Low-dose effects of bisphenol A on mammary gland development in rats

Egebjerg, Karen Mandrup; Boberg, Julie; Isling, Louise Krag; Christiansen, Sofie; Hass, Ulla

Published in:
International Journal of Andrology

Link to article, DOI:
10.1111/andr.12193

Publication date:
2016

Document Version
Publisher's PDF, also known as Version of record

ANDROLOGY

ISSN: 2047-2919

ANDROLOGY

ORIGINAL ARTICLE

Correspondence:
Julie Boberg, National Food Institute, Division of Diet, Disease Prevention and Toxicology, Technical University of Denmark, Mørkhøj Bygade 19, DK-2860 Søborg, Denmark.
E-mail: jubo@food.dtu.dk

Keywords:
animal models, development, endocrine disrupters, environmental factors, estrogens/oestrogens, mammary cancer, microscopy, rat, toxicological evaluation

Received: 9-Oct-2015
Revised: 16-Feb-2016
Accepted: 1-Mar-2016

doi: 10.1111/andr.12193

Low-dose effects of bisphenol A on mammary gland development in rats

K. Mandrup, J. Boberg, L. K. Isling, S. Christiansen and U. Hass

Division of Diet, Disease Prevention and Toxicology, National Food Institute, Technical University of Denmark, Søborg, Denmark

SUMMARY

Bisphenol A (BPA) is widely used in food contact materials, toys, and other products. Several studies have indicated that effects observed at doses near human exposure levels may not be observed at higher doses. Many studies have shown effects on mammary glands at low doses of BPA, however, because of small number of animals or few doses investigated these data have not been used by EFSA as point of departure for the newly assessed tolerable daily intake (TDI). We performed a study with perinatal exposure to BPA (0, 0.025, 0.25, 5, and 50 mg/kg bw/day) in rats (n = 22 mated/group). One of the aims was to perform a study robust enough to contribute to the risk assessment of BPA and to elucidate possible biphasic dose–response relationships. We investigated mammary gland effects in the offspring at 22, 100, and 400 days of age. Male offspring showed increased mammary outgrowth on pup day (PD) 22 at 0.025 mg/kg BPA, indicating an increased mammary development at this low dose only. Increased prevalence of intraductal hyperplasia was observed in BPA females exposed to 0.25 mg/kg at PD 400, but not at PD 100, and not at higher or lower doses. The present findings support data from the published literature showing that perinatal exposure to BPA can induce increased mammary growth and proliferative lesions in rodents. Our results indicate that low-dose exposure to BPA can affect mammary gland development in male and female rats, although higher doses show a different pattern of effects. The observed intraductal hyperplasia in female rats could be associated with an increased risk for developing hyperplastic lesions, which are parallels to early signs of breast neoplasia in women. Collectively, current knowledge on effects of BPA on mammary gland at low doses indicates that highly exposed humans may not be sufficiently protected.

INTRODUCTION

The European Food Safety Authority (EFSA) concluded in an opinion on bisphenol A (BPA) in January 2015 that the substance is 'likely' to induce proliferative changes in mammary glands, based on a weight-of-evidence approach (EFSA 2015). However, EFSA evaluated that most of the studies on mammary gland effects of BPA include too few doses or are in other ways not robust and they could therefore not be used for deriving a tolerable daily intake (TDI) for BPA.

Quite a few rodent studies have been performed on mammary gland effects of pre- or perinatal exposure of BPA showing proliferative lesions in the glands (Table 1). Although most of the studies include few animals or only one or two BPA doses, several studies showed effects at BPA levels around 0.025–0.25 mg/kg bw/day. In general, effects were found in the lowest doses investigated, but not at higher doses. Although non-monotonic dose–response relationships are accepted in some scientific fields today, it is still a controversial subject in other scientific areas (Myers *et al.*, 2009; Vandenberg *et al.*, 2012, 2013a). The evidence on low-dose effects of BPA was further challenged by the results of a large study by Delclos *et al.* (2014) where significant mammary effects were only reported in the highest doses investigated, and according to the authors, not at lower doses around 0.025–0.25 mg/kg. As endpoints showing low-dose effects in previous BPA studies (i.e. intraductal hyperplasia and epithelial proliferation) were not evaluated in adult offspring in the Delclos study, it was considered necessary to conduct this study on these (and other) endpoints in a large-

Table 1 Proliferative changes of mammary glands observed in rodent studies with prenatal or perinatal exposure to bisphenol A

