic endometrial hyperplasia in the uterus. The effect of high dose BPA on the incidence of mammary gland ductal hyperplasia in females was discussed at low dose BPA effects above.

EE_2, a positive estrogen control, reacted as expected.

As aforementioned, observed effects of high dose BPA partially overlapped with those of EE2 due to its expected estrogenic activity. As expected, the target organ(s) of BPA would be similar to that of estrogen, primarily reproductive organs/system in both sexes.

Taken together, we have concluded that no clear treatment-related effects were observed in the low-dose range of the study. A possibly statistically significant increase in the incidence of ductal

hyperplasia in the female mammary gland was observed in animals in the 2,700 µg BPA/kg bw/day dose group necropsied at PND 21 and 90. However, the magnitude of the increase was slight, and significance was not observed across all statistical tests. The observations were not considered clearly treatment-related by the original study pathologists, nor in subsequent review of the study report by CFSAN Pathology; therefore, this observation may be considered equivocal. High dose BPA (100,000 and 300,000 µg/kg bw/day) exhibited this and many other adverse effects, often in both males and females. Most of the effects of BPA at the high doses, but not all, overlapped with the reference compound, EE_2. The chronic oral toxicity study currently being conducted should clarify whether the effect observed in mammary gland duct hyperplasia in the specialized developmental toxicity study is equivocal or treatment-related.

JRWG OVERALL CONCLUSIONS

General Considerations

Contamination

Uncertainty is inherent in any scientific study or evaluation. Hazard identification and risk assessments use criteria and methods to identify, reduce, or address uncertainty to provide confidence in weight-of-evidence conclusions. A major source of uncertainty in the field of "low-dose" BPA research has been the many discrepancies and conflicts in reported effects within and across species, even when studies are conducted over the same dose range. Following our extensive review of "low-dose" BPA literature, we are unable to construct a plausible or logical comprehensive toxicological profile or explanation for the many claimed effects of BPA, largely due to the inconsistencies that currently exist within this literature. As an example, while one study may report an effect on a specific endpoint, contradictory results are reported in other studies examining related endpoints that share pathways or mechanisms of action with the first reported effect. Similar issues arise when trying to compare various doses, routes of administration, and internal dosimetry. This has not been the case for understanding the effects of BPA at *high* doses. The pattern of effects of BPA at high doses compares well with the reported effects for estrogens, and supports the hypothesis that BPA acts through estrogen receptor mediated pathways. This conclusion is additionally supported by our understanding of the pharmacokinetics of BPA and solid data on internal dosimetry.

To provide a comprehensive biological and toxicological evaluation of the effects of BPA in the low-dose range, very few studies (*e.g.*, Delclos *et al.* 2014) have evaluated an extensive range of endpoints (both discrete and related) across multiple systems. However, a few recent studies (reviewed herein) have employed new methods to elucidate the underlying causes of the observed variability in the BPA literature and thereby improve HI and RAs. The ability to correlate observed effects of BPA on various organ systems with levels of BPA in the affected tissues is crucial for demonstrating biological plausibility of adverse effects of low-dose BPA. Consequently, one of the most important findings was that the likelihood of sample contamination and inadvertent exposure by trace levels of environmental BPA is high in low-dose studies. For this reason, the criterion *Environmental Contamination* (described in the Methods section of this memorandum), which is based on previous experiences with background contamination in testing for other compounds (*e.g.*, dioxin, acrylamide, genistein), was explicitly considered in this review. However, the extent to which biological samples can be contaminated with native aglycone BPA is only now being fully understood. Assessment of sample contamination and potential for inadvertent exposure must remain important considerations in performing laboratory and/or clinical studies, collecting and analyzing samples, and finally in the acceptance and interpretation of data. Ye *et al.* (2013) identified multiple sources of BPA in the hospital and laboratory environment, including solvents and equipment, which may contaminate biomonitoring samples. The findings and recommendations from Ye *et al.* (2013) are further supported by the analyses in Teeguarden *et al.* (2013), in which comparison of various biomonitoring reports and methods for estimating human exposures were used to calculate expected human internal dosimetry and serum/blood ratios. The Teeguarden analysis also identifies which published studies used methods that were likely susceptible to contamination.

Thigpen *et al.* (2013) examined whether general estrogenic background levels and/or contamination could be a source of variability in the low-dose BPA literature. The study correlated higher and variable levels of estrogenic contamination sources with the inconsistencies in reported effects in the "low-dose" literature. The results of this study suggest that, if in a low-dose study examining endocrine effects of a weakly estrogenic chemical, background phytoestrogen and environmental estrogenic levels are not well controlled and/or measured, it becomes very difficult to exclude background contamination as a possible explanation for the reported effects. To minimize environmental BPA exposure as a source of variability, these issues were considered and stringent controls were incorporated into the rodent subchronic toxicity study by Delclos *et al.* (2014). These included reducing and measuring sources of background estrogenic and BPA contamination, measuring serum levels of BPA, examining a wide range of low doses, and measuring large numbers of endpoints. Despite these very strict efforts at minimizing environmental contamination, the serum BPA measurements (Churchwell *et al.*, 2014) showed similar levels of BPA in controls *and* the two lowest doses, thus demonstrating the difficulty of eliminating all sources of BPA exposure, the need for vigilance in study conduct, and the high potential for unintended or unexpected exposure. In the design of the Delclos *et al.* study, selection of 2.5 µg/kg bw/d as the lowest dose was based on reports of effects in this range. Analytical limitations in dose preparation, reproducibility, and stability, while maintaining an acceptable level of standard error were also considered in this dose selection. Based on the findings from these two studies, we conclude that studies conducted at doses of 2.5 µg/kg bw/d and below must be interpreted with extreme caution due to the high probability of inadvertent exposure or contamination of samples by exogenous sources of BPA.

The studies reviewed in this *Contamination* section support the need for strict criteria in hazard identification and risk assessment and in building weight-of-evidence evaluations. The high potential for inadvertent exposure or contamination by native BPA, confounding due to high or variable environmental estrogenic contamination or background, and methodological limitations in dose preparation significantly limits interpretation of studies that did not address these issues, leading to a high degree of uncertainty when trying to incorporate a given study's findings into an overall assessment. The issues cited here do, however, stress the need that studies reporting many of the "low-dose" and "non-monotonic" effects of BPA require sufficient methodological detail to exclude artifacts of environmental BPA exposure and sample contamination.

PK conclusions and PBPK model application

Published studies and NCTR work reviewed herein continue to support previous conclusions from this workgroup and have further contributed to a more complete understanding of pharmacokinetic properties following BPA exposure. The previous workgroup PK conclusions are summarized below with specific points updated based on the current review:

Cross-species and age-related differences

- Presystemic metabolism of low-dose BPA in the gastrointestinal (GI) tract and liver of adults of all species attenuates internal exposures to aglycone BPA following oral administration to <1% of total.

- Neonatal rodent metabolic and excretory capacities are immature as compared to adult rodents, whereas neonatal monkeys are very near adult metabolic competence. The same oral BPA low-dose would produce approximately 10-fold higher aglycone BPA levels in a newborn rodent vs. a newborn monkey.
- 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.

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.

PBPK models

Additional data on BPA and metabolite metabolism, distribution, and elimination were identified that can be considered for refining current PBPK models, providing interspecies comparisons, and estimating human internal exposures. PBPK models should also account for BPA metabolism through availability of multiple UGTs that can catalyze the glucuronidation of BPA.

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.

   Endpoints to be maintained for hazard identification include the following. These endpoints are maintained with low confidence due to study limitations, conflicting reports, and current understanding of potential for unintended exposure or contamination.

   a) Developmental neurotoxicity related to molecular or neuroanatomical endpoints and correlated behaviors with varying routes of administration.
   b) Cardiovascular disease-related factors based on human epidemiology studies.
   c) Sperm/testicular/hormone related parameters based on very limited supporting animal data.

The studies cited in support of hazard identification often report conflicting data and study design limitations. Many other studies report no effects in these endpoints including studies with gestational and neonatal oral low dose exposure. Findings in large, multigenerational rodent studies, as well as the large extent of reproductive, sperm, and hormone parameters evaluated in the NCTR 90-day subchronic rat toxicity study, have not demonstrated decreased reproductive function or related parameters at low doses.

