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CLARITY-BPA Academic Laboratory Studies Identify Consistent Low-Dose Bisphenol A Effects on Multiple Organ Systems^a

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Abstract

Bisphenol A (BPA) is a high-production chemical used in a variety of applications worldwide. While BPA has been documented as an endocrine disrupting chemical (EDC) having adverse health-related outcomes in multiple studies, risk assessment for BPA has lagged due to reliance on guideline toxicology studies over academic ones with endpoints considered more sensitive and appropriate. To address current controversies on BPA safety, the United States National Institute of Environmental Health Sciences (NIEHS), the National Toxicology Program (NTP) and the Food and Drug Administration (FDA) established the Consortium Linking Academic and Regulatory Insights on BPA Toxicity (CLARITY-BPA) using the NCTR-Sprague-Dawley rats. The goal of CLARITY-BPA is to perform a traditional regulatory toxicology study (Core study) in conjunction with multiple behavioural, molecular and cellular studies by academic laboratories focused on previously identified BPA-sensitive organ systems (Academic studies). Combined analysis of the data from both study types will be undertaken by the NTP with the aim of resolving uncertainties on BPA toxicity. To date, the Core study has been completed and a draft report released. Most of the academic studies have also been finalized and published in peer reviewed journals. In light of this important milestone, the PPTOX-VI meeting held in the Faroe Islands, 27–30 May 2018 devoted a plenary session to CLARITY-BPA with presentations by multiple investigators with the purpose of highlighting key outcomes. This MiniReview synthesizes the results of three academic studies presented at this plenary session, evaluates recently published findings by other CLARITY-BPA academic studies to provide an early combined overview of this emerging data, and places this in the context of the Core study findings. This coordinated effort revealed a plethora of significant BPA effects across multiple organ systems and BPA doses with non-monotonic responses across the dose-range utilized. Remarkably consistent across most studies, including the Core study, are low-dose effects (2.5, 25 and 250 µg BPA/kg body weight). Collectively, the

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findings highlighted herein corroborate a significant body of evidence that documents adverse effects of BPA at doses relevant to human exposures and emphasizes the need for updated risk assessment analysis.

Keywords

Bisphenol A; CLARITY-BPA; endocrine disruptor; EDC; estrogen

INTRODUCTION:

Despite a wealth of data showing that Bisphenol A (BPA) is an endocrine-disrupting chemical (EDC), there remains controversy as to whether exposure poses a risk to human health. The incongruence between the results from academic laboratories and the results of traditional regulatory toxicity studies has contributed to ongoing debate regarding the human health risks posed by BPA. Recognizing this controversy, an inter-agency collaboration was initiated between the National Institutes of Environmental Health Sciences (NIEHS), the National Toxicology Program and the US Food and Drug Administration (FDA). Together, they established the “*Consortium Linking Academic and Regulatory Insights on BPA Toxicity*” (CLARITY-BPA). The goal of CLARITY-BPA is to combine a traditional regulatory toxicology study, which would evaluate validated guideline endpoints, with detailed behavioural, cellular and molecular research, designed and performed by academic researchers, to ultimately resolve uncertainties on BPA toxicity [1]. This MiniReview highlights papers presented at a dedicated session of the PPTOX-VI meeting that aimed to highlight emerging data from academic laboratories that participated in CLARITY-BPA.

BPA is a high-production chemical with >5 million tons produced annually worldwide, for use in epoxy resins, polycarbonate plastics and other consumer goods. Its production and use continues to increase and contributes to widespread environmental contamination of waters, sediments, soil, air, wildlife and humans [2]. Because of frequent contact with consumer products manufactured with BPA, there are widespread human exposures. Most humans have detectable levels of BPA and BPA metabolites in their urine [3], indicating chronic exposure. Although controversial, estimates of daily intake range from 0.01 to > 5 µg/kg BW/day for adults and 0.01 to 13 µg/kg BW/day for children in westernized countries with higher exposures in Asia [2, 4–6]. BPA crosses the placental barrier [7], and detection of BPA and its metabolites in cord blood, foetal tissue, placenta and amniotic fluid indicates that foetal exposures occur [8–10]. Because foetuses and newborns have limited ability to transform BPA to its metabolites, exposures and potential risks may be greater than for adults [11].

BPA is an EDC that interferes with multiple hormone actions [12, 13]. Specifically, BPA acts as an agonist of membrane and nuclear estrogen receptors (ERs), and an antagonist of thyroid hormone receptor and androgen receptor signalling [14]. There is also some evidence that it can disrupt other signalling pathways including neuropeptide and GABA signalling [15].

More than 100 human epidemiology studies have examined associations between BPA exposures and human diseases [16]. Although these studies individually have limitations, they collectively suggest that current levels of exposure are associated with adverse health outcomes. Hundreds of controlled laboratory animal studies have also identified adverse health outcomes in mice, rats, zebrafish, non-human primates and other model species after exposures to BPA (reviewed in [17–19]). Endpoints as diverse as changes to the structure of the brain, behavioural and cognitive decrements, disruptions to metabolism and obesity, altered reproductive function (male and female), shifted age at pubertal onset, cardiac remodelling, and pre-cancerous and cancerous lesions in the mammary gland and prostate, have been reported, often following exposures below the US Environmental Protection Agency (EPA)'s reference dose of 50 µg/kg/day [20]. This extensive literature has demonstrated that there are vulnerable windows of exposure specific to each organ/endpoint, that many deficits induced by BPA become apparent only later in life, that effects can be sex-specific, and that BPA can sensitize animals to environmental insults encountered after the original period of BPA exposure.

Despite the extensive peer-reviewed literature, regulatory action for BPA has relied on the results of guideline studies, i.e. studies conducted according to internationally agreed upon protocols that include validated endpoints such as body weight, organ weight and histopathological evaluation of tissues. These studies are typically used by risk assessors because of their compliance with Good Laboratory Practices (GLP), a series of record-keeping methodologies that were implemented in response to research misconduct by chemical-testing laboratories [21]. To date, guideline studies have failed to identify effects of BPA at low doses [22–25]; instead, these studies have been used to identify a no-observed-adverse-effect-level (NOAEL) of 5 mg/kg/day, based on changes in body weight observed in rat dams exposed to higher doses [22].

For evaluation of EDCs, both academic and industry-affiliated scientists have noted that the endpoints included in guideline studies are not comprehensive [26, 27]; concerns have also been raised that guideline endpoints may not appropriately 'map' to human diseases of concern and may not be sensitive enough to determine 'safe' doses for human exposures (e.g., reference doses) [28]. To address these concerns, CLARITY-BPA included two parallel components: 1) A core GLP-compliant guideline study using 1 and 2-year chronic or developmental BPA exposures conducted at the FDA National Center for Toxicological Research (NCTR) laboratories, and 2) Hypothesis-driven studies evaluating non-guideline endpoints previously identified as sensitive to BPA exposure completed in academic laboratories by 14 NIEHS-funded research teams. By design, the academic studies allowed for comprehensive evaluations across a range of tissues and BPA doses to augment the classical guideline endpoints [29]. Importantly, all animals were dosed and handled at the GLP-compliant FDA-NCTR laboratories using a common protocol and study design.

In brief, NCTR Sprague-Dawley rats were gavaged daily with 0.3% aqueous carboxymethylcellulose (CMC) vehicle, one of five BPA doses (2.5 to 25,000 µg/kg BW/day), or ethinyl estradiol (0.05 or 0.5 µg/kg BW/day, a positive control for estrogenicity). Two exposure periods were examined: "continuous exposure" from gestation day 6 (GD6) to the time of sacrifice, and a "stop-dose" with exposure from GD6 to postnatal day (PND) 21

to examine the effects of developmental exposure. Tissues were collected at necropsy at PND 1, 21, 90, 180 and at 1 year by NCTR staff, coded and shipped to the academic laboratories for analysis. Although the pathologists that evaluated the guideline endpoints at the NCTR were unblinded to the control groups, all academic laboratory experiments were conducted in a fully blinded manner with identity of all control and exposure groups unknown throughout data collection. After study completion in the independent laboratories, the primary data were submitted to the Chemical Effects in Biological Systems Database (CEBS) maintained by the NIEHS in a read-only format so they could not be altered. After data were archived and quality review was performed by the Decoding Team, the decoded information was shared with the investigators to permit statistical analysis and publication of findings.