Paper	Doses in mg/kg	Exposure period	Exposure route	Strain, species	Effects
Markey *et al.* (2001)	**0.025**, **0.25**	Prenatal	Osmotic pump	CD-1 mice	Increased ductal & alveolar structures, 6 months (♀)
Markey *et al.* (2003)	**0.000025**, **0.00025**	Prenatal	Osmotic pump	CD-1 mice	Increased lobular structures at 6 months (♀)
Muñoz-de-Toro *et al.* (2005)	**0.000025**[a], **0.00025**[b]	Perinatal	Osmotic pump	CD-1 mice	Increased number of lateral branches[a] and number of TEBs/mammary gland area[b] ($p = 0.054$ at 0.000025 mg/kg) PND 30 (♀)
Durando *et al.* (2007)	**0.025**	Prenatal	Osmotic pump	Wistar rats	Intraductal hyperplasia PND 110 and 180 (♀) Increased proliferative index PND 50
Murray *et al.* (2007)	**0.0025**, 0.025, 0.25, 1	Prenatal	Osmotic pump	Wistar-furth rats	Intraductal hyperplasia PND 50 and 95 (♀)
Vandenberg *et al.* (2008)	**0.00025**, 0.0025, 0.025	Perinatal	Osmotic pump	CD-1 mice	Increased number of alveolar buds, 3 months (♀)
Betancourt *et al.* (2010)	**0.25**	Prenatal	Gavage	SD rats	Proliferation of epithelial cells PND 100 (♀)
Durando *et al.* (2011)	**0.025**, 0.25	Prenatal	Osmotic pump	Wistar rats	Intraductal hyperplasia PND 110 but not PND 50 (♀)
Acevedo *et al.* (2013)	0.00025, 0.0025, 0.025, 0.25	Prenatal or perinatal	Osmotic pump	SD rats	Adenocarcinomas observed PND 90, 140 and 200 (five animals in total in different BPA exposure groups) but not statistically significant
Vandenberg *et al.* (2013b)	**0.00025**[c], **0.0025**[b,c], **0.025**[a], 0.25	Perinatal	Osmotic pump	CD-1 mice	Increased ductal area at 3–4 months[b]. Increased branching points at 3–4 months[c]. Proliferation of epithelial cells at 8 months[a] (two lowest doses not investigated) (♂)
Delclos *et al.* (2014)	0.0025, 0.008, 0.025, 0.08, 0.26, 0.84, **2.7**[a], **100**[a], **300**[b]	Perinatal and postnatal	Gavage	SD rats	Increased ductal structures PND 21[a] and 90[b] (♀) Branching and budding
DTU evaluation of Delclos *et al.* (2014)[c]	0.0025, 0.008, 0.025, **0.08**, **0.26**, 0.84, **2.7**, **100**, 300	Perinatal and postnatal	Gavage	SD rats	Evaluation of increased ductal structures PND 90 (with Fisher's exact test for incidence of mild ductal hyperplasia compared to naïve and vehicle controls) (♀)
Kass *et al.* (2015)	A: 0.025, **0.25** B: **0.064**	A: prenatal B: perinatal	A: Osmotic pump B: Water	Wistar rats	Increased outgrowth PND 5 (♂, A)
This study	**0.025**[b], **0.25**[a], 5, 50	Perinatal	Gavage	Wistar rats	[a]Intraductal hyperplasia PD 400 (♀), [b]increased outgrowth PD22 (♂)

Doses in bold are doses where statistically significant changes were observed for the effects listed in the column to the right. PND, postnatal day; TEBs, terminal end buds; TDs, terminal ducts. [a,b]Refer to specific effects explained in the column to the right. [c]See National Food Institute DTU (2015).

scale study. This study aimed to strengthen the overall weight of evidence regarding possible non-monotonic effects of perinatal low-dose BPA exposure on rodent mammary glands.

Proliferative effects of BPA were seen in several studies: intraductal hyperplasia, epithelial proliferation, and development of more complex structures of the glands. The effects were mainly found in adult female offspring, but in three studies proliferative changes were also observed in prepubertal mammary glands (Muñoz-de-Toro *et al.*, 2005; Delclos *et al.*, 2014; Kass *et al.*, 2015) and in male mammary glands (Vandenberg *et al.*, 2013b; Kass *et al.*, 2015). Thus, the presently available data point to proliferative effects of BPA in both female and male mammary glands of offspring exposed perinatally and the changes may be observable at different time points.