The current review does not provide evidence that the following endpoints be included in hazard identification.
a) Anxiety and learning and memory
b) Developmental prostatic changes
c) Mammary gland carcinogenicity
d) Perturbations in glucose homeostasis
These endpoints may be revisited with completion of the FDA/NIEHS two-year chronic toxicity study or other relevant data.

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

a) A few studies reviewed herein satisfied the criteria for risk assessment. The results of these new toxicity data and studies do not affect the dose-effect level and the existing NOAEL (5 mg/kg bw/day; oral exposure).

b) The current state of pharmacokinetic data and PBPK models may be sufficient to estimate dose-effect levels 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 models continue to contribute to the improvement of BPA risk assessments by clarifying the PK activities related to BPA metabolism and distribution; providing interspecies, age-dependent, and route of exposure dependent extrapolations, and by estimating internal exposures of both the conjugated and unconjugated forms of BPA through adult and infant exposures. Additional assessment may be needed in the future to potentially address subjects with impaired BPA metabolism and/or higher exposure levels via non-dietary sources.

b) The reviewed PK data and analyses of biomonitoring data and methods have identified a high potential for inadvertent exposure or contamination by BPA. These findings provide additional context for interpretation of low-dose studies and identified limitations in preparing accurate and reproducible doses at very low dose levels.

c) Hazard identification endpoints identified in this and previous reviews are currently being investigated in a FDA/NIEHS chronic oral toxicity study, and the results of this study should further address risk assessment issues.

d) Some improvements in study reporting have been noticed in this cycle of review; however, incomplete reporting of details in study design and procedures remains a significant problem in most of the exploratory studies reviewed. Other commonly identified deficiencies included inadequate accounting for litter effects, over-interpretation of marginally significant results, and inappropriate extension of findings to humans. Problems with measurement of exposure in epidemiological studies remain a significant limitation, *e.g.*, appropriate timing of BPA exposure, BPA measurement, analytical methods, and endpoint measurement.

On Behalf of the BPA Joint Emerging Science Working Group:

Jason Aungst, Ph.D.	Steven Anderson, Ph.D., M.P.P.
Co-Chair	Co-Chair

ATTACHMENTS

ATTACHMENT 1: Y.Gu and R.Mitkus/J.Aungst, 8/2/2013. Review of a specialized oral developmental toxicity study on bisphenol A (BPA), entitled "Evaluation of the toxicity of bisphenol A (BPA) in male and female Sprague-Dawley rats exposed orally from gestation day 6 through postnatal day 90"

ATTACHMENT 2: S.Mog and S.Francke-Carroll/J.Aungst, 7/19/2013. Request for CFSAN Pathology comments on mammary gland hyperplasia in the NCTR subchronic (PND 21 and 90) oral rat study with BPA

ATTACHMENT 3: NCTR GLP/NTP Technical Report Project No. E2176.01: Evaluation of the toxicity of bisphenol A (BPA) in male and female Sprague-Dawley rats exposed orally from gestation day 6 through postnatal day 90

REFERENCE LIST

Acevedo N, Davis B, Schaeberle CM, Sonnenschein C, Soto AM. Perinatally Administered Bisphenol A Acts as a Mammary Gland Carcinogen in Rats. Environ Health Perspect. 2013. Retraction/Resubmission: Acevedo N, Davis B, Schaeberle CM, Sonnenschein C, Soto AM. Perinatally Administered Bisphenol A Acts as a potential Mammary Gland Carcinogen in Rats. Environ Health Perspect. 2013, 121(9):1040-6.

Adibi *et al.*, Maternal urinary metabolites of di-(2-ethylhexyl) phthalate in relation to timing of labor in a US multicenter pregnancy cohort study. Am J Epidemol. 2009, 169(8):1015-1024.

Anderson OS, Nahar MS, Faulk C, Jones TR, Liao C, Kannan K, Weinhouse C, Rozek LS, Dolinoy DC. Epigenetic responses following maternal dietary exposure to physiologically

relevant levels of bisphenol A. Environ Mol Mutagen 2012, 53:334-42.

Anderson OS, Peterson KE, Sanchez BN, Zhang Z, Mancuso P, Dolinoy DC. Perinatal bisphenol A exposure promotes hyperactivity, lean body composition, and hormonal responses across the murine life course. FASEB J. 2013, 27(4):1784-92.

Aylward LL, Kirman CR, Adgate JL, McKenzie LM, Hays SM. Interpreting variability in population biomonitoring data: role of elimination kinetics. J Expo Sci Environ Epidemiol. 2012, 22(4):398-408.

Bae S, Kim JH, Lim YH, Park HY, Hong YC. Associations of bisphenol A exposure with heart rate variability and blood pressure. Hypertension 2012, 60(3):786-93.

Batista TM, Alonso-Magdalena P, Vieira E, Amaral ME, Cederroth CR, Nef S, Quesada I, Carneiro EM, Nadal A. Short-term treatment with bisphenol-a leads to metabolic abnormalities in adult male mice. PLoS One. 2012, 7(3):e33814.

Betancourt AM, Wang J, Jenkins S, Mobley J, Russo J, Lamartiniere CA. Altered carcinogenesis and proteome in mammary glands of rats after prepubertal exposures to the hormonally active chemicals bisphenol a and genistein. J Nutr. 2012, 142(7):1382S-8S.

Bhandari R, Xiao J, Shankar A. Urinary bisphenol A and obesity in U.S. children. Am J Epidemiol. 2013, 177(11):1263-70.

Brannick KE, Craig ZR, Himes AD, Peretz JR, Wang W, Flaws JA, Raetzman LT. Prenatal exposure to low doses of bisphenol a increases pituitary proliferation and gonadotroph number in female mice offspring at birth. Biol Reprod. 2012, 87(4):82.

Braun JM, Kalkbrenner AE, Calafat AM, Yolton K, Ye X, Dietrich KN. Impact of Early-Life Bisphenol A Exposure on Behavior and Executive Function in Children. Pediatrics 2011, 128:873-882.

Braun JM, Smith KW, Williams PL, Calafat AM, Berry K, Ehrlich S, Hauser R. Variability of urinary phthalate metabolite and bisphenol A concentrations before and during pregnancy. Environ Health Perspect. 2012, 120(5):739-45.

Brucker-Davis F, Wagner-Mahler K, Delattre I, Ducot B, Ferrari P, Bongain A, Kurzenne JY, Mas JC, Fénichel P. Cryptorchidism at birth in Nice area (France) is associated with higher prenatal exposure to PCBs and DDE, as assessed by colostrum concentrations, Hum Reprod 2008, 23(8):1708-18.

Buck Louis GM, Peterson CM, Chen Z, Croughan M, Sundaram R, Stanford J, Varner MW, Kennedy A, Giudice L, Fujimoto VY, Sun L, Wang L, Guo Y, Kannan K. Bisphenol A and phthalates and endometriosis: the Endometriosis: Natural History, Diagnosis and Outcomes

Study. Fertil Steril. 2013, 100(1):162-169.e2.

Buck Louis GM, Hediger ML, Peterson CM, Croughan M, Sundaram R, Stanford J, Chen Z, Fujimoto VY, Varner MW, Trumble A, Giudice LC. Incidence of endometriosis by study population and diagnostic method: the ENDO study. Fertil Steril. 2011, 96(2):360-5.

Buttke DE, Sircar K, Martin C. Exposures to endocrine-disrupting chemicals and age of menarche in adolescent girls in NHANES (2003-2008). Environ Health Perspect. 2012, 120(11):1613-8.

Cagampang FR, Torrens C, Anthony FW, Hanson MA. Developmental exposure to bisphenol A leads to cardiometabolic dysfunction in adult mouse offspring. Journal of Developmental Origins of Health and Disease 2012, 3(4):287-292.

Calafat AM, Koch HM, Swan SH, Hauser R, Goldman LR, Lanphear BP, Longnecker MP, Rudel RA, Teitelbaum SL, Whyatt RM, Wolff MS. Misuse of blood serum to assess exposure to bisphenol A and phthalates. Breast Cancer Res. 2013, 15(5):403.

Cao J, Mickens JA, McCaffrey KA, Leyrer SM, Patisaul HB. Neonatal Bisphenol A exposure alters sexually dimorphic gene expression in the postnatal rat hypothalamus. Neurotoxicology. 2012, 33(1):23-36.