The CLARITY-BPA Consortium had multiple strengths but also some limitations (highlighted in Table 1) that should be taken under consideration when evaluating the utility of the studies. Ultimately, any final conclusions regarding the safety and potential health effects of BPA are expected to be derived from the combined analysis of the Core and Academic study components [29, 30]. Completion of this study will also shed light on the prior concerns raised about the ability of guideline studies to detect low-dose effects, and the comparative sensitivity and utility of guideline and non-guideline endpoints [13, 31].

Remarkably, although these studies were conducted on varied tissues and endpoints, significant effects at the lowest BPA doses (2.5, 25 and 250 µg/kg/day) were consistently identified, with variable and, at times absent effects at the higher BPA doses (Table 2). We discuss these findings in the context of other CLARITY-BPA published studies and a similarly designed Danish guideline study examining BPA toxicity.

NEUROBIOLOGY and BEHAVIOR ENDPOINTS

Over a half century of work has established that perinatal estrogen, aromatized from testicular androgens, is essential for masculinization of the rodent brain [32, 33], and that gonadal hormones also contribute to human neural and behavioural sex differences [34, 35]. Manipulation of estrogen signalling during these critical periods of sexual differentiation can produce profound effects on brain architecture and behaviour. For example, female rodents exposed to estrogens during a tight, well-defined perinatal critical period spanning just before and after birth subsequently display male-like copulatory behaviour, while male rodents in which ER signalling is blocked fail to display male-typical mating behaviours and can instead display lordosis under the right conditions [33]. The location and density of estrogen and other hormonal receptors are critically important for the coordination of sexual differentiation throughout the brain and can radically change across neonatal life [36–38]. Studies using ER knockout mice have revealed a particularly critical role for ER α [39, 40], with ER β and other hormones, including the neuropeptides oxytocin and vasopressin, playing a supporting role and facilitating social recognition and other behaviours fundamental to partner preference, courtship, social anxiety and copulation [41, 42]. Morphological sex differences also emerge in response to perinatal estrogen, most notably a larger SDN-POA in males and a larger AVPV in females. Thus, behaviour, ER expression,

and subregion volume are all reliable endpoints of developmental endocrine disruption via interference with estrogen signalling.

Work by Patisaul and others using multiple rodent models has shown that developmental exposure to BPA can alter the structure and sexual differentiation of many brain regions including the AVPV, amygdala (Fig. 1) and medial preoptic area, and result in altered non-reproductive behaviors, particularly elevated anxiety [43–47]. Most significantly, Patisaul and colleagues have repeatedly shown, in multiple rat strains, that developmental BPA exposure can alter the expression of ERs in brain regions, such as the amygdala and hypothalamus, that coordinate reproductive and other sexually dimorphic behaviours [38, 48]. Two of these studies were done in the same animal strain as CLARITY-BPA, with rats treated at the NCTR-FDA labs under nearly identical conditions, with a similar dose range, as a prelude to the CLARITY-BPA studies at that facility.

The first of these studies [49] was conducted in collaboration with Sherry Ferguson at NCTR and used two doses of BPA (2.5 and 25 µg/kg BW) and two doses of EE (5 and 10 µg/kg BW) as well as a vehicle (CMC) and naïve control (which was removed from the cage but not gavaged). Dams were exposed prenatally from GD6 through the day of birth. The postnatal day (PND) 1 offspring were decapitated and the frozen heads sent to the Patisaul laboratory for quantification of ER α and ER β expression in the hypothalamus and amygdala via *in situ* hybridization (Fig. 1). Effects were region-, dose- and sex-specific with some known sex differences in ER expression eliminated at the lowest BPA dose. BPA- and EE-related effects were directionally similar and exposure resulted in ER up-regulation in most cases. Notably, ER levels were markedly lower, particularly in the amygdala, in the vehicle controls compared to the naïve animals suggesting a suppressive effect of gavage on ER expression. This observation is significant, because gavage is traditionally the dosing method of choice for guideline toxicity studies. Additionally, exposure-related increases in ER expression generally returned expression levels to a range typical of the naïve animals. Thus, it was concluded that increased expression levels in the exposed animals likely reflected the interaction of BPA exposure and stress.

The second study [50] used animals obtained from a 90-day subchronic study conducted by NCTR as a prelude to CLARITY-BPA [51, 52]. For this project, only females were examined but vehicle controls of both sexes were included to 1) ensure known sex differences could be reliably detected and 2) establish the degree to which BPA and EE could “masculinize” the female brain. Four doses of BPA (2.5, 25, 260, 2700 µg/kg bw), and two EE doses were used, (0.5, 5 µg/kg BW) were used. Two exposure windows were used: GD6 through PND21 and GD6 through PND90. Offspring brains were collected on PND21 or PND90 and sent to the Patisaul laboratory for analysis of ER expression in the preoptic area via *in situ* hybridization. As in the PND1 study, effects were region- and sex-specific. In this case, BPA exposure generally decreased ER expression, which is opposite of what was observed on PND 1. This is not surprising given the dramatic age-dependent differences in baseline ER expression levels in the rodent brain [36–38]. Similar to the PND 1 study, ER β appeared to be more sensitive. Interestingly, effects were not always monotonic, with some effects observable at the lowest two, but not the highest two, doses. BPA and EE were

concordant in direction but not necessarily dose, with some cases where low-dose effects of BPA were observed but not low-dose EE.

The CLARITY-BPA studies in the Patisaul laboratory used two different groups of animals. One, collected on PND1, had five doses of BPA (2.5, 25, 250, 2,500 and 25,000 µg/kg BW) and two doses of EE (0.05 and 0.5 µg/kg BW). Exposure was exclusively prenatal. The second group, used for behavioural analyses at NCTR by the Patisaul laboratory and the laboratory of Cheryl Rosenfeld. In collaboration with Sherry Ferguson, CLARITY-BPA rats were transferred to a separate building at weaning for assessment of anxiety, exploratory behaviour and spatial navigation. In this second group, exposure spanned GD6 to PND21 and included only a subset of the dose groups (vehicle, 2.5, 25 and 2,500 µg/kg BW BPA, 0.5 µg/kg BW EE). One group of animals was tested as juveniles and another was tested in adulthood. Despite numerous prior studies showing robust and reproducible effects of developmental BPA exposure on anxiety and exploratory behaviors, effects in this case were subtle and sporadic [53, 54]. Some sex differences were either not detected or the opposite of expected in the vehicle controls leading to the conclusion that some behavioural sex differences may be uniquely different in this strain compared to other Sprague-Dawley strains [53].

The brains from the juvenile animals were then collected and subjected to morphometric analysis to look for evidence of abrogated volumetric sex differences [55]. All expected sex differences were detected in the vehicle controls and there was no instance where exposure eliminated those differences. Interestingly, all doses of BPA enlarged the female AVPV and a similar enlargement was observed in males at the 25 and 2,500 µg/kg BW dose levels. Because endogenous estrogen, via action on ER α , acts to reduce the volume of the AVPV [32, 33], the effect of BPA observed here is consistent with anti-estrogenic activity. An effect of BPA on MePD volume was also detected, but only in the right MePD in males exposed to the 2,500 µg BPA/kg BW/day exposure group. Because the left and right MePD have numerous structural and functional differences, an effect on only one side is biologically plausible, although the functional significance is not entirely clear.