Bisphenol A is known to act via estrogenic and anti-androgenic modes of action and has been shown to act via several other toxicological modes of action (Bonefeld-Jørgensen *et al.*, 2007; Vandenberg *et al.*, 2009; Zhang *et al.*, 2011), but it is not the scope of this manuscript to describe this in detail. The mammary gland effects of BPA correspond to effects observed by other estrogenic compounds, as for example, enhanced mammary development prepubertally in female offspring has been shown for several estrogenic compounds such as diethylstilbestrol, 17β-estradiol, and genistein (Cotroneo *et al.*, 2002; Hovey *et al.*, 2005; Thomsen *et al.*, 2006). However, estrogenic compounds can affect the glands in different ways other than the ones described above. Enhanced lobular development has been described previously for female rats exposed to estrogenic compounds such as ethinyl estradiol and genistein (Murrill *et al.*, 1996; Takagi *et al.*, 2004) and in our previous study on perinatal exposure to phytoestrogens, a trend to an increased prevalence of lobuloalveolar pattern of the adult female mammary glands was observed (Boberg *et al.*, 2013). Moreover, enhanced mammary development and morphological changes such as hypertrophy have been reported in males exposed to estrogenic compounds (Mandrup *et al.*, 2012; Boberg *et al.*, 2013). Thus, such changes may also be relevant to investigate in mammary glands of offspring exposed to BPA.

The aim of this study was to supplement the existing data available on mammary gland effects of BPA, to better establish whether there are low-dose effects of BPA on mammary glands and to better understand the dose–response relationships of BPA on mammary glands. To do this, we applied perinatal exposure of a sufficient number of rats to doses in the low-dose range (μg/kg bw), where mammary changes have been reported by others, as well as dose levels of BPA covering regulatory NOAELs (mg/kg bw). The investigated endpoints included early mammary gland development and different morphological changes in mammary glands of young adult or aging male and female offspring exposed in the perinatal period. Proliferative changes of pituitary glands were evaluated in the 1-year-old rats as previous studies have shown that prolactinomas of the pituitary glands of

elderly female rats may influence morphological changes in the mammary glands (Trouillas *et al.*, 1982).

Other endpoints were also evaluated in this study and results on reproductive parameters and prepubertal data on offspring are published in Christiansen *et al.* (2014) and data on adult reproductive organs and behavioral effects are published in Hass *et al.* (submitted to the current issue of Andrology).

MATERIALS AND METHODS

Chemicals

Bisphenol A (purity ≥99% pure, CAS no. 80-05-7, no. 239658) was purchased from Sigma-Aldrich. Corn oil in glass bottles (no. C8267) also purchased from Sigma-Aldrich was used as vehicle and negative control. The dosing solutions were stirred continuously, kept in glass bottles in the dark at room temperature and verified by chemical analysis (reported in Christiansen *et al.* 2014).

Animals

A total of 110 time-mated female Wistar rats (HanTac : WH, SPF from Taconic Europe, Ejby, Denmark) with a body weight of 200 ± 20 g arrived on gestation day (GD) 3 and on GD 4. The dams were distributed to five groups (n = 22 per group) with similar weight distributions in all groups. The animals arrived in three blocks with 1 week separating each block. The dose groups were equally represented in the different blocks. Dams were housed in pairs until GD 17. From this day and on dams were housed alone. After weaning, offspring were housed in pairs. Dams were housed in polysulfone cages (PSU 80-129HOOSU Type III; Techniplast, Buguggiate, Italy, 15 × 27 × 43 cm) with Aspen wood chip bedding (Tapvei, Gentofte, Denmark) and Enviro Dri nesting material (Brogaarden, Lynge, Denmark) and wooden shelters (Tapvei Arcade 17, Aspen wood; Brogaarden). Animals had access to acidified tap water in BPA-free polysulfone water bottles (84-ACBT0702SU) and soy- and alfalfa free diet (Altromin 1314, Altromin GmbH, Lage, Germany.) for breeding animals. The PSU bottles and cages as well as the aspen wood shelters (instead of plastic) were used to reduce the risk of migration of BPA that potentially could confound the study results. The animals were kept at 22 ± 1 °C with a humidity of 55% ±5, 10 air changes per hour and a light–dark cycle of 12–12 h with lights on from 9 PM to 9 AM. Light and dark cycle was reversed because of behavioral testing of the adult offspring, which was done during their active period, that is, during the dark cycle. Behavioral data are reported in Hass *et al.* (2016).

Dams were gavaged once daily with 0, 0.025, 0.25, 5, or 50 mg BPA/kg bw/day from GD 7 to the day before expected birth (GD 21) and from the day after birth until pup day (PD) 22. PD 0 was defined as the day of expected birth. The animal studies were performed under conditions approved by the Danish Animal Experiments Inspectorate (Council for Animal Experimentation, authorization number: 2012-15-2934-00089) and by the in-house Animal Welfare Committee of the National Food Institute at the Technical University of Denmark.