Cao J, Rebuli ME, Rogers J, Todd KL, Leyrer SM, Ferguson SA, Patisaul HB. Prenatal bisphenol a exposure alters sex-specific estrogen receptor expression in the neonatal rat hypothalamus and amygdala. Toxicol Sci. 2013, 133(1):157-73.

Cao XL, Zhang J, Goodyer CG, Hayward S, Cooke GM, Curran IH. Bisphenol A in human placental and fetal liver tissues collected from Greater Montreal area (Quebec) during 1998-2008. Chemosphere 2012, 89(5):505-11.

Carlsen T. Exposure Parameters for the Deer Mouse and Rock Wren at Lawrence Livermore National Laboratory Site 300. USDOE, 2012. http://www-erd.llnl.gov/library/LLNL_AR_562674_B812_Deer_Mouse_Rock_Wren_2012.pdf

Carlsson L, Lind L, Larsson A. Reference values for 27 clinical chemistry tests in 70-year-old males and females. Gerontology 2010, 56:259–265.

Caserta D, Bordi G, Ciardo F, Marci R, La Rocca C, Tait S, Bergamasco B, Stecca L, Mantovani A, Guerranti C, Fanello EL, Perra G, Borghini F, Focardi SE, Moscarini M. The influence of endocrine disruptors in a selected population of infertile women. Gynecol Endocrinol. 2013, 29(5):444-7.

Caserta D, Ciardo F, Bordi G, Guerranti C, Fanello E, Perra G, Borghini F, La Rocca C, Tait S, Bergamasco B, Stecca L, Marci R, Lo Monte G, Soave I, Focardi S, Mantovani A, Moscarini M.

Correlation of endocrine disrupting chemicals serum levels and white blood cells gene expression of nuclear receptors in a population of infertile women. Int J Endocrinol. 2013, 2013:510703.

Castro B, Sánchez P, Torres JM, Preda O, del Moral RG, Ortega E. Bisphenol A exposure during adulthood alters expression of aromatase and 5α-reductase isozymes in rat prostate. PLoS One. 2013, 8(2):e55905.

Chahoud I, Paumgartten FJ. Influence of litter size on the postnatal growth of rat pups: is there a rationale for litter-size standardization in toxicity studies? Environ Res 2009, 109:1021-7.

Chao HH, Zhang XF, Chen B, Pan B, Zhang LJ, Li L, Sun XF, Shi QH, Shen W. Bisphenol A exposure modifies methylation of imprinted genes in mouse oocytes via the estrogen receptor signaling pathway. Histochem Cell Biol. 2012, 137(2):249-59.

Chen X, Chen M, Xu B, Tang R, Han X, Qin Y, Xu B, Hang B, Mao Z, Huo W, Xia Y, Xu Z, Wang X. Parental phenols exposure and spontaneous abortion in Chinese population residing in the middle and lower reaches of the Yangtze River. Chemosphere 2013, 93(2):217-22.

Chen M, Tang R, Fu G, Xu B, Zhu P, Qiao S, Chen X, Xu B, Qin Y, Lu C, Hang B, Xia Y, Wang X. Association of exposure to phenols and idiopathic male infertility. Hazard Mater. 2013, 250-251:115-21.

Chevrier J, Gunier RB, Bradman A, Holland NT, Calafat AM, Eskenazi B, Harley KG. Maternal Urinary Bisphenol A during Pregnancy and Maternal and Neonatal Thyroid Function in the CHAMACOS Study. Environ Health Perspect. 2013, 121(1):138-44.

Chou WC, Chen JL, Lin CF, Chen YC, Shih FC, Chuang CY. Biomonitoring of bisphenol A concentrations in maternal and umbilical cord blood in regard to birth outcomes and adipokine expression: a birth cohort study in Taiwan. Environ Health. 2011, 10:94.

Christensen KL, Lorber M, Koch HM, Kolossa-Gehring M, Morgan MK. Population variability of phthalate metabolites and bisphenol A concentrations in spot urine samples versus 24- or 48-h collections. J Expo Sci Environ Epidemiol. 2012, 22(6):632-40.

Christensen KL, Lorber M, Koslitz S, Brüning T, Koch HM. PK The contribution of diet to total bisphenol A body burden in humans: Results of a 48hour fasting study. Environ Int. 2012, 50C:7-14.

Churchwell MI, Camacho L, Vanlandingham M, Twaddle NC, Sepehr E, Delclos KB, Fisher JW, Doerge DR. Is lifestage-dependent internal dosimetry for bisphenol A consistent with an estrogenic mode of action in Sprague-Dawley rats when compared with a reference estrogen, ethinyl estradiol, and endogenous estradiol? Toxicol Sci. 2014. PK analysis of NCTR study E02176.01 manuscript submitted to FDA BPA working group.

Corbel T, Gayrard V, Viguié C, Puel S, Lacroix MZ, Toutain PL, Picard-Hagen N. Bisphenol a disposition in the sheep maternal-placental-fetal unit: mechanisms determining fetal internal exposure. Biol Reprod. 2013, 89(1):11.

Coughlin JL, Winnik B, Buckley B. Measurement of bisphenol A, bisphenol A ß-D-glucuronide, genistein, and genistein 4'-ß-D-glucuronide via SPE and HPLC-MS/MS. Anal Bioanal Chem. 2011, 401(3):995-1002.

Cubitt HE, Bouston JB, Galetin A. Prediction of human drug clearance by multiple metabolic pathways: Integration of hepatic and intestinal microsomal and cytosolic data. Drug Metabol. 2011, 39:861-873.

D'Cruz SC, Jubendradass R, Jayakanthan M, Rani SJ, Mathur PP. Bisphenol A impairs insulin signaling and glucose homeostasis and decreases steroidogenesis in rat testis: an *in vivo* and in silico study. Food Chem Toxicol. 2012, 50(3-4):1124-33.

D'Cruz SC, Jubendradass R, Mathur PP. Bisphenol A induces oxidative stress and decreases levels of insulin receptor substrate 2 and glucose transporter 8 in rat testis. Reprod Sci. 2012, 19(2):163-72.

Decatanzaro D, Berger RG, Guzzo AC, Thorpe JB, Khan A. Perturbation of male sexual behavior in mice (Mus musculus) within a discrete range of perinatal bisphenol-A doses in the context of a high- or low-phytoestrogen diet. Food Chem Toxicol. 2013, 55:164-71.

Deis RP. Inhibition of Milk Ejection by Exogenous Oxytocin in Lactating Rats. Proc Soc Exp Biol Med. 1971, 137(3):1006-12.

Delclos KB, Camacho L, Lewis SM, Vanlandingham MM, Latendresse JR, Olson GR, Davis KJ, Patton RE, da Costa GG, Woodling KA, Bryant MS, Chidambaram M, Trbojevich R, Juliar BE, Felton RP, Thorn BT. Toxicity Evaluation of Bisphenol A Administered by Gavage to Sprague Dawley Rats From Gestation Day 6 Through Postnatal Day 90. Toxicol Sci. 2014.

Diaz Weinstein S, Villafane JJ, Juliano N, Bowman RE. Adolescent exposure to Bisphenol-A increases anxiety and sucrose preference but impairs spatial memory in rats independent of sex. Brain Res. 2013, 1529:56-65.

Doerge DR, Vanlandingham M, Twaddle NC, Delclos KB. Lactational transfer of bisphenol A in Sprague-Dawley rats. Toxicol Lett. 2010, 199(3):372-6.

Doerge DR, Twaddle NC, Vanlandingham M, Brown RP, Fisher JW. Distribution of bisphenol A into tissues of adult, neonatal, and fetal Sprague-Dawley rats. Toxicol Appl Pharmacol. 2011, 255:261-270.

Doerge DR, Twaddle NC, Vanlandingham M, Fisher JW. Pharmacokinetics of bisphenol A in serum and adipose tissue following intravenous administration to adult female CD-1 mice. Toxicol Lett. 2012, 211(2):114-9.

Dolinoy DC, Huang D, Jirtle RL. Maternal nutrient supplementation counteracts bisphenol A-induced DNA hypomethylation in early development. Proc Natl Acad Sci USA 2007, 104(32):13056-61.