The PND1 brains sent to the Patisaul laboratory were analysed by a combination of transcriptomics and qRT-PCR with the hypothesis that exposure would alter ER expression levels and other targets in the ER signalling cascade. Although the prior two studies with NCTR rats used *in situ* hybridization, here the three regions of interest (hypothalamus, hippocampus and amygdala) were isolated by micropunch (Fig. 1) and analysed by targeted and untargeted transcriptomics to obtain a richer picture of the suite of genes impacted by exposure. Overall, the greatest number of differentially expressed genes were found in the male hypothalamus and female amygdala [56, 57]. Heightened ER α and ER β expression was observed in the hypothalamus of both sexes at 2.5, 25 and 2,500 µg BPA/kg bw. In the hippocampus, the only evidence of ER disruption was heightened ER β expression in males at the 25,000 µg/kg bw dose. Similarly, only ER β was altered in the amygdala with expression levels non-monotonically heightened in both sexes.

The CLARITY transcriptome data are consistent with the data obtained in the first study the Patisaul laboratory did in conjunction with NCTR, which also found heightened ER

expression in BPA-exposed animals. Because the CLARITY-BPA study used micropunched tissue containing the entire region of interest, it lacks the resolution obtainable via *in situ* hybridization and thus could not recapitulate the subregional differences observed in that first study (Fig. 2). Nevertheless, it successfully reproduced evidence that prenatal BPA exposure disrupts neonatal ER expression in the hypothalamus and amygdala. Other genes altered by BPA included oxytocin and GABA vesicular transporter (Slc32a1) in the hypothalamus, oxytocin in the hippocampus, and oxytocin and vasopressin receptors in the amygdala. Nearly identical outcomes were found in adult hippocampus and hypothalamus by the Rosenfeld laboratory working on CLARITY-BPA animals dosed at 2,500 µg/kg BW from GD 6 through PND 21 [58]. Numerous genes involved in glutamate signalling were also found to be disrupted in the amygdala. Collectively, these data are consistent with prior work by Patisaul and others showing that BPA impacts estrogen, oxytocin and vasopressin pathways throughout the brain [15, 45, 48, 59–61].

In summary, the CLARITY-BPA studies, and the two collaborative studies with NCTR that proceeded them, confirm that developmental exposure to BPA, at doses as low as 2.5 µg/kg BW, can alter ER expression in the brain. Directionality differs with age with heightened expression generally observed in neonates and suppressed expression observed in adolescents. Disruption of brain ER is one of the most consistently observed outcomes of developmental BPA exposure. Additionally, the CLARITY-BPA studies from the Patisaul and Rosenfeld laboratories provide further confirmatory evidence that developmental BPA exposure alters oxytocin- and vasopressin-related signalling pathways, and the volume of the AVPV.

CARDIAC ENDPOINTS

The heart of both males and females express estrogen receptors [62]. Estrogens (i.e. 17β-estradiol) normally act to regulate heart function and vascular tone through direct actions on cardiac and vascular tissues, and by regulating autonomic nervous system activity. The impacts of endogenous estrogens and BPA are sex-specifically regulated and differ in males and females [63–67]. Prior work by the Belcher laboratory demonstrated that sub-nanomolar concentrations of BPA and 17β-estradiol could sex-specifically alter rapid estrogen-signalling in cultured adult rodent cardiomyocytes [68]. Those effects of BPA were found only in females and mediated through an ERα and ERβ-dependent mechanism that disrupted Ca²⁺ handling and modified excitation–contraction coupling. In both rats and wildtype and ER knockout mice, those estrogen-like effects of BPA were shown to sex specifically increase arrhythmia frequencies in response to β-adrenergic stress in females [65].

Following up on those *in vitro* findings, *in vivo* studies using a continuous life-long dietary exposure protocol of BPA or control 17α-ethinyl estradiol exposures were performed in CD-1 mice [64, 69–72]. BPA exposure spanned five orders of magnitude from ~4 µg/kg BW/day to ~50,000 µg/kg BW/day with a 10-fold dose interval. Three doses of 17α-ethinyl estradiol resulting in exposures of ~0.02, 0.2 and 0.15 µg/kg BW/day were also included. These studies demonstrated significant, and most often, sex-specific effects of BPA on a variety of reproductive, metabolic, immune and cardiovascular related endpoints [64, 69–

72]. In the heart, only minor exposure-related increases in heart weight, left ventricular (LV) wall thickness, and modest changes in collagen content in the extracellular matrix of hearts were found in males exposed to 430 and 4400 µg BPA/kg BW/day. In contrast, modest decreases in LV wall thickness and a remarkable decrease in collagen were observed in hearts of females at the lowest BPA dose (5 µg/kg BW/day). In females, BPA exposure resulted in an extraordinary increase in sensitivity of the heart to isoproterenol-induced ischaemic damage and hypertrophy that was likely due to alterations in the collagen extracellular matrix and modified cardiac fat metabolism. This conclusion was supported by cardiac transcriptome analysis that revealed BPA exposure caused sex-specific changes in expression of components of the extracellular matrix and in dysregulation of fatty acid metabolism and glycolytic metabolism. These alterations were consistent with the observed histological changes in the collagen extracellular matrix and pathological remodelling observed in BPA-exposed hearts [64].

The published finding analysing cardiac effects in CLARITY-BPA NCTR-Sprague Dawley rat hearts [73], found limited evidence of BPA or EE alterations on gross cardiac endpoints related to hypertrophy. While alterations in LV wall thickness were not observed at any dose in either sex, a significant decrease in heart weight and heart weight normalized to body weight was observed in females exposed to 2.5 µg/kg BW, a finding consistent with previous findings in mice [64]. Exposure-related changes in collagen accumulation in males were limited to the highest EE dose group with increased collagen accumulation in PND21 males. In female hearts, decreased collagen content was observed in rats treated with 25,000 µg/kg BW BPA and 0.5 µg/kg BW EE at PND90 and 6 months, respectively.

The lack of overt morphology phenotypes in the CLARITY-BPA or EE exposed hearts was not surprising since pathology associated with the majority of cardiac insults typically becomes evident only after adverse cardiovascular events such as cardiac ischaemia or myocardial infarction [64, 66]. For experimental studies with rodents, it is well-accepted that an intervention resulting in increased β-adrenergic stress, ischaemic injury or genetic manipulations are necessary to reveal cardiac fibrosis, hypertrophy or phenotypes indicative of overt cardiac pathology [74]. Such manipulations were not possible in the CLARITY-BPA study and limit any interpretations resulting from negative data. Additionally, compared to control mice, the hearts of control NCTR-Sprague Dawley rats had relatively higher levels of collagen due to known species-specific differences in the proportions of myocytes and fibroblasts present in murine and rat hearts [75].

Progressive cardiac myopathy (PCM) is a common background lesion in some rat strains that is suspected to arise from localized microvascular dysfunction. The common occurrence of PCM lesions in Sprague-Dawley rats has presented challenges for analysing cardiotoxicity in regulatory toxicology studies of chemicals and pharmaceuticals [76–80]. Previous studies had not analysed PCM lesions in hearts of young animals. In the CLARITY-BPA study, a remarkable abundance of early PCM lesions was detected in the hearts of most control and exposed animals analysed at PND21 [73]. Consistent with PCM found in adults, the lesion incidence and severity was greater in control males than in females. In BPA- or EE-treated females at PND21, cardiomyopathy incidence was increased compared to control females and a significant increase in severity was found for 2.5, 250 or

25,000 µg BPA/kg BW/day and both EE groups. In a male exposed to 250 µg BPA/kg BW/day and a female from each of the two lowest BPA dose groups (2.5 and 25 µg/kg BW/day), a diffuse degeneration phenotype involving much of the myocardium was also observed [73]. This high level of cardiac pathology across much of the myocardium is an accepted indication of exposure-related cardiotoxicity [76].