Necropsies

Offspring were killed on PD 22, at 3–4 months of age (approximately PD 100) and at 13 months of age (approximately PD 400) for mammary gland analysis. At necropsies at all ages, the animals were anesthetized with O_2/CO_2 and decapitated. In each age group, one offspring per sex per litter were selected for necropsies. On PD 22, one abdominal (4th) mammary gland per animal was dissected from the skin for whole mounting. Adult males were killed on PD 90–115 and adult females on PD 104–129 (both males and females referred to as PD 100 in the following) and on PD 398–416 (referred to as PD 400 in the following). No males were killed on PD 400. In adults, male and female abdominal (4th) mammary glands with adjacent lymph nodes were dissected and fixed in formalin for histology. On PD 400, pituitary glands were additionally removed and gross lesions in the pituitary glands were described. The pituitary glands were weighted and fixed in formalin for histopathological evaluation. Females were selected for necropsies when assessed to be in estrous or proestrous, evidenced by vaginal smears in the morning showing macroscopically evident clots of epithelial cells as described in OECD (2011b). As some females were killed hours after selection in the morning, an additional vaginal smear was performed at the time of killed for a more precise evaluation of the stage of estrous cycle at sample selection. Assessment of the estrous cycle at killed was assessed based on vaginal smears only. Smears were stained with Papanicolaou stain as described in Isling *et al.* (2014) and evaluated microscopically and classified in estrous or not in estrous. Estrous was defined as the presence of large amounts of cornified cells in the smear.

Mammary gland whole mounts

Whole mounts were fixed in formalin and stained with alum carmine as described in Mandrup *et al.* (2015). Whole mounts were scanned (4800 dpi) and analyzed also as described in Mandrup *et al.* (2015), using IMAGE PRO PLUS 7.0 software (Media Cybernetics, Bethesda, MD, USA). Mammary glands (n = 13–17 per dose group for females and n = 13–19 per group for males) were evaluated for outgrowth (outer area defined as the smallest polygon enclosing the gland, longitudinal growth (LG), transverse growth (as defined by Mandrup *et al.*, 2012), distance to the lymph node and distance to the fifth gland), the number of terminal end buds (TEBs) in zone C (end buds with a diameter ≥100 μm), and density (mean of scores given for area, number of buds, number of branches, branch generations, and TEBs). Density scores were defined differently for males and females and were therefore not comparable between the sexes.

Histology of pituitary and mammary glands

Fixed tissue samples were routinely processed, embedded in paraffin, sectioned (3 μm), stained with hematoxylin and eosin (H&E), and examined blindly to treatment groups by light microscopy. One section of pituitary glands from all female offspring PD 400 was examined with emphasis on presence of nodular hyperplasia and adenoma in *pars distalis* (MacKenzie & Boorman,1990). From females with gross lesions, an extra section was examined if histological lesions were not observed in the first section in the pituitary gland.

Histological examination of adult mammary glands (males and females PD 100 and females PD 400) was performed on one section of mammary gland. The glands were sectioned horizontally as described in Hvid *et al.* (2011) and all ductular and alveolar structures in the section were evaluated for each animal (Hvid *et al.*, 2011). The sections used for histological evaluation

were representative of the mammary gland and deep enough to include primary to tertiary ducts and a large number of both ducts and lobular structures. Histological examination of adult female mammary glands included the evaluation of lobular development (lobule types ranging from single alveoli to well-developed lobuli and lobular density), lobule morphology (shift to male-like morphology with no lumens or small lumens and multilayered epithelium), intraductal hyperplasia (four or more layers of duct epithelium). To evaluate the extent of branching, the density of ducts was evaluated in females PD 100. Four females on PD 100 and 21 females on PD 400 were not found to be in estrous at the time of killed. These were omitted from the histological evaluation of lobular development because these changes could be related to the estrous stage (Schedin *et al.*, 2000). Intraductal hyperplasia and changes in lobular morphology toward a lobuloalveolar pattern (male-like) are not regarded as normal cyclical changes in females, and all females were evaluated for such changes. Male mammary glands were evaluated for tubuloalveolar pattern (female-like morphology with lumens and single layered epithelium), secretory activity (dilated ducts with secretory material in ductal or alveolar lumens and vacuolated alveolar epithelium), and hypertrophy.

Statistics

Statistical analysis was performed in the statistical software SAS Enterprise Guide 6.1 or GRAPHPAD PRISM 5. The statistical unit (alpha level) was set at 0.05. Continuous data were evaluated for homogeneity of variance before performing the statistical tests. Data not fulfilling the criteria for homogeneity of variance (i.e. distance to the lymph nodes and transverse growth in males) were transformed accordingly. Continuous data (growth measurements in whole mounts) were analyzed using an analysis of variance (ANOVA) with body weight (bw) as a covariate and a Dunnett's test to identify the effects of single treatments compared to controls. To increase the power of the statistical analysis, data for outer area were analyzed with an ANOVA with body weight as a covariate and gender and litter as random factors. Testing for non-monotonic dose–response relationships was performed by at Tukey's test for LSMeans.

Counting data and multi-leveled scoring data (i.e. density scores and TEB data) were analyzed using a non-parametric test (Kruskal–Wallis) with Dunn's post hoc test correcting for multiple comparisons. Histological scoring data were analyzed using Fisher's exact test ($r \times k$ for overall assessments and $2 \times k$ for differences between two groups) or Chi-squared test unless otherwise stated.