Doshi T, D'Souza C, Dighe V, Vanage G. Effect of neonatal exposure on male rats to bisphenol a on the expression of DNA methylation machinery in the postimplantation embryo. J Biochem Mol Toxicol. 2012, 26(9):337-43.

Doshi T, D'souza C, Vanage G. Aberrant DNA methylation at Igf2-H19 imprinting control region in spermatozoa upon neonatal exposure to bisphenol A and its association with post implantation loss. Mol Biol Rep. 2013, 40(8):4747-57.

Duan B, Hu X, Zhao H, Qin J, Luo J. The relationship between urinary bisphenol A levels and meningioma in Chinese adults. Int J Clin Oncol. 2013, 18(3):492-7.

Duty SM, Mendonca K, Hauser R, Calafat AM, Ye X, Meeker JD, Ackerman R, Cullinane J, Faller J, Ringer S. Potential sources of bisphenol A in the neonatal intensive care unit. Pediatrics 2013, 131(3):483-9.

Edlow AG, Chen M, Smith NA, Lu C, McElrath TF. Fetal bisphenol A exposure: Concentration of conjugated and unconjugated bisphenol A in amniotic fluid in the second and third trimesters. Reprod Toxicol. 2012, 34(1):1-7.

Ehrlich S, Williams PL, Hauser R, Missmer SA, Peretz J, Calafat AM, Flaws JA. Urinary Bisphenol A Concentrations and Cytochrome P450 19 A1 (Cyp19) Gene Expression in Ovarian Granulosa Cells: An *in vivo* Human Study. Reprod Toxicol. 2013, 42:18-23.

Ehrlich S, Williams PL, Missmer SA, Flaws JA, Berry KF, Calafat AM, Ye X, Petrozza JC, Wright D, Hauser R. Urinary Bisphenol A Concentrations and Implantation Failure among Women Undergoing *In vitro* Fertilization. Environ Health Perspect. 2012, 120(7):978-83.

Ehrlich S, Williams PL, Missmer SA, Flaws JA, Ye X, Calafat AM, Petrozza JC, Wright D, Hauser R. Urinary bisphenol A concentrations and early reproductive health outcomes among women undergoing IVF. Hum Reprod. 2012, 27(12):3583-92.

Elsworth JD, Jentsch JD, Vandevoort CA, Roth RH, Jr DE, Leranth C. Prenatal exposure to bisphenol A impacts midbrain dopamine neurons and hippocampal spine synapses in non-human primates. Neurotoxicology 2013, 35:113-20.

Ema M, Fujii S, Furukawa M, Kiguchi M, Ikka T, Harazono A. Rat two-generation reproductive

toxicity study of bisphenol A. Reproductive Toxicology 2001, 15:505-523.

Fénichel P, Déchaux H, Harthe C, Gal J, Ferrari P, Pacini P, Wagner-Mahler K, Pugeat M, Brucker-Davis F. Unconjugated bisphenol A cord blood levels in boys with descended or undescended testes. Hum Reprod. 2012, 27(4):983-90.

Ferguson SA, Law CD, Abshire JS. Developmental treatment with bisphenol A or ethinyl estradiol causes few alterations on early preweaning measures. Toxicol Sci. 2011, 124:149–160.

Ferguson SA, Boctor SY. Use of food wafers for multiple daily oral treatments in young rats. J Amer Assoc Lab Animal Sci 2009, 96:292-295.

Ferguson SA, Law CD, Abshire JS. Developmental treatment with bisphenol A causes few alterations on measures of postweaning activity and learning. Neurotoxicol Teratol. 2012, 34(6):598-606.

Fernández M, Bianchi M, Lux-Lantos V, Libertun C. Neonatal exposure to bisphenol a alters reproductive parameters and gonadotropin releasing hormone signaling in female rats. Environ Health Perspect. 2009, 117(5):757-62.

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

Fox SD, Falk RT, Veenstra TD, Issaq HJ. Quantitation of free and total bisphenol A in human urine using liquid chromatography-tandem mass spectrometry. J Separation Science 2011, 34(11):1268–1274.

Fujimoto T, Kubo K, Nishikawa Y, Aou S. Postnatal exposure to low-dose bisphenol A influences various emotional conditions. J Toxicol Sci. 2013, 38(4):539-46.

Gayrard V, Lacroix MZ, Collet SH, Viguié C, Bousquet-Melou A, Toutain PL, Picard-Hagen N. High Bioavailability of Bisphenol A from Sublingual Exposure. Environ Health Perspect. 2013, 121(8):951-6.

Geens T, Neels H, Covaci A. Distribution of bisphenol-A, triclosan and n-nonylphenol in human adipose tissue, liver and brain. Chemosphere 2012, 87(7):796-802.

Gioiosa L, Parmigiani S, Vom Saal FS, Palanza P. The effects of bisphenol A on emotional behavior depend upon the timing of exposure, age and gender in mice. Horm Behav. 2013, 63(4):598-605.

Goodman JE. An updated weight of evidence evaluation of reproductive and developmental effects of low doses of bisphenaol A. Crit Rev Toxicol 2006, 36:387-457.

Groothuis GMM, de Graff AM. Precision-cut intestinal slices as *In vitro* Tool for studies on drug metabolism. Current Drug Metabol. 2013, 14:112-119.

Gundert-Remy U, Mielke H, Bernauer U. Commentary: dermal penetration of bisphenol A--consequences for RA. Toxicol Lett. 2013, 217(2):159-61.

Halade GV, Rahman MM, Williams PJ, Fernandes G. High fat diet-induced animal model of age-associated obesity and osteoporosis. J Nutr Biochem. 2010, 21(12):1162-9.

Hammond KA, Kristan DM. Responses to lactation and cold exposure by deer mice (Peromyscus maniculatus). Physiol Biochem Zool 2000, 73:547-556.

Harley KG, Aguilar Schall R, Chevrier J, Tyler K, Aguirre H, Bradman A, Holland NT, Lustig RH, Calafat AM, Eskenazi B. Prenatal and postnatal bisphenol A exposure and body mass index in childhood in the CHAMACOS cohort. Environ Health Perspect. 2013, 121(4):514-20, 520e1-6.

Harley KG, Gunier RB, Kogut K, Johnson C, Bradman A, Calafat AM, Eskenazi B. Prenatal and early childhood bisphenol A concentrations and behavior in school-aged children. Environ Res. 2013, 126:43-50.

Harthé C, Rinaldi S, Achaintre D, de Ravel MR, Mappus E, Pugeat M, Déchaud H. Bisphenol A-glucuronide measurement in urine samples. Talanta. 2012, 100:410-3.

Haseman JK. Data analysis: statistical analysis and use of historical control data. Regul Toxicol Pharmacol 1995, 21(1):52-9.

Haseman JK, Bailer AJ, Kodell RL, Morris R, Portier K. Statistical issues in the analysis of low-dose endocrine disruptor data. Toxicol Sci 2001, 61:201-10.

He Z, Paule MG, Ferguson SA. Low oral doses of bisphenol A increase volume of the sexually dimorphic nucleus of the preoptic area in male, but not female, rats at postnatal day 21. Neurotoxicol Teratol. 2012, 34(3):331-337.

Holson RR, Freshwater L, Maurissen JP, Moser VC, Phang W. Statistical issues and techniques appropriate for developmental neurotoxicity testing: a report from the ILSI Research Foundation/Risk Science Institute expert working group on neurodevelopmental endpoints. Neurotoxicol Teratol 2008, 30:326-48.

Hong SB, Hong YC, Kim JW, Park EJ, Shin MS, Kim BN, Yoo HJ, Cho IH, Bhang SY, Cho SC. Bisphenol A in relation to behavior and learning of school-age children. J Child Psychol Psychiatry 2013, 54(8):890-9.

Horstman KA, Naciff JM, Overmann GJ, Foertsch LM, Richardson BD, Daston GP. Effects of transplacental 17-α-ethynyl estradiol or bisphenol A on the developmental profile of steroidogenic acute regulatory protein in the rat testis. Birth Defects Res B Dev Reprod Toxicol. 2012, 95(4):318-25.