At PND90 and 6 months, cardiomyopathy in both males and females was observed in 100% of control samples from both the stop dose and the continuous dose arms of the CLARITY-BPA study [73]. At PND90, the diffuse degeneration phenotype was again observed in males and females from the continuous (males: BPA 25, 250, 25,000; EE 0.5 µg/kg/day; females: BPA 2.5, 25; EE 0.05, 0.5 µg/kg/day) and stop dose (males: BPA 250, 25,000; EE 0.05, 0.5 µg/kg/day; Females 250 µg/kg/day) exposure groups.

The increases in PCM observed in females at PND21 and the notable increase in myocardial degeneration at PND90 suggests that BPA and EE impact cardiovascular health resulting in an early onset of vascular dysfunction and progression of cardiomyopathy. These findings are clear indicators of exposure-related cardiotoxicity in the 2.5 µg/kg/day BPA group resulting from increases in adverse vascular events, findings that support a NOAEL of <2.5 µg/kg/day for effects in the heart.

It is also notable that the largest observed morphometric effects in the CLARITY-BPA study animals analysed were due to treatment duration (“stop-dose” *versus* “continuous dose”) which altered body weight and cardiac collagen accumulation in control animals, an effect arising from experimental differences between the two different study designs [73]. At PND90, mean body weight of stop dose control males was 9.5% greater than in the continuously exposed control group; at 6 months, mean body weight of stop dose control males was 9.1% greater than continuously exposed controls. Those observed differences in body weights between continuously dosed males and males dosed only until weaning at PND21 indicate that there were effects of post-weaning dosing procedures and/or the CMC vehicle. The sex-specific decreased weight of males dosed daily with vehicle by gavage is consistent with previous studies showing that prolonged postnatal stress in males decreases weight gain over time, and that female SD rats are resistant to these effects of stress [81–83].

PROSTATE ENDPOINTS

It is well established that the developing prostate gland has heightened sensitivity to estrogenic exposures which can reprogram the organ to elevated disease risk in adulthood [84–86]. In this context, previous work from the Prins, Ho and Walker laboratories determined that early-life, low-dose BPA exposure reprogrammed the rat prostate epigenome and increased its susceptibility to hormonal carcinogenesis with ageing, a finding that was subsequently confirmed in a humanised prostate model [87–94]. This is germane to human disease since elevated estrogen levels rise in ageing men [95] and together with androgens, induce prostate cancer in the rat and human epithelium [96, 97]. As part of the CLARITY-BPA consortium, the Prins laboratory had two main goals: 1) re-assess whether developmental BPA exposures across multiple doses could alter susceptibility to estrogen-

driven carcinogenesis with ageing, and 2) determine whether chronic low-dose BPA exposures might target prostate epithelial stem and progenitor cells [98]

For goal 1, NCTR Sprague-Dawley rats were gavaged daily with vehicle, EE (0.5 µg/kg BW), 2.5, 25, 250, 2500 or 25,000 µg BPA/kg BW from GD6 to one year (continuous dose), from GD6 to PND21 (stop-dose), or from GD6 to PND21 (stop dose) with testosterone plus estradiol-17β (T+E) implants given at PND90 to drive carcinogenesis with ageing. The T+E treatment resulted in normal circulating testosterone levels and a 2-fold estradiol-17β elevation to model rising estrogen levels in ageing men. Prostates were collected at one-year necropsy, coded and analysed for histopathology by Maarten Bosland, DVM, PhD who was blinded to treatments and controls. As previously reported by the Prins laboratory [87, 94], developmental or continuous exposure to BPA alone at any dose did not produce prostate pathology that differed from vehicle controls. However, in response to T+E, rats given developmental EE, 2.5, 250 or 25000 µg BPA/kg BW showed significant increases in lateral prostate prostatic intraepithelial neoplasia (PIN) severity compared to vehicle controls at one year, shifting from low-grade PIN in controls to high-grade PIN in rats developmentally exposed to BPA with the highest PIN score observed at the 2.5 µg BPA/kg BW dose. PIN could not be evaluated in the rats given 25 or 2500 µg BPA/kg BW due to limited sample size. Of note, in humans, high-grade PIN is a precursor lesion of prostate cancer, whereas low-grade PIN is not considered clinically relevant. Importantly, there was a four-fold increase in dorsolateral ductal adenocarcinoma multiplicity in rats developmentally exposed to 2.5 µg BPA/kg BW plus adult T+E. Together, these CLARITY-BPA findings confirm previous reports that low-dose BPA exposures augment prostate cancer risk.

To address goal 2, NCTR Sprague-Dawley rats were gavaged daily with vehicle, ethinyl estradiol (EE; 0.5 µg/kg BW), 2.5, 25 or 250 µg BPA/kg BW from GD6 to 6 months whereupon prostates were removed and shipped on ice overnight to the Prins laboratory for stem cell isolation and culture [98]. Dorsolateral lobe epithelial stem cells were isolated by direct prostatesphere 3-D culture and passaged 3 times in the absence of hormone or BPA to enhance stem cell purification. Dose-specific responses to BPA exposures were observed for stem and progenitor cells. *In vivo* exposure to EE and 2.5 µg BPA doubled the total prostatesphere number, reflecting increased stem cell numbers in the adult prostates. Prostatesphere size, a marker of progenitor cell proliferation in the cultured spheroids, was increased steeply by EE and 25 µg BPA/kg BW and to a lesser degree by 250 µg BPA/kg BW compared to vehicle-treated rats. To assess the effects of BPA on prostate stem cell lineage commitment, progenitor lineage markers were quantified by q-RT-PCR in the prostatesphere cells. Tightly paralleling prostatesphere size effects, exposure to EE and 25 µg/kg BW BPA significantly increased CK5, *Sox2* and *HoxB13* expression while EE, 25 and 250 µg/kg BW BPA suppressed CK8, *Trop2* and *Thx3* mRNA. This indicates that chronic BPA exposures permanently modify the lineage commitment of the prostate stem cell progeny, increasing basal progenitors and suppressing luminal progenitor cells. Together, these results show that chronic low-dose BPA exposure alters adult prostate stem cell homeostasis in a dose-dependent manner, increasing stem cell numbers at the lowest dose and elevating progenitor cell proliferation while also shifting lineage commitment to favour basal progenitor cells at a 10- and 100-fold higher dose. That the 0.5 µg EE/kg BW exposure resulted in similar stem and progenitor cell effects indicates that they are mediated through

ER pathways. The dose-specific responses observed over a 100-fold BPA dose range is likely due to differential engagement of ER populations and membrane *versus* nuclear signalling pathways. These findings are particularly relevant in light of reports that cancer risk is strongly correlated with the number of normal stem cell divisions across multiple human tissues, including the prostate [99]. Furthermore, since tumour initiating cells for human prostate cancer are largely localized to the basal cell compartment [100], it is possible that increased basal cell numbers in the prostate may increase the opportunity for tumour initiation by secondary hormone exposures as occurs in our animal model.

In summary, the CLARITY-BPA findings show the most significant effects on the prostate occur at the lowest doses examined, with peak effects observed at the 2.5 µg BPA/kg BW dose. Together, our results confirm that low-dose developmental BPA exposure increases prostate cancer susceptibility to ageing-associated increases circulating estrogens which may be underpinned, in part, by reprogrammed stem cells.

OVARIAN ENDPOINTS

Prior studies have demonstrated that low-dose BPA exposures during perinatal development affect three types of endpoints in the ovary: 1) exposures at the onset of meiosis increase the likelihood that a female will ovulate chromosomally abnormal eggs in adulthood; 2) exposures during follicle formation induce an increase in multi-oocyte follicles and reduce the total pool of oocytes; and 3) exposures throughout gestation affect the adult ovary including the induction of polycystic ovaries. These effects have been observed in several mammalian species including rat, mouse, sheep and monkey [101–108] as well as in nematodes [109] and in human foetal ovaries exposed *in vitro* [110].