RESULTS

As reported in Christiansen *et al.* (2014), no general maternal toxicity was observed at any of the administered doses. Furthermore, BPA exposure had no significant effect on maternal weight gain, mean gestational length, post-implantation loss, litter size, birth weights, sex ratio, perinatal loss, neonatal deaths, or offspring body weights in the postnatal period (Christiansen *et al.*, 2014).

Mammary gland whole mounts in prepubertal offspring

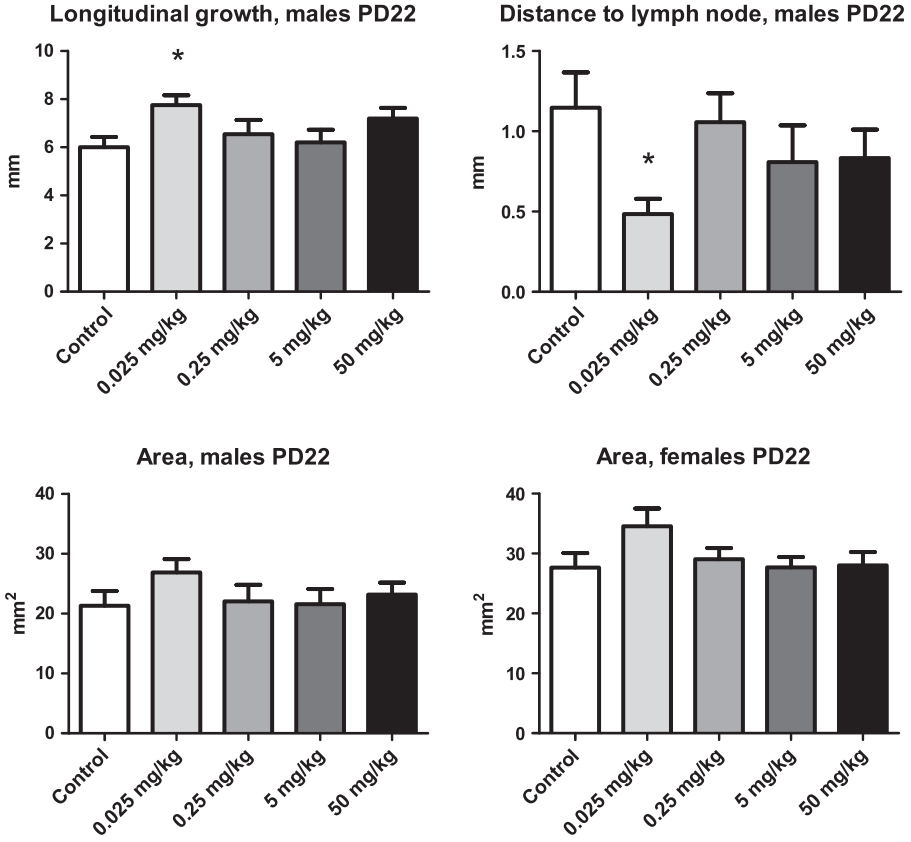
In prepuberty, BPA affected male, but not female, mammary glands. An increased longitudinal growth and a decreased distance to the lymph node was observed in males in the lowest dose group (0.025 mg/kg bw/day) ($p = 0.05$ and $p = 0.02$ in Dunnett's test, respectively) (Fig. 1), although this was not statistically significant in the overall ANOVA ($p = 0.10$ and $p = 0.07$ for longitudinal growth and distance to the lymph node, respectively). These signs of increased outgrowth were supported by a slight increase in the area of the gland in that dose group for males and females ($p = 0.07$ in Dunnett's test for pooled data from males and females). No changes in density scores were observed. Although the longitudinal growth and the distance to the lymph node in males in the higher dose groups were somewhat similar to control levels, they were not statistically significantly different from the 0.025 mg/kg exposure group when analyzed with a Tukey's test.