Hunt PA, Lawson C, Gieske M, Murdoch B, Smith H, Marre A, Hassold T, Vandevoort CA. Bisphenol A alters early oogenesis and follicle formation in the fetal ovary of the rhesus monkey. Proc Natl Acad Sci USA 2012, 109(43):17525-30.

Inagaki T, Frankfurt M, Luine V. Estrogen-induced memory enhancements are blocked by acute bisphenol A in adult female rats: role of dendritic spines. Endocrinology 2012, 153(7):3357-67.

Jang YJ, Park HR, Kim TH, Yang WJ, Lee JJ, Choi SY, Oh SB, Lee E, Park JH, Kim HP, Kim HS, Lee J. High dose bisphenol A impairs hippocampal neurogenesis in female mice across generations. Toxicology 2012, 296(1-3):73-82.

Jašarević E, Williams SA, Vandas GM, Ellersieck MR, Liao C, Kannan K, Roberts RM, Geary DC, Rosenfeld CS. Sex and dose-dependent effects of developmental exposure to bisphenol A on anxiety and spatial learning in deer mice (Peromyscus maniculatus bairdii) offspring. Horm Behav. 2013, 63(1):180-9.

Jin P, Wang X, Chang F, Bai Y, Li Y, Zhou R, Chen L. Low dose bisphenol A impairs spermatogenesis by suppressing reproductive hormone production and promoting germ cell apoptosis in adult rats. J Biomed Res. 2013, 27(2):135-44.

Jones BA, Watson NV. Perinatal BPA exposure demasculinizes males in measures of affect but has no effect on water maze learning in adulthood. Horm Behav. 2012, 61(4):605-10.

Jung H, Hong Y, Lee D, Pang K, Kim Y. The association between some endocrine disruptors in human plasma and the occurrence of congenital hypothyroidism. Environ Toxicol Pharmacol. 2013, 35(2):278-83.

Karavan JR, Pepling ME. Effects of estrogenic compounds on neonatal oocyte development. Reprod Toxicol. 2012, 34(1):51-6.

Kass L, Altamirano GA, Bosquiazzo VL, Luque EH, Muñoz-de-Toro M. Perinatal exposure to xenoestrogens impairs mammary gland differentiation and modifies milk composition in Wistar rats. Reprod Toxicol. 2012, 33(3):390-400.

Kendig EL, Buesing DR, Christie SM, Cookman CJ, Gear RB, Hugo ER, Kasper SN, Kendziorski JA, Ungi KR, Williams K, Belcher SM. Estrogen-like disruptive effects of dietary exposure to bisphenol A or 17α-ethinyl estradiol in CD1 mice. Int J Toxicol. 2012, 31(6):537-50.

Kendziorski JA, Kendig EL, Gear RB, Belcher SM. Strain specific induction of pyometra and

differences in immune responsiveness in mice exposed to 17α-ethinyl estradiol or the endocrine disrupting chemical bisphenol A. Reprod Toxicol. 2012, 34(1):22-30.

Kim K, Park H. Association between urinary concentrations of bisphenol A and type 2 diabetes in Korean adults: A population-based cross-sectional study. Int J Hyg Environ Health 2013, 216(4):467-71.

Krieter DH, Canaud B, Lemke HD, Rodriguez A, Morgenroth A, von Appen K, Dragoun GP, Wanner C. Bisphenol A in chronic kidney disease. Artif Organs 2013, 37(3):283-90.

Kobayashi K, Kubota H, Ohtani K, Hojo R, Miyagawa M. Lack of effects for dietary exposure of bisphenol A during *in utero* and lactational periods on reproductive development in rat offspring. J Toxicol Sci. 2012, 37(3):565-73.

Komada M, Asai Y, Morii M, Matsuki M, Sato M, Nagao T. Maternal bisphenol A oral dosing relates to the acceleration of neurogenesis in the developing neocortex of mouse fetuses. Toxicology 2012, 295(1-3):31-8.

Kosarac I, Kubwabo C, Lalonde K, Foster W. A novel method for the quantitative determination of free and conjugated bisphenol A in human maternal and umbilical cord blood serum using a two-step solid phase extraction and gas chromatography/tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci. 2012, 898:90-4.

Krotz SP, Carson SA, Tomey C, Buster JE. PK Phthalates and bisphenol do not accumulate in human follicular fluid. J Assist Reprod Genet. 2012, 29(8):773-7.

Kumamoto T, Oshio S. Effect of fetal exposure to bisphenol A on brain mediated by X-chromosome inactivation. J Toxicol Sci. 2013, 38(3):485-94.

Kundakovic M, Gudsnuk K, Franks B, Madrid J, Miller RL, Perera FP, Champagne FA. Sex-specific epigenetic disruption and behavioral changes following low-dose *in utero* bisphenol A exposure. Proc Natl Acad Sci USA 2013, 110(24):9956-61.

Kuzutani K, Shibanushi T, Kangawa Y, Kihara T. Spontaneous Mammary Adenocarcinoma in a Twelve-week-old Female Sprague-Dawley Rat. J Toxicol Pathol. 2012, 25(3):221-4.

Lakind JS, Goodman M, Naiman DQ. Use of NHANES Data to Link Chemical Exposures to Chronic Diseases: A Cautionary Tale. PLoS One 2012, 7(12):e51086.

Latini G, De Felice C, Presta G, Del Vecchio A, Paris I, Ruggieri F, Mazzeo P. *In utero* exposure to di-(2-ethylhexyl) phthalate and duration of human pregnancy. Environ Health Perspect. 2003, 111:1783-1785.

Lee SH, Kang SM, Choi MH, Lee J, Park MJ, Kim SH, Lee WY, Hong J, Chung BC. Changes in

steroid metabolism among girls with precocious puberty may not be associated with urinary levels of bisphenol A. Reprod Toxicol. 2013, pii: S0890-6238(13)00073-7.

Lee SG, Kim JY, Chung JY, Kim YJ, Park JE, Oh S, Yoon YD, Yoo KS, Yoo YH, Kim JM. Bisphenol A Exposure during Adulthood Causes Augmentation of Follicular Atresia and Luteal Regression by Decreasing 17β-Estradiol Synthesis via Downregulation of Aromatase in Rat Ovary. Environ Health Perspect. 2013, 121(6):663-9.

Leranth C, Hajszan T, Szigeti-Buck K, Bober J, MacLusky NJ. Bisphenol A prevents the synaptogenic response to estradiol in hippocampus and prefrontal cortex of ovariectomized nonhuman primates. Proc Natl Acad Sci USA. 2008, 105(37):14187-91.

Leung K, Solomon S. Metabolism of progesterone in the fetus and placenta of the rhesus monkey (Macaca mulatta). Endocrinology 1972, 91(2):341-349.

Li DK, Miao M, Zhou Z, Wu C, Shi H, Liu X, Wang S, Yuan W. Urine bisphenol-a level in relation to obesity and overweight in school-age children. PLoS One 2013, 8(6):e65399.

Li M, Bi Y, Qi L, Wang T, Xu M, Huang Y, Xu Y, Chen Y, Lu J, Wang W, Ning G. Exposure to bisphenol A is associated with low-grade albuminuria in Chinese adults. Kidney Int. 2012, 81(11):1131-9.

Liao C, Kannan K. Determination of free and conjugated forms of bisphenol a in human urine and serum by liquid chromatography-tandem mass spectrometry. Environ Sci Technol. 2012, 46(9):5003-9.

Linzell JL, Peaker M, Taylor JC. The effects of prolactin and oxytocin on milk secretion and on the permeability of the mammary epithelium in the rabbit. J Physiol. 1975, 253(2):547–563.

Littell RC, Milliken GA, Stroup WW, Wolfinger R. SAS system for mixed models. Cary, NC: SAS Publishing 1996.

Liu C, Duan W, Li R, Xu S, Zhang L, Chen C, He M, Lu Y, Wu H, Pi H, Luo X, Zhang Y, Zhong M, Yu Z, Zhou Z. Exposure to bisphenol A disrupts meiotic progression during spermatogenesis in adult rats through estrogen-like activity. Cell Death Dis. 2013, 4:e676.

Liu J, Yu P, Qian W, Li Y, Zhao J, Huan F, Wang J, Xiao H. Perinatal bisphenol A exposure and adult glucose homeostasis: identifying critical windows of exposure. PLoS One 2013, 8(5):e64143.