As part of the CLARITY-BPA consortium, Flaws and colleagues examined the effects of exposure to EE (0.05 or 0.5 µg/kg BW), or BPA (2.5, 25, 250, 2500 or 25,000 µg BPA/kg BW) from GD6 to one year (continuous), or from GD6 to PND21 (stop-dose), on ovarian morphology and sex steroid hormone production [111]. Ovarian endpoints including follicle health, follicle number, and follicle stage were evaluated at PND1, PND21, PND90, 6 months and 12 months of age. Effects of BPA were only observed at PND21, prior to puberty; BPA significantly decreased the number of primordial follicles (250 µg/kg/day), number of primary follicles (2.5 and 250 µg/kg/day), and number of total healthy follicles (2.5 and 250 µg/kg/day). These same outcomes were not disrupted by EE, the positive control. In fact, the lower dose of EE (0.05 µg/kg/day) did not affect any ovarian endpoints at any age.

Another important role for the ovary is sex steroid production. Prior work from the Flaws laboratory and others has demonstrated that BPA can disrupt ovarian steroidogenesis [112]. An *in vitro* study found that exposure to 10 and 100 µg/mL concentrations of BPA disrupted expression of steroidogenesis genes in mouse antral follicles, ultimately decreasing production of progesterone, androstenedione, testosterone and estradiol [113].

Within the CLARITY-BPA study, Flaws and colleagues also examined steroidogenesis in females from both the stop-dose and continuous-dose groups at PND21, PND90, 6 months

and 1 year of age [111]. Continuous exposure to EE (0.5 µg/kg BW) significantly decreased serum progesterone levels at PND21, PND90 and 6 months of age, whereas the lower dose of EE and all doses of BPA had no effect on this hormone. Continuous exposure to either dose of EE, or the two highest doses of BPA (2500 and 25000 µg/kg BW), significantly decreased serum estradiol levels. Similar declines were also observed in the three lower BPA groups, but these did not reach statistical significance, likely due to small sample sizes that diminished statistical power.

In summary, these results suggest that the ovary is an important target of BPA, with effects that occur both at the level of reproductive function (e.g., health and number of follicles) and endocrine function (e.g., production of sex steroids). Although the CLARITY-BPA study has limitations due to small sample sizes for some measures, the effects observed in this study are consistent with prior studies showing harm after low-dose exposures [112].

IMMUNE ENDPOINTS

Prior studies examining the effects of BPA on immune cells have been conducted both *in vitro* and *in vivo*, and epidemiological evidence suggests associations between BPA and immune cell mediated diseases including asthma [114]. *In vitro* studies suggested that BPA could alter expression of cytokines and chemokines from a range of different immune cell types, alter mast cell degranulation, and enhance antigen presentation. In rodents, significant variability in responses was observed across studies; these studies examined different exposure periods, doses, routes of exposure and health outcomes. Some studies revealed effects of BPA on inflammatory cytokines in the spleen without effects in the lung [115], whereas others observed lung inflammation and airway hyperactivity [116, 117]. Effects of BPA on innate immune responses were also observed [118]. Prior to CLARITY, an expert panel concluded that there was credible evidence that BPA could induce abnormal immune responses after relatively low levels of exposure (5 µg/kg/day) during development [119].

Within the CLARITY-BPA consortium, the Kaminski laboratory examined immune cell populations residing in the spleen and thymus [120]. Only animals that were continuously exposed to BPA throughout life were utilized in two published studies resulting from this work. At PND21, the composition of the spleen and thymus were not affected by BPA exposure, and neither the lymphoid nor the myeloid populations were altered in their composition within the spleen. At PND90, there were similarly no effects of BPA, with the exception of the spleen-associated myeloid populations, which showed increases in the number of cDC cells in males exposed to 2.5 µg/kg/day. By 6 months of age, more disruptions to immune cell populations became evident: there was an increase in total spleen cellularity and a decrease in B-cell populations in females exposed to 25 µg/kg/day; males treated with either 2.5 or 2500 µg/kg/day also displayed decreases in the number of mature cDC cells at this age. By 12 months of age, low-dose effects of BPA were observed only in males, with decreases in the number of APC cells (after treatment with 2.5 or 25 µg/kg/day) and decreases in the number of macrophages (after treatment with 2500 µg/kg/day) in the spleen.

The second study from the Kaminski group examined the response of the immune system to stimuli administered to cells *ex vivo* from animals exposed continuously to BPA [121]. Spleen cell proliferation was not affected by BPA treatment alone, but after induction by an activating agent, PWM, heightened responses were seen in females at 6 months of age (exposed to 2.5 or 2500 µg BPA/kg/day) whereas diminished responses were seen in males of the same age (exposed to 2.5, 250 or 2500 µg BPA/kg/day). Spleen cell proliferation was also affected by BPA exposure at 12 months of age, where males from the 2.5, 25, 2500 and 25,000 µg/kg/day groups had heightened responses to the PWM activating agent; females were not affected by PWM, but did respond to another activating agent (Anti-CD3/28), with diminished responses that were significant in the 2.5 µg/kg/day group. Finally, the study authors isolated specific kinds of immune cells including T-cells and Natural Killer Cells. Sex-specific effects of low-dose BPA were observed, although the timing of evaluation (6 *versus* 12 months of age) also influenced these outcomes.

Overall, the CLARITY-BPA study authors downplay the significance of their results, noting that a large number of individual outcomes were examined (530 in the first study, 630 in the second study) with few that were deemed statistically significant. Here, we have merged many of these individual outcomes to instead see the overall effect (e.g., 40 individual measurements were taken to evaluate the myeloid cell lineage: 5 different cells types, 2 sexes, at 4 different ages; we combined these to a single measure: disruption of the myeloid cell lineage). When viewed in this way, a clear pattern emerges for several parameters: low doses of BPA alter how immune cells respond to stimulation, with effects that are different in males and females.

SPERM ENDPOINTS

Because sperm are relatively easy to access, prior studies have conducted *in vitro* investigations into the effects of BPA on a range of outcomes including proliferation and survival, motility, velocity, ATP content and gene expression, among others. These studies have found effects of BPA at low concentrations (10^{-12} – 10^{-9} M) [122–124]. In rodents, developmental exposures to BPA have been shown to disrupt a number of male reproductive functions including alterations to sperm count, motility and density [125].

One report from the CLARITY-BPA study from the Boekelheide laboratory found no effect of BPA on sperm endpoints in males exposed continuously and evaluated at PND90 [126]. Outcomes that were evaluated included testis weight, epididymal weight, sperm production (estimated from testicular homogenization-resistant spermatid heads), disruptions to spermiation (retained spermatid heads in the seminiferous tubules), and apoptotic germ cells. BPA also did not disrupt sperm mRNA levels or DNA methylation. Unfortunately, this CLARITY-BPA study did not include any positive control groups treated with EE, making it difficult to evaluate the sensitivity of the measurements or the outcomes. Since only the continuous BPA exposures were assessed and not the stop-dose group, we cannot directly compare these findings to an independent CLARITY-like study where exposure to 25 µg BPA/kg BW/day from GD7 to PND22 decreased epididymal sperm counts. Furthermore, as some estrogenic effects on male reproduction are best observed beyond young adulthood

[127], it would have been useful to evaluate the rats at the one year necropsy to determine whether BPA exposures affected sperm endpoints throughout adulthood.