Mammary gland histology in adults

In females, histological examination of adult mammary glands showed a statistically significant increase in intraductal hyperplasia in females PD 400, but not PD 100. At PD 400, intraductal hyperplasia was observed in all dose groups, but the distribution and severity was increased in some BPA-dosed groups compared to controls (Fig. 2). In controls, a mild degree of intraductal hyperplasia affecting less than 30% of the mammary tissue was seen in five of 20 animals. Extensive intraductal hyperplasia (more than 30% of the mammary tissue) was observed in five of 18 females in the group exposed to 0.25 mg/kg BPA, but not in controls ($p = 0.017$, Fig. 2). The prevalence of extensive intraductal hyperplasia seemed lower at 5 and 50 mg/kg BPA than at 0.25 mg/kg (affecting one of 18 females at 5 mg/kg and two of 18 females at 50 mg/kg; Fig. 2). However, the difference between 0.25 and 5 mg/kg was not statistically significant ($p = 0.18$). Presence of adenomas or hyperplasia in the *pars distalis* of pituitary glands were not correlated with intraductal hyperplasia or lobuloalveolar pattern, as the prevalences of intraductal hyperplasias were comparable in animals with and without pituitary hyperplasia (36 and 26%, respectively). In 1-year-old females, fewer (yet not statistically significant) exposed animals were irregularly cycling compared to controls (Hass *et al.*, 2016). It is therefore improbable that the hyperplastic changes are related to changes in cycling. No statistically significant changes were observed in lobular development, lobular morphology in females PD 100 or 400 or in branching in females PD 100.

In adult males, lobuloalveolar morphology is the typical morphology of mammary glands (Cardy,1991; Wang *et al.*, 2006). In this study, some males showed lobuloalveolar morphology with basally situated nuclei, resulting in a tubular-like orientation of the epithelium, although a tubuloalveolar pattern with lumens was not present. These changes were considered as an early shift toward a female-like morphology. Areas of female-like morphology (tubuloalveolar pattern or basal nuclei) of the male mammary glands were seen in a few controls at PD 100. In the highest dose group, this could be observed in the majority of male mammary glands. A trend to increased frequency of males with female-like morphology was observed in the two highest dose groups (Fig. 3; $p = 0.05$ and 0.1 for the two highest doses in a two-sided 2×2 Fisher's exact test). Female-like structures can appear in small amounts in controls as a normal background variation in young males before full differentiation of the glands to the male-like morphology (OECD, 2011a). On PD 100, male mammary glands should be fully differentiated (Cardy, 1991),

Figure 1 Mammary gland outgrowth in offspring at PD 22 after perinatal exposure to 0, 0.025, 0.25, 5, or 50 mg/kg bw/day of bisphenol A (BPA). A statistically significant increase in the longitudinal growth and decrease in the distance to the lymph node were seen in males exposed to BPA 0.025 mg/kg (top). A related trend ($p = 0.07$) to an increased area was also observed in both males and females (bottom), although not statistically significant and with large variations within groups. Individual values and means ± SEM are shown. $N = 13–16$ for females and $n = 13–19$ for males.



however, as the female-like changes in the current study were observed in very few structures in each gland they may be chance findings.

Histological evaluation of pituitary glands at PD 400

No statistically significant differences were observed between exposed groups and controls in the incidence of pituitary gland nodular hyperplasia and/or adenoma in *pars distalis*. A slightly increased incidence of pituitary adenoma was found in the high-dose BPA group (group 5), but this was not statistically significant (Fig. 4).

DISCUSSION

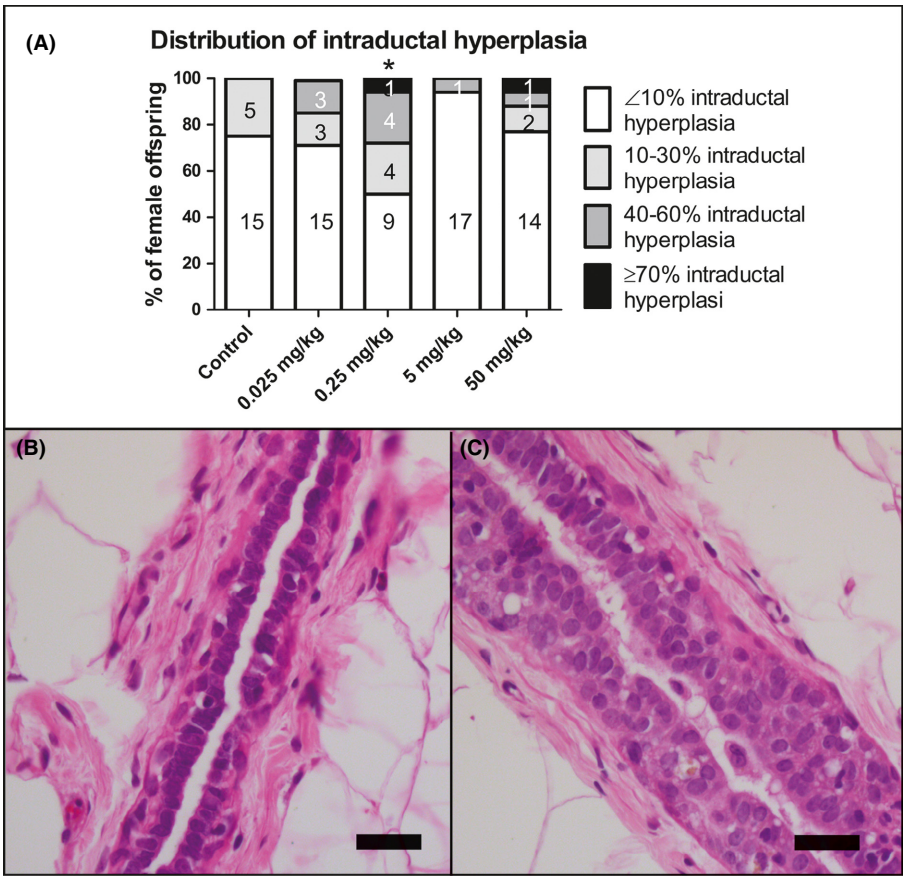
Previous studies on mammary gland effects of perinatal BPA exposure have shown proliferative changes in young and adult female and male rodent offspring (Table 1). Just as in our study, some of these changes are only seen at low, but not at high doses, whereas other changes are seen mainly at the high-dose levels. Data from the present and other mammary gland studies on BPA all together suggest that perinatal exposure to BPA induce effects that do not follow a classical monotonic dose–response relationship. This cannot be readily explained, but a pattern seems to appear when evaluating all available data together. Table 1 lists the findings in this study together with previous studies using more or less comparable study designs for evaluation of BPA toxicity.