López-Casas PP, Mizrak SC, López-Fernández LA, Paz M, de Rooij DG, del Mazo J. The effects of different endocrine disruptors defining compound-specific alterations of gene expression profiles in the developing testis. Reprod Toxicol. 2012, 33(1):106-15.

Losa-Ward SM, Todd KL, McCaffrey KA, Tsutsui K, Patisaul HB. Disrupted organization of RFamide pathways in the hypothalamus is associated with advanced puberty in female rats neonatally exposed to bisphenol A. Biol Reprod. 2012, 87(2):28.

Lušin T, Roškar R, Mrhar A. Evaluation of bisphenol A glucuronidation according to UGT1A1*28 polymorphism by a new LC-MS/MS assay. Toxicology 2012, 292(1):33-41.

Ma W, Kannan K, Wu Q, Bell EM, Druschel CM, Caggana M, Aldous KM. Analysis of polyfluoroalkyl substances and bisphenol A in dried blood spots by liquid chromatography tandem mass spectrometry. Anal Bioanal Chem. 2013, 405(12):4127-38.

Ma Y, Xia W, Wang DQ, Wan YJ, Xu B, Chen X, Li YY, Xu SQ. Hepatic DNA methylation modifications in early development of rats resulting from perinatal BPA exposure contribute to insulin resistance in adulthood. Diabetologia 2013, 56(9):2059-67.

Mackay H, Patterson ZR, Khazall R, Patel S, Tsirlin D, Abizaid A. Organizational effects of perinatal exposure to bisphenol-A and diethylstilbestrol on arcuate nucleus circuitry controlling food intake and energy expenditure in male and female CD-1 mice. Endocrinology 2013, 154(4):1465-75.

Mahalingaiah S, Hauser R, Patterson DG Jr, Woudneh M, Racowsky C. Bisphenol A is not detectable in media or selected contact materials used in IVF. Reprod Biomed Online 2012, 25(6):608-11.

Matsuda S, Matsuzawa D, Ishii D, Tomizawa H, Sutoh C, Nakazawa K, Amano K, Sajiki J, Shimizu E. Effects of perinatal exposure to low dose of bisphenol A on anxiety like behavior and dopamine metabolites in brain. Prog Neuropsychopharmacol Biol Psychiatry 2012, 39(2):273-9.

Matsuda S, Matsuzawa D, Ishii D, Tomizawa H, Sajiki J, Shimizu E. Perinatal exposure to bisphenol A enhances contextual fear memory and affects the serotoninergic system in juvenile female mice. Horm Behav. 2013, 63(5):709-16.

Matsumoto A, Kunugita N, Kitagawa K, Isse T, Oyama T, Foureman GL, Morita M, Kawamoto T. Bisphenol A levels in human urine. Environ Health Perspect. 2003, 111(1):101-4.

Matsumoto A, Kunugita N, Kitagawa K, Isse T, Oyama T, Foureman GL, Morita M, Kawamoto T. Bisphenol A levels in human urine. Environmental Health Perspectives 2013, 111(1):101-4.

Mazur C, Marrchitti S, Fisher J, Kenneke J, Lumen A, Dimova M. Human and Rat ABC Transporter Efflux of Bisphenol A and Bisphenol A Glucuronide: Interspecies Comparison and Implications for Pharmacokinetic Assessment. Toxicol Sci. 2012, 128(2):317-25.

McCaffrey KA, Jones B, Mabrey N, Weiss B, Swan SH, Patisaul HB. Sex specific impact of perinatal bisphenol A (BPA) exposure over a range of orally administered doses on rat

hypothalamic sexual differentiation. Neurotoxicology 2013, 36:55-62.

Meeker JD1, Hu H, Cantonwine DE, Lamadrid-Figueroa H, Calafat AM, Ettinger AS, Hernandez-Avila M, Loch-Caruso R, Téllez-Rojo MM. Urinary phthalate metabolites in relation to preterm birth in Mexico City. Environ Health Perspect. 2009, 117:1587-1592.

Melzer D, Gates P, Osborn NJ, Henley WE, Cipelli R, Young A, Money C, McCormack P, Schofield P, Mosedale D, Grainger D, Galloway TS. Urinary bisphenol a concentration and angiography-defined coronary artery stenosis. PLoS One 2012, 7(8):e43378.

Melzer D, Osborne NJ, Henley WE, Cipelli R, Young A, Money C, McCormack P, Luben R, Khaw KT, Wareham NJ, Galloway TS. Urinary bisphenol A concentration and risk of future coronary artery disease in apparently healthy men and women. Circulation 2012, 125(12):1482-90.

Mendiola J, Jørgensen N, Andersson AM, Calafat AM, Ye X, Redmon JB, Drobnis EZ, Wang C, Sparks A, Thurston SW, Liu F, Swan SH. Are environmental levels of bisphenol a associated with reproductive function in fertile men? Environ Health Perspect 2010, 118(9):1286-91.

Mendonca K, Hauser R, Calafat AM, Arbuckle TE, Duty SM. Bisphenol A concentrations in maternal breast milk and infant urine. Int Arch Occup Environ Health 2014, 87(1):13-20.

Mita L, Baldi A, Diano N, Viggiano E, Portaccio M, Nicolucci C, Grumiro L, Menale C, Mita DG, Spugnini EP, Viceconte R, Citro G, Pierantoni R, Sica V, Marino M, Signorile PG, Bianco M. Differential accumulation of BPA in some tissues of offspring of Balb-C mice exposed to different BPA doses. Environ Toxicol Pharmacol. 2012, 33(1):9-15.

Moon MK, Kim MJ, Jung IK, Koo YD, Ann HY, Lee KJ, Kim SH, Yoon YC, Cho BJ, Park KS, Jang HC, Park YJ. Bisphenol A impairs mitochondrial function in the liver at doses below the no observed adverse effect level. J Korean Med Sci. 2012, 27(6):644-52.

Nachman RM, Fox SD, Golden WC, Sibinga E, Veenstra TD, Groopman JD, Lees PS. Urinary free bisphenol A and bisphenol A-glucuronide concentrations in newborns. J Pediatr. 2013, 162(4):870-2.

Nah WH, Park MJ, Gye MC. Effects of early prepubertal exposure to bisphenol A on the onset of puberty, ovarian weights, and estrous cycle in female mice. Clin Exp Reprod Med. 2011, 38(2):75-81.

Nahar MS, Liao C, Kannan K, Dolinoy DC. Fetal Liver Bisphenol A Concentrations and Biotransformation Gene Expression Reveal Variable Exposure and Altered Capacity for Metabolism in Humans. J Biochem Mol Toxicol. 2013, 27(2):116-23.

Nakamura K, Itoh K, Dai H, Han L, Wang X, Kato S, Sugimoto T, Fushiki S. Prenatal and

lactational exposure to low-doses of bisphenol A alters adult mice behavior. Brain Dev. 2012, 34(1):57-63.

Nanjappa MK, Simon L, Akingbemi BT. The industrial chemical bisphenol A (BPA) interferes with proliferative activity and development of steroidogenic capacity in rat Leydig cells. Biol Reprod. 2012, 86(5):135, 1-12.

Neese SL, Bandara SB, Schantz SL. Working memory in bisphenol-A treated middle-aged ovariectomized rats. Neurotoxicol Teratol. 2013, 35:46-53.

Nelson J F, Felicio LS, Randall PK, Sims C, Finch CE. A longitudinal study of estrous cyclicity if aging C57BL/6J mice: I. cycle frequency, length and vaginal cytology. Biol Reprod 1982, 26:327-339.

O'Dowd R, Kent JC, Moseley JM, Wlodek ME. Effects of uteroplacental insufficiency and reducing litter size on maternal mammary function and postnatal offspring growth. Am J Physiol Regul Integr Comp Physiol 2008, 294:R539-48

Ogi H, Itoh K, Fushiki S. Social behavior is perturbed in mice after exposure to bisphenol A: a novel assessment employing an IntelliCage. Brain Behav. 2013, 3(3):223-8.

Olsén L, Lind L, Lind PM. Associations between circulating levels of bisphenol A and phthalate metabolites and coronary risk in the elderly. Ecotoxicol Environ Saf. 2012, 80:179-83.