RESULTS OF THE DRAFT CORE STUDY:

Here, we will focus on the effects of BPA that were observed at the three lowest doses in the CLARITY core study [128]; additional effects were observed at higher doses as well, but the seriousness of the adverse outcomes disrupted at these three lowest doses is telling (see Table 2). At the lowest dose, 2.5 µg/kg/day, females that were exposed only during early development displayed increased incidence of mammary adenocarcinoma and abnormal infiltration of the liver with mononuclear cells at 1 year of age and an increased incidence of renal tubule cysts at 2 years; males displayed an increased incidence of pancreas pigmentation at 2 years of age. In animals that were continuously exposed, there was an increased incidence of cysts in the kidney renal tubule in females at 1 year of age; increased chronic inflammation of the dorsal and lateral prostate and decreased liver weight in males at 1 year of age; increased kidney neuropathy in females at 2 years of age; and increased mammary alveolar dilation, and increased incidence of liver angiectasis in males at 2 years of age.

In animals exposed to 25 µg/kg/day during development only, males displayed decreased serum protein levels and total bile acids at 1 year of age. In animals that were continuously exposed to this dose, females had increased hemoglobin concentrations at 1 year of age; increased vaginal epithelial hyperplasia at 2 years of age; and at 2 years of age, males had increased hyperplasia of the transitional epithelium of the kidney and increased hyperplasia of the parathyroid gland.

Finally, exposure to 250 µg/kg/day during development increased thyroid ultimobranchial cysts in females at 2 years of age; increased spleen pigmentation in males at 1 year of age; and increased pituitary gland cysts, bone marrow hypercellularity, and lymphoid hyperplasia in the spleen of males at 2 years of age. Continuous exposure to this dose decreased eosinophil number and increased serum alkaline phosphatase levels in females at 1 year of age; decreased the percentage of blood cells that were eosinophils and increased infiltration of the liver with mononuclear cells in males at 1 year of age; increased the body weight of females starting at 96 weeks of age; and increased the incidence of cysts in the kidney renal tubule of males at 2 years of age.

In analysing the ~36 significant changes as a function of BPA exposures in the Core Study, 50% were found in only low-dose BPA exposure groups, 30% in only the 2 high-dose groups and 20% found with both low-and-high-dose exposures (Table 2). Although the authors of the draft core report dismiss these findings because they were seen in only one arm of the study (developmental exposure *versus* continuous exposure), or because they were only seen at the lower doses (e.g., indicative of non-monotonic responses), the number and severity of many of these endpoints with the majority occurring at low-dose exposures raises serious concern about the safety of BPA in the low dose range.

ANOTHER CLARITY-LIKE STUDY:

A previous guideline study of BPA was conducted at the National Food Institute of Denmark [129]. Wistar rats were gavaged with one of four BPA doses (25, 250, 5000 or 50,000 µg/kg/day) from GD 7 through lactational day 22; this period is similar to the stop-dose group in the CLARITY-BPA study, except that the postnatal treatment continued to be given to the mother rather than the pup. Like CLARITY, the Danish study also included additional non-guideline endpoints including evaluations of behaviours (sugar preference, spatial learning ability) [130], male reproductive outcomes (sperm count) [130], and mammary growth parameters (male epithelial outgrowths, female mammary intraductal hyperplasias) [131].

Remarkably, the authors found that male offspring in the lowest dose group (25 µg/kg/day) had significantly fewer sperm and larger mammary gland epithelial trees compared to controls. Female offspring from this dose group had masculinized neurobehaviours (in spatial learning and preference for sugar) and increased body weights in later life (9–13 months of age). Females exposed to 250 µg/kg/day also had increased mammary intraductal hyperplasias. These effects were observed without seeing changes to ‘core’ outcomes like organ weight. The adverse effects seen in the Danish study are mostly consistent with what was observed in CLARITY-BPA, with the exception of sperm outcomes, increasing confidence in these findings. Considering their results, the Danish study authors concluded that the European reference dose of 4 µg/kg/day is not sufficiently protective [130]. (The US reference dose is even higher: 50 µg/kg/day.)

CONCLUSIONS:

Many datasets within the CLARITY-BPA study have documented effects of BPA in the low-dose range, often at the lowest dose provided, i.e. 2.5 µg/kg BW/day and frequently in the stop-dose cohorts, specifically suggesting heightened sensitivity to developmental exposures. For carcinoma risk, heightened mammary cancer incidence and prostate cancer multiplicity were uniquely seen in the cohort developmentally exposed to 2.5 µg/kg BW/day, which is cause for great concern. These low-dose effects were observed not only in the Academic Studies as highlighted herein, but in the Core Study itself; significant effects on mammary cancer, prostatic inflammation, kidney cysts and increased body weight within the Core Study are especially notable. It is also noteworthy that many of these CLARITY outcomes have been observed across multiple previous studies, including similar low-dose effects on neurobehaviours that have been observed both in CLARITY and in the Danish study, effects on sexually dimorphic brain regions that have been observed in CLARITY and across numerous prior academic studies, and enhanced prostatic carcinogenic susceptibility observed in CLARITY and previous academic laboratories.

There are numerous outcomes, across different organ systems that were disrupted in animals exposed to 2.5, 25 and 250 µg BPA/kg/day. If these effects at the lowest doses were observed less frequently or limited to single system, their importance could be questioned. However, when results from the Core study and Academic studies published thus far are combined, we identify a total of 75 endpoints with significant BPA effects noted relative to controls (Table 2). Of those, 51% appear only in low-dose groups (at or below 250 µg/kg BW/day), 21% in

high-dose only rats (2,500 and 25,000 µg/kg BW/day) and 27% across both low and high-dose exposure groups. Thus, the large majority of identified effects are observed in low-dose exposed animals. This is best visualized using a heat-map approach (Fig. 2) where BPA effects are found across a range of doses with a preponderance in the three low-dose groups. These results are particularly noteworthy in the context of several limitations of the study design (summarized in Table 1).

As discussed in the Introduction, BPA effects are known to be mediated through multiple cellular receptors and signalling pathways [14, 15]. As BPA engagement of these multiple receptors/pathways occurs at widely different doses (low-dose for membrane receptor pathways; high-dose activation/antagonism for nuclear receptors), one would expect to observe non-linear BPA effects across increasing doses [132], as is seen in a considerable number of endpoints in the CLARITY-BPA studies. Further, expression of the different receptors varies widely across organs and cell types which can lead to dose-selective responses in different organs within the same animals. This argues strongly against dismissing significant BPA effects observed at single doses or in non-monotonic patterns and, in fact, supports the patterns observed thus far as scientifically sound.

Collectively, the CLARITY results highlighted herein contribute to and strongly corroborate a significant body of evidence that documents adverse effects of BPA at low doses. Additional studies are anticipated from the academic participants over the next year. As academic investigators who have long studied BPA, we propose that the cumulative results of the CLARITY study provide solid documentation of BPA effects in the low-dose range, below the current NOAEL, and conclude that the TDI dose is not reasonably protective of public health.

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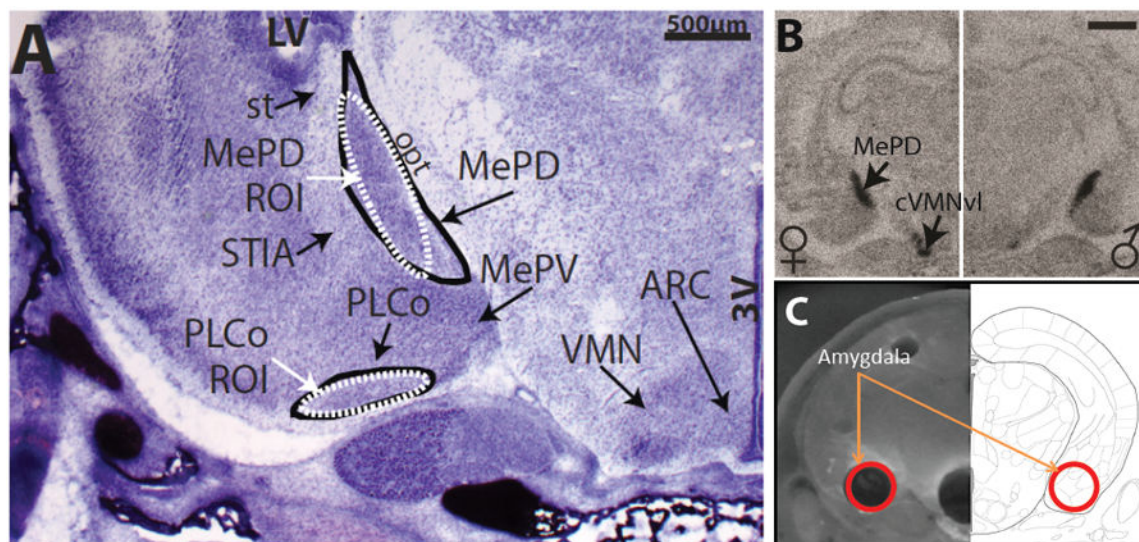