Female mammary gland effects

In this study, the main finding in female mammary glands was an increased incidence of intraductal hyperplasia. In other studies, the proliferative changes reported in female rats span from increased ductal area and number of ductal and alveolar structures to hyperplasia or proliferation of ductal epithelium (Table 1). Interestingly, effects of BPA on intraductal hyperplasia and proliferation have previously been seen in the lower dose range up to 0.25 mg/kg in adults (Durando *et al.*, 2007, 2011; Murray *et al.*, 2007; Betancourt *et al.*, 2010) but not at higher doses (Murray *et al.*, 2007; Durando *et al.*, 2011). Taken together with the data from our study, it appears that the effects of BPA on mammary gland hyperplasia and proliferation do not follow a typical monotonic dose–response pattern. In contrast, effects on growth and development are seen at 0.025 mg/kg and at higher doses at prepuberty and in adults (Markey *et al.*, 2001; Delclos *et al.*, 2014).

Strikingly, the hyperplastic changes were observed in adult females although exposure did not continue into adulthood, whereas effects in prepubertal female mammary glands were weak or absent in this study. Accordingly, Markey *et al.* (2001) and Durando *et al.* (2007) showed that changes in development and epithelial proliferation of the female glands exposed to BPA prenatally were only apparent after sexual maturation for exposures corresponding to the low-dose range of our study (0.025 and 0.25 mg/kg) (Markey *et al.*, 2001; Durando *et al.*, 2007). In

Figure 2 Female mammary gland histology at PD 400 after perinatal exposure to 0, 0.025, 0.25, 5, or 50 mg/kg bw/day of bisphenol A (BPA). (A) Distribution of scores for intraductal hyperplasia. Extensive intraductal hyperplasia (more than 30% of the gland) was observed in a significantly higher prevalence of females in the group exposed to 0.25 mg/kg BPA compared to controls (Fisher's exact test $p = 0.017$). *$p < 0.05$ for high intraductal hyperplasia (more than 30% of tissue), Fisher's exact 2 × 2 test. (B) Typical duct from control female with a single layer of duct epithelium. (C) Example of duct with intraductal hyperplasia in a female exposed perinatally to 0.25 mg/kg BPA. Bars in the lower right corner of pictures B and C represent 25 μm. $N = 18–21$.



contrast, higher doses of BPA-induced effects on growth and development in both prepubertal and adult female mammary glands (Delclos *et al.*, 2014). It is possible that different mechanisms of action are prevailing at different dose levels. Estrogens are known to have different effects at different doses at the cellular level (Amara & Dannies,1983; Soto & Sonnenschein,1987). Overall, low doses of BPA appear to increase the risk for developing hyperplasia later in life, whereas higher doses appear to increase early growth and development of the glands.

In accordance with our findings, previous studies have shown intraductal hyperplasia in controls as well as in exposed animals, but exposure to BPA increases the incidence (Durando *et al.*, 2007, 2011). In this study, the increased incidence of intraductal hyperplasia was observed in BPA-exposed females from the dose group exposed to 0.25 mg/kg bw/day, when the animals were 14 months of age, but not on PD 100. Such ductal changes have also been reported in previous BPA studies at doses down to 0.025 and 0.0025 mg/kg and in younger females on PD 50, 110, and PD 180 (Durando *et al.*, 2007, 2011; Murray *et al.*, 2007). In those studies, a quantitative technique was used for histological evaluation, and it is possible that using a quantitative technique instead of a scoring technique could have improved our ability to detect small changes at the lower dose (0.025 mg/kg) or at an earlier age. When evaluating histological data, it is highly relevant to consider that even perinatally induced lesions may become more severe at increasing ages. For example, studies in female rats exposed to a carcinogen on PND21 revealed an increased incidence of intraductal hyperplasia with increasing age (Thompson *et al.*, 1998, 2000). Thus, at increasing ages it may become easier to detect treatment-related effects with crude methods such as scoring evaluation of only one section per tissue per animal, as long as control levels are relatively low. Despite a risk of more background 'noise' at increasing age, it seems warranted to continue investigating mammary glands in older females, and at the same time to continue development of more sensitive methods for mammary gland evaluation in younger animals. Further development of quantitative methods may increase the sensitivity of mammary gland examination using the whole-mount method or histological examination.