Otsuka H, Sugimoto M, Ikeda S, Kume S. Effects of bisphenol A administration to pregnant mice on serum Ca and intestinal Ca absorption. Anim Sci J. 2012, 83(3):232-7.

Pant J, Pant MK, Deshpande SB. Bisphenol A attenuates phenylbiguanide-induced cardio-respiratory reflexes in anaesthetized rats. Neurosci Lett. 2012, 530(1):69-74.

Partosch F, Mielke H, Gundert-Remy U. Functional UDP-glucuronyltransferase 2B15 polymorphism and bisphenol A concentrations in blood: results from physiologically based kinetic modelling. Arch Toxicol. 2013, 87(7):1257-64.

Patel BB, Raad M, Sebag IA, Chalifour LE. Lifelong exposure to bisphenol a alters cardiac structure/function, protein expression, and DNA methylation in adult mice. Toxicol Sci. 2013, 133(1):174-85.

Patisaul HB, Sullivan AW, Radford ME, Walker DM, Adewale HB, Winnik B, Coughlin JL, Buckley B, Gore AC. Anxiogenic effects of developmental bisphenol a exposure are associated with gene expression changes in the juvenile rat amygdala and mitigated by soy. PLoS One 2012, 7(9):e43890.

Patterson TA, Twaddle NC, Roegge CS, Callicott RJ, Fisher JW, Doerge DR. Concurrent

determination of bisphenol A pharmacokinetics in maternal and fetal rhesus monkeys. Toxicol Appl Pharmacol. 2013, 267(1):41-8.

Pelch KE, Carleton SM, Phillips CL, Nagel SC. Developmental exposure to xenoestrogens at low doses alters femur length and tensile strength in adult mice. Biol Reprod. 2012, 19;86(3):69.

Perera F, Vishnevetsky J, Herbstman JB, Calafat AM, Xiong W, Rauh V, Wang S. Prenatal Bisphenol A Exposure and Child Behavior in an Inner City Cohort. Environ Health Perspect. 2012, 120(8):1190-4.

Qiu LL, Wang X, Zhang XH, Zhang Z, Gu J, Liu L, Wang Y, Wang X, Wang SL. Decreased androgen receptor expression may contribute to spermatogenesis failure in rats exposed to low concentration of bisphenol A. Toxicol Lett. 2013, 219(2):116-24.

Rabinovici J, Goldsmith PC, Roberts VJ, Vaughan J, Vale W, Jaffe RB. Localization and secretion of inhibin/activin subunits in the human and subhuman primate fetal gonads. J Clin Endocrinol Metab. 1991, 73(5):1141-1149.

Ramirez MC, Bourguignon NS, Bonaventura MM, Lux-Lantos V, Libertun C, Becu-Villalobos D. Neonatal xenoestrogen exposure alters growth hormone-dependent liver proteins and genes in adult female rats. Toxicol Lett. 2012, 213(3):325-31.

Riesen JW, Meyer RK, Wolf RC. The Effect of Season on Occurrence of Ovulation in the Rhesus Monkey. Biology of Reproduction 1971, 5(2):111-114.

Rodríguez HA, Santambrosio N, Santamaría CG, Muñoz-de-Toro M, Luque EH. Neonatal exposure to bisphenol A reduces the pool of primordial follicles in the rat ovary. Reproductive Toxicology 2010, 30:550-557.

Rönn M, Kullberg J, Karlsson H, Berglund J, Malmberg F, Orberg J, Lind L, Ahlström H, Lind PM. Bisphenol A exposure increases liver fat in juvenile fructose-fed Fischer 344 rats. Toxicology 2013, 303:125-32.

Roy A, Gaylo A, Cao W, Saubermann LJ, Lawrence BP. Neither direct nor developmental exposure to bisphenol A alters the severity of experimental inflammatory colitis in mice. J Immunotoxicol. 2013, 10(4):334-40.

Sabanayagam C, Teppala S, Shankar A. Relationship between urinary bisphenol A levels and prediabetes among subjects free of diabetes. Acta Diabetol. 2013, 50(4):625-31.

Sathyanarayana S, Alcedo G, Saelens BE, Zhou C, Dills RL, Yu J, Lanphear B. Unexpected results in a randomized dietary trial to reduce phthalate and bisphenol A exposures. J Expo Sci Environ Epidemiol. 2013, 23(4):378-84.

Shankar A, Teppala S, Sabanayagam C. Bisphenol A and Peripheral Arterial Disease: Results from the NHANES. Environ Health Perspect. 2012, 120(9):1297-300.

Shankar A, Teppala S. Urinary bisphenol A and hypertension in a multiethnic sample of US adults. J Environ Public Health. 2012, 2012:481641.

Signorile PG, Spugnini EP, Citro G, Viceconte R, Vincenzi B, Baldi F, Baldi A. Endocrine disruptors *in utero* cause ovarian damages linked to endometriosis. Front Biosci (Elite Ed). 2012, 4:1724-30.

Silver MK, O'Neill MS, Sowers MR, Park SK. Urinary bisphenol A and type-2 diabetes in U.S. adults: data from NHANES 2003-2008. PLoS One. 2011, 6(10):e26868.

Snijder CA, Heederik D, Pierik FH, Hofman A, Jaddoe VW, Koch HM, Longnecker MP, Burdorf A. Fetal growth and prenatal exposure to bisphenol A: the generation R study. Environ Health Perspect. 2013, 121(3):393-8.

Sprague BL, Trentham-Dietz A, Hedman CJ, Wang J, Hemming JD, Hampton JM, Buist DS, Bowles EJ, Sisney GS, Burnside ES. Circulating serum xenoestrogens and mammographic breast density. Breast Cancer Res. 2013, 15(3):R45.

Sriphrapradang C, Chailurkit LO, Aekplakorn W, Ongphiphadhanakul B. Association between bisphenol A and abnormal free thyroxine level in men. Endocrine 2013, 44(2):441-7.

Takahashi O, Oishi S. Disposition of orally administered 2,2-Bis(4-hydroxyphenyl)propane (Bisphenol A) in pregnant rats and the placental transfer to fetuses. Environ Health Perspect. 2000, 108(10):931-5.

Takahashi O, Oishi S. Testicular toxicity of dietarily or parenterally administered bisphenol A in rats and mice. Food Chem Toxicol. 2003, 41(7):1035-44.

Tan W, Huang H, Wang Y, Wong TY, Wang CC, Leung LK. Bisphenol A differentially activates protein kinase C isoforms in murine placental tissue. Toxicol Appl Pharmacol. 2013, 269(2):163-8.

Tang R, Chen MJ, Ding GD, Chen XJ, Han XM, Zhou K, Chen LM, Xia YK, Tian Y, Wang XR. Associations of prenatal exposure to phenols with birth outcomes. Environ Pollut. 2013, 178:115-20.

Tarantino G, Valentino R, Di Somma C, D'Esposito V, Passaretti F, Pizza G, Brancato V, Orio F, Formisano P, Colao A, Savastano S. Bisphenol A in Polycystic Ovary Syndrome and its Association with Liver-Spleen Axis. Clin Endocrinol (Oxf). Clin Endocrinol (Oxf). 2013, 78(3):447-53.

Teeguarden J, Hanson-Drury S, Fisher JW, Doerge DR. Are Typical Human Serum BPA Concentrations Measureable and Sufficient to be Estrogenic in the General Population? Food Chem Toxicol. 2013, 62:949-63.

Teppala S, Madhavan S, Shankar A. Bisphenol A and Metabolic Syndrome: Results from NHANES. Int J Endocrinol. 2012, 2012:598180.

Tharp AP, Maffini MV, Hunt PA, VandeVoort CA, Sonnenschein C, Soto AM. Bisphenol A alters the development of the rhesus monkey mammary gland. Proc Natl Acad Sci USA 2012, 109(21):8190-5.

Thigpen JE, Setchell KD, Kissling GE, Locklear J, Caviness GF, Whiteside T, Belcher SM, Brown NM, Collins BJ, Lih FB, Tomer KB, Padilla-Banks E, Camacho L, Adsit FG, Grant M. The estrogenic content of rodent diets, bedding, cages, and water bottles and its effect on bisphenol a studies. J Am Assoc Lab Anim Sci. 2013, 52(2):130-41.