Figure 1:

(A) The neonatal rat amygdala is a complex structure containing multiple subnuclei readily discernable with a NISSL stain. Two regions of particular interest (MePD and PLCo) are depicted (circled in black) along with the areas selected within those subregions for quantification by *in situ* hybridization (white ovals labelled ROI). (B) In situ hybridization allows for mRNA quantification on a subregional, and even individual cell, scale. In the representative autoradiograph, ER β mRNA is clearly labelled in the MePD and neighboring VMN in both sexes. The two Patisaul studies done in collaboration with NCTR prior to CLARITY used this approach to quantify ERs in multiple brain regions including the hypothalamus and amygdala. (C) For the CLARITY study, regions including the amygdala were isolated by micropunch and the whole transcriptome sequenced. With this approach, anatomical resolution is reduced in exchange for the capacity to sequence many more genes. In all three studies, ER expression was found to be affected by BPA at doses as low as 2.5 $\mu\text{g}/\text{kg}$ bw. Scale bar in B = 1000 μm ; 3V = third ventricle; ARC = arcuate nucleus; LV = lateral ventricle; MePD = medial postdorsal portion of the amygdala; opt = optic track; PLCo = posterolateral cortical amygdaloid nucleus; ROI = region of interest; st = stria terminalis; VMN = ventromedial nucleus; cVMNvl = caudal portion of the ventrolateral ventromedial nucleus.

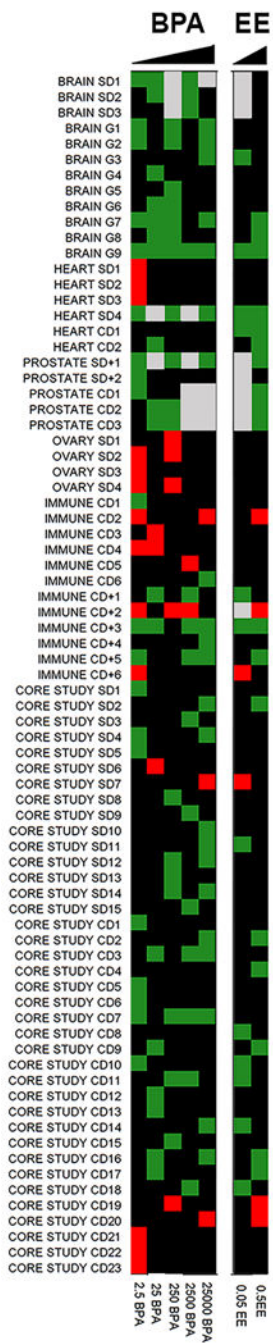


Figure 2: Significant BPA effects from CLARITY-BPA Academic and Core studies available to date are visually represented as a heat map over 5 BPA doses (25,000, 2,500, 250, 25 and 2.5 µg/kg BW/day) and 2 EE doses (0.5 and 0.05 µg/kg BW/day).. The specific endpoints for various organ systems are numbered at left with corresponding details provided in Table 2. Green indicates increased effects, red indicates decreased effects and black represents no

significant difference relative to vehicle controls for each group. Areas of grey represent doses or treatments that were not examined.

Table 1:**CLARITY-BPA strengths and weaknesses in study design and implementation**

STRENGTHS:

- GLP-compliant across all animal handling and experiments
- Single, common facility for all animal treatments and common animal protocols
- Allows guideline and non-guideline endpoints to be compared in “the same” animals. Also allows many different endpoints to be compared in “the same” animals and, in several instances, sharing of tissues in the same animals
- All academic experimenters were blind to treatments and controls
- Environmental BPA levels were measured (food, water)
- Locked CEBS database for experimental results

LIMITATIONS:

- Concerns about the rat strain utilized (NCTR Sprague-Dawley rat)
 - Positive control unexpectedly did not affect some endpoints
 - Differences in controls from the stop-dose group and the continuous exposure group suggest that the animals are stressed by gavage
 - Contamination documented in pre-CLARITY, but then not evaluated in the Core Study or Academic Study animals
 - Many Academic Studies were severely underpowered
 - Use of historical controls by Core Study
 - Pathologists for the guideline endpoints were not blind to control groups
-

Table 2: Significant BPA and EE effects observed across multiple experiments, target organs and doses in CLARITY-BPA

TARGET ORGANS	TREATMENT GROUPS	ENDPOINTS ASSESSED	BPA DOSE (µg/kg BW/day)							Ethinyl Estradiol (µg/kg BW/day)	
			2.5	25	250	2,500	25,000	0.05 EE	0.5 EE		
BRAIN											
SD1	Stop-dose; PND 28 examined	AVPV size increase; females	<0.01	<0.05	ne	<0.001	ne	ne	ne	0	
SD2		AVPV size increase; males	0	<0.001	ne	<0.001	0	ne	0		
SD3		MePD volume; males	0	0	ne	<0.01	0	ne	0		
G1	G6 to DOB: PND 1 examined	Hypothalamic ERα increase; females only	<0.01	0	<0.01	0	<0.01	0	0		
G2		Hypothalamic ERβ increase; females only	<0.01	0	<0.05	0	<0.05	0	0		
G3		Hippocampus ERβ increase; males only	0	0	0	0	<0.01	<0.01	0		
G4		Amygdala ERβ increase; males	0	<0.01	0	0	0	0	0		
G5		Amygdala ERβ increase; females	0	0	<0.05	0	0	0	0		
G6		Amygdala oxytocin receptor increase; males	0	<0.01	<0.05	0	0	0	0		
G7		Amygdala oxytocin receptor increase; females	<0.05	<0.01	<0.05	0	<0.05	0	<0.01		
G8		Amygdala vasopressin increase; males	0	<0.01	<0.05	0	0	0	<0.05		
G9		Amygdala genes for GABA/glutamate signaling; females	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01		
HEART											
SD1	Stop-dose	Heart weight decrease; females at 6 months	<0.02	0	0	0	0	0	0		
SD2		Heart weight/body weight decrease; females at 6 months	<0.06	0	0	0	0	0	0		
SD3		LV wall thickness decrease; females at 3 months	<0.05	0	0	0	0	0	<0.01		
SD4		Cardiomyopathy, increase severity; females at PND 21	<0.05	0	<0.05	0	<0.02	<0.0005	0		
CD1	Continuous-dose	LV wall thickness increase; females at 6 months	0	0	0	0	0	<0.05	0		
CD2		Heart weight/body weight increase; females at 6 months	0	<0.05	0	0	0	0	<0.001		
PROSTATE											
SD+1	Stop-dose: T+E at PND 90	Lateral high-grade prostatic intraepithelial neoplasia (HGPIN) at 1 year	<0.01	na	<0.05	na	<0.05	ne	<0.05		
SD+2		Dorsolateral ductal adenocarcinoma, multiplicity increase at 1 year	<0.01	0	0	0	0	ne	0		