BPA-induced intraductal hyperplasia (Durando *et al.*, 2007, 2011; Murray *et al.*, 2007) and increased mammary development (Muñoz-de-Toro *et al.*, 2005; Moral *et al.*, 2008; Ayyanan *et al.*, 2011) may not be rodent specific. A study in Rhesus monkeys also showed increased mammary development in female offspring few days after birth following prenatal exposure to BPA (Tharp *et al.*, 2012). In general, the intraductal hyperplasia observed in these studies is comparable to hyperplasia observed in human breasts (Singh *et al.*, 2000) and are likely to be preneoplastic lesions (Thompson *et al.*, 1998). Several studies have shown increased susceptibility to mammary carcinogens after perinatal exposure to

Figure 3 Male mammary gland histology at PD 100 after perinatal exposure to 0, 0.025, 0.25, 5, or 50 mg/kg bw/day of bisphenol A (BPA). (A) Histological evaluation showed exposed males with female-like morphology of the mammary glands (tubuloalveolar morphology or epithelium with basally situated nuclei) in all groups. A trend to increased number of males with female-like pattern was observed in the two highest dose groups ($p = 0.05$ for 5 mg/kg and $p = 0.1$ for 50 mg/kg BPA in a 2 × 2 Fisher exact test). $N = 17–22$. (B) Control male mammary gland with lobuloalveolar pattern. (C) Male from the highest exposure group (50 mg/kg bw/day) with tubuloalveolar morphology of the mammary gland. Bars in B and C represent 100 μm.

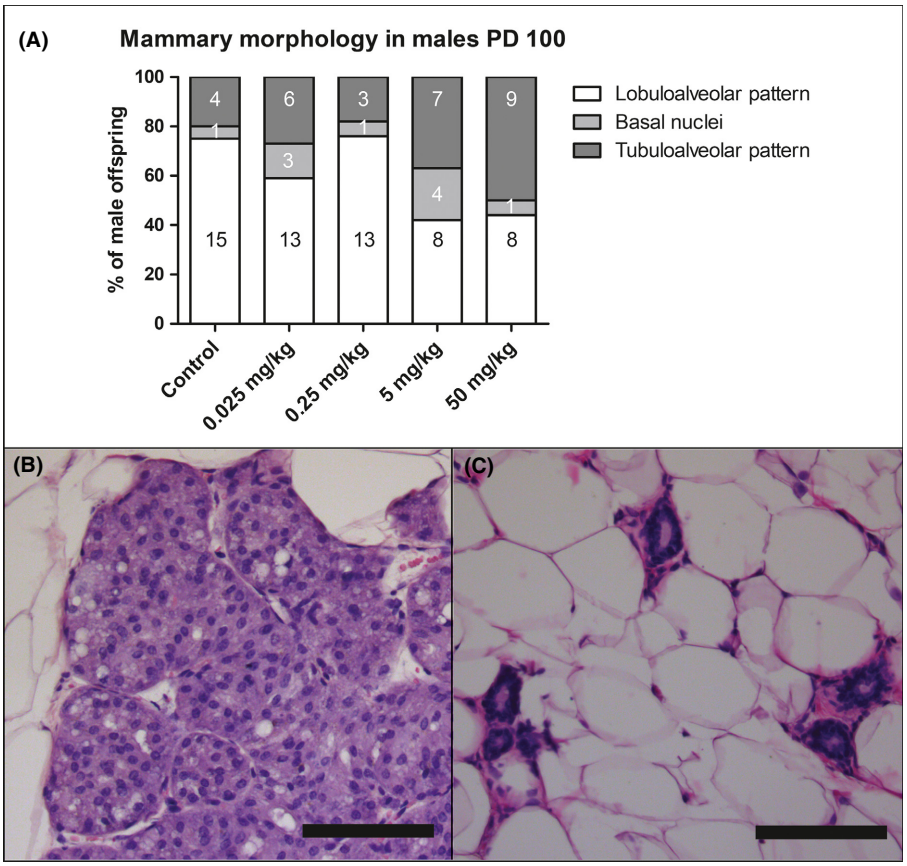