Tiwari D, Vanage G. Mutagenic effect of Bisphenol A on adult rat male germ cells and their fertility. Reprod Toxicol. 2013, 40:60-8.

Trasande L, Attina TM, Blustein J. Association between urinary bisphenol A concentration and obesity prevalence in children and adolescents. JAMA. 2012, 308(11):1113-21.

Editorial: Brent RL. Bisphenol A and obesity in children and adolescents. JAMA 2013, 309(2):134.

Editorial: Trasande L, Attina TM, Blustein J. Bisphenol A and obesity in children and adolescents--reply. JAMA 2013, 309(2):134-5.

Trasande L, Attina TM, Trachtman H. Bisphenol A exposure is associated with low-grade urinary albumin excretion in children of the United States. Kidney Int. 2013, 83(4):741-8.

Tyl RW, Myers CB, Marr MC, Thomas BF, Keimowitz AR, Brine DR, Veselica MM, Fail PA, Chang TY, Seely JC, Joiner RL, Butala JH, Dimond SS, Cagen SZ, Shiotsuka RN, Stropp GD, Waechter JM. Three generation reproductive toxicity study of dietary bisphenol A in CD Sprague-Dawley rats. Toxicological Sciences 2002, 68:121-146.

Unal ER, Lynn T, Neidich J, Salazar D, Goetzl L, Baatz JE, Hulsey TC, Van Dolah R, Guillette LJ Jr, Newman R. Racial disparity in maternal and fetal-cord bisphenol A concentrations. J Perinatol. 2012, 32(11):844-50.

Vandenberg LN, Schaeberle CM, Rubin BS, Sonnenschein C, Soto AM. The male mammary gland: a target for the xenoestrogen bisphenol A. Reprod Toxicol. 2013, 37:15-23.

Veiga-Lopez A, Luense LJ, Christenson LK, Padmanabhan V. Developmental programming: gestational bisphenol-A treatment alters trajectory of fetal ovarian gene expression. Endocrinology 2013, 154(5):1873-84.

Viberg H, Lee I. A single exposure to bisphenol A alters the levels of important neuroproteins in adult male and female mice. Neurotoxicology 2012, 33(5):1390-5.

Viguié C, Collet SH, Gayrard V, Picard-Hagen N, Puel S, Roques BB, Toutain PL, Lacroix MZ. Maternal and fetal exposure to bisphenol a is associated with alterations of thyroid function in pregnant ewes and their newborn lambs. Endocrinology 2013, 154(1):521-8.

Wang F, Hua J, Chen M, Xia Y, Zhang Q, Zhao R, Zhou W, Zhang Z, Wang B. High urinary bisphenol A concentrations in workers and possible laboratory abnormalities. Occup Environ Med. 2012, 69(9):679-84.

Wang HX, Zhou Y, Tang CX, Wu JG, Chen Y, Jiang QW. Association between bisphenol a exposure and body mass index in Chinese school children: a cross-sectional study. Environ Health 2012, 11(1):79.

Wang T, Li M, Chen B, Xu M, Xu Y, Huang Y, Lu J, Chen Y, Wang W, Li X, Liu Y, Bi Y, Lai S, Ning G. Urinary bisphenol A (BPA) concentration associates with obesity and insulin resistance. J Clin Endocrinol Metab. 2012 97(2):E223-7.

Wang T, Lu J, Xu M, Xu Y, Li M, Liu Y, Tian X, Chen Y, Dai M, Wang W, Lai S, Bi Y, Ning G. Urinary bisphenol a concentration and thyroid function in Chinese adults. Epidemiology 2013, 24(2):295-302.

Weinberger B, Vetrano AM, Archer FE, Marcella SW, Buckley B, Wartenberg D, Robson MG, Klim J, Azhar S, Cavin S, Wang L, Rich DQ. Effects of maternal exposure to phthalates and bisphenol A during pregnancy on gestational age. J Matern Fetal Neonatal Med. 2014, 27(4):323-7.

Wells EM, Jackson LW, Koontz MB. Association between bisphenol A and waist-to-height ratio among children: National Health and Nutrition Examination Survey, 2003-2010. Ann Epidemiol. 2014, 24(2):165-7.

Williams SA, Jasarevic E, Vandas GM, Warzak DA, Geary DC, Ellersieck MR, Roberts RM, Rosenfeld CS. Effects of developmental bisphenol A exposure on reproductive-related behaviors in California mice (Peromyscus californicus): a monogamous animal model. PLoS One 2013, 8(2):e55698.

Wolstenholme JT, Edwards M, Shetty SR, Gatewood JD, Taylor JA, Rissman EF, Connelly JJ. Gestational exposure to bisphenol a produces transgenerational changes in behaviors and gene expression. Endocrinology 2012, 153(8):3828-38.

Xi W, Wan HT, Zhao YG, Wong MH, Giesy JP, Wong CK. Effects of perinatal exposure to bisphenol A and di(2-ethylhexyl)-phthalate on gonadal development of male mice. Environ Sci

Pollut Res Int. 2011, 19(7):2515-27.

Xu J, Knowler WC, Devereux RB, Yeh J, Umans JG, Begum M, Fabsitz RR, Lee ET. Albuminuria within the "normal" range and risk of cardiovascular disease and death in American Indians: the Strong Heart Study. Am J Kidney Dis 2007, 49(2):208-16.

Xu X, Hong X, Xie L, Li T, Yang Y, Zhang Q, Zhang G, Liu X. Gestational and lactational exposure to bisphenol-A affects anxiety- and depression-like behaviors in mice. Horm Behav. 2012, 62(4):480-90.

Xu X, Liu X, Zhang Q, Zhang G, Lu Y, Ruan Q, Dong F, Yang Y. Sex-specific effects of bisphenol-A on memory and synaptic structural modification in hippocampus of adult mice. Horm Behav. 2013a 63(5):766-75.

Xu X, Xie L, Hong X, Ruan Q, Lu H, Zhang Q, Zhang G, Liu X. Perinatal exposure to bisphenol-A inhibits synaptogenesis and affects the synaptic morphological development in offspring male mice. Chemosphere 2013b, 91(8):1073-81.

Yang X, Doerge DR, Fisher JW. Prediction and evaluation of route dependent dosimetry of BPA in rats at different life stages using a physiologically based pharmacokinetic model. Toxicol Appl Pharmacol. 2013, 270(1):45-59.

Ye X, Zhou X, Hennings R, Kramer J, Calafat AM. Potential external contamination with bisphenol A and other ubiquitous organic environmental chemicals during biomonitoring analysis: an elusive laboratory challenge. Environ Health Perspect. 2013, 121(3):283-6.

Yi B, Kim C, Park M, Han Y, Park JY, Yang M. Association between Endocrine Disrupting Phenols in Colostrums and Maternal and Infant Health. Int J Endocrinol. 2013, 2013:282381.

Yum T, Lee S, Kim Y. Association between precocious puberty and some endocrine disruptors in human plasma. J Environ Sci Health A Tox Hazard Subst Environ Eng. 2013, 48(8):912-7.

Zhang GL, Zhang XF, Feng YM, Li L, Huynh E, Sun XF, Sun ZY, Shen W. Exposure to bisphenol A results in a decline in mouse spermatogenesis. Reprod Fertil Dev. 2013, 25(6):847-59.

Zhang T, Sun H, Kannan K. Blood and urinary bisphenol A concentrations in children, adults, and pregnant women from china: partitioning between blood and urine and maternal and fetal cord blood. Environ Sci Technol. 2013, 47(9):4686-94.

Zhao HY, Bi YF, Ma LY, Zhao L, Wang TG, Zhang LZ, Tao B, Sun LH, Zhao YJ, Wang WQ, Li XY, Xu MY, Chen JL, Ning G, Liu JM. The effects of bisphenol A (BPA) exposure on fat mass and serum leptin concentrations have no impact on bone mineral densities in non-obese premenopausal women. Clin Biochem. 2012, 45(18):1602-6.

Zhou R, Chen F, Chang F, Bai Y, Chen L. Persistent overexpression of DNA methyltransferase 1 attenuating GABAergic inhibition in basolateral amygdala accounts for anxiety in rat offspring exposed perinatally to low-dose bisphenol A. J Psychiatr Res. 2013, 47(10):1535-44.