TARGET ORGANS	TREATMENT GROUPS	ENDPOINTS ASSESSED	BPA DOSE (µg/kg BW/day)						Ethinyl Estradiol (µg/kg BW/day)		
			2.5	25	250	2,500	25,000	0.05 EE	0.5 EE	0.5 EE	
CD1	Continuous-dose: 6 months	Stem cell # increase	<0.02	0	0	ne	ne	ne	<0.02		
CD2		Progenitor cell proliferation increased	0	<0.01	<0.02	ne	ne	ne	<0.01		
CD3		Stem cell lineage commitment shift to basal cells	0	<0.001	<0.01	ne	ne	ne	<0.001		
Ovary											
SD1	Stop-dose: PND 21 effects	Primordial follicle # decrease	0	0	<0.05	0	0	0	0		
SD2		Primary follicle # decrease	<0.05	0	<0.05	0	0	0	0		
SD3		Preantral follicle # decrease	<0.05	0	0	0	0	0	0		
SD4		Healthy follicle # decrease	<0.05	0	<0.05	0	0	0	0		
CD1	Continuous-dose: 1 year	Serum estradiol	0	P=0.08	0	<0.05	<0.05	<0.05	<0.05		
Immune											
CD1	Continuous-dose: Spleenic effects	Myoid cDC cell increase; males at 3 months	<0.05	0	0	0	0	0	0		
CD2		cDC cell decrease; males at 6 months	<0.01	0	0	0	<0.01	0	<0.001		
CD3		B-cell decrease; females at 6 months	0	<0.05	0	0	0	0	0		
CD4		APC cell decrease; males at 1 year	<0.05	<0.05	0	0	0	0	0		
CD5		Macrophage decrease; males at 1 year	0	0	0	<0.05	0	0	0		
CD6		CD8 T cells and NKT cell increase; males at 1 year	0	0	0	0	<0.05	0	0		
CD+1	Continuous-dose: Spleenic response to activating stimuli <i>ex vivo</i>	Proliferation increase (to PWM); females at 6 months	0	<0.01	0	<0.0001	0	<0.001	0		
CD+2		Proliferation decrease (to PWM); males at 6 months	<0.05	0	<0.05	<0.01	0	0	<0.001		
CD+3		Proliferation increase (to PWM); males at 1 year	<0.01	<0.05	0	<0.01	<0.01	<0.01	<0.001		
CD+4		Proliferation increase (to LPS); females at 1 year	0	0	0	0	<0.05	0	0		
CD+5		Proliferation increase (to LPS); males at 1 year	<0.05	0	0	<0.05	<0.01	0	<0.0001		
CD+6		Proliferation decrease (to anti-CD3); females at 1 year	<0.05	0	0	0	0	<0.01	0		
Core Study											
SD1	Stop-dose	Mammary adenocarcinoma incidence increase; females at 2 years	<0.02	0	0	0	0	0	0		

TARGET ORGANS	TREATMENT GROUPS	ENDPOINTS ASSESSED	BPA DOSE (µg/kg BW/day)						Ethinyl Estradiol (µg/kg BW/day)		
			2.5	25	250	2,500	25,000	0.05 EE	0.5 EE	0.5 EE	
SD2		Ovarian follicular cysts; 1 year	0	0	0	0	<0.001	0	<0.001	0	<0.001
SD3		Aberrant estrous cycle, early onset	0	0	0	<0.03	0	0	0	0	0
SD4		Liver infiltration with mononuclear cells; females at 1 year	<0.05	0	0	0	<0.05	0	0	0	0
SD5		Kidney tubule cyst increase; females at 2 years	<0.01	0	0	0	0	0	0	0	0
SD6		Serum protein and total bile acids decrease; males at 1 year	0	<0.05	0	0	0	0	0	0	0
SD7		Platelets decrease; females at 1 year	0	0	0	0	<0.05	<0.002	0	0	0
SD8		Pancreas pigmentation increase; males at 1 year	0	0	X	0	0	0	0	0	0
SD9		Adrenal hyperplasia; males at 2 years	0	0	0	X	0	0	0	0	0
SD10		Pituitary cysts; males at 2 years	0	0	0	0	<0.05	0	0	0	0
SD11		Pituitary gland hyperplasia; males at 2 years	0	0	0	0	<0.05	<0.05	<0.05	0	0
SD12		Bone marrow hypercellularity; males at 2 years	0	0	X	0	0	X	0	0	0
SD13		Spleen lymphoid hyperplasia; males at 2 years	0	0	X	0	0	0	0	0	0
SD14		Thyroid cysts; females at 2 years	0	0	<0.05	0	<0.05	<0.05	0	0	0
SD15		Epididymis, exfoliated germ cells increase; 2 years	0	0	0	<0.05	0	0	0	0	0
CD1	Continuous-dose	Mammary gland alveoli dilation; females at 2 years	X	0	0	0	0	0	0	0	0
CD2		Uterine apoptosis increase	0	0	0	0	<0.05	<0.05	0	<0.001	<0.001
CD3		Vaginal epithelial hyperplasia increase; females at 2 years	0	<0.05	0	<0.05	<0.05	<0.05	0	0	0
CD4		Vaginal epithelial hyperplasia increase; females at 1 year	0	0	0	0	0	0	0	0	<0.001
CD5		Liver weight decrease; males at 1 year	<0.05	0	0	0	0	0	0	0	0
CD6		Liver angiectasis incidence increase; males at 2 years	X	0	0	0	0	0	0	0	0
CD7		Liver mononuclear infiltration; males at 1 year	<0.05	0	<0.05	<0.05	<0.05	<0.05	0	0	0
CD8		Liver mononuclear infiltration; males at 2 years	0	0	0	0	0	0	<0.05	<0.05	0
CD9		Liver fatty acid increase; males at 1 year	0	<0.05	0	0	0	0	0	0	<0.05
CD10		Kidney tubule cysts; females at 1 year	<0.02	0	0	0	0	0	0	<0.05	0
CD11		Kidney tubule cysts; males at 1 year	0	0	X	X	0	0	X	0	0
CD12		Kidney epithelial hyperplasia, males at 2 years	0	<0.01	0	0	0	0	0	0	0
CD13		Hemoglobin increase; females at 1 year	0	<0.01	0	0	0	0	0	0	0
CD14		Hemoglobin increase; males at 1 year	0	0	0	0	0	<0.05	<0.05	<0.05	0
CD15		Body weight increase; females at 2 years	0	0	<0.05	0	0	0	0	0	0

TARGET ORGANS	TREATMENT GROUPS	ENDPOINTS ASSESSED	BPA DOSE (µg/kg BW/day)						Ethinyl Estradiol (µg/kg BW/day)		
			2.5	25	250	2,500	25,000	0.05 EE	0.5 EE	0.5 EE	
CD16		Pituitary hyperplasia; males at 2 years	0	<0.05	0	0	<0.05	0	<0.05	0	<0.05
CD17		Parathyroid gland hyperplasia; males at 2 years	0	<0.01	0	0	0	0	<0.05	0	<0.05
CD18		Thyroid hyperplasia; males at 2 years	0	0	0	<0.01	0	<0.01	0	<0.01	0
CD19		Eosinophil decrease; males and females at 1 year	0	0	<0.05	0	0	0	0	0	<0.05
CD20		Epididymis, exfoliated germ cells & lymphocytic infiltration increase; 1 year	0	0	0	0	<0.05	0	<0.05	0	<0.05
CD21		Fat pad, retroperitoneal; females at 1 year	<0.05	0	0	0	0	0	0	0	0
CD22		Prostate DLP chronic lymphocytic inflammation increase; 1 year	<0.05	0	0	0	0	0	0	0	0
CD23		Prostate DLP chronic intraluminal inflammation increase; 2 years	<0.05	0	0	0	0	0	0	0	0

Significant effects observed for each organ system were sequentially numbered (column at left): (SD) Stop-dose; (CD) Continuous-dose; (G) Gestation exposure only; (+) indicates secondary exposures in vivo or in vitro as described in the text. (na) not available; (ne) not examined; (O) no significant effect; (X) significant effect noted on Core Study Overview Table; data not available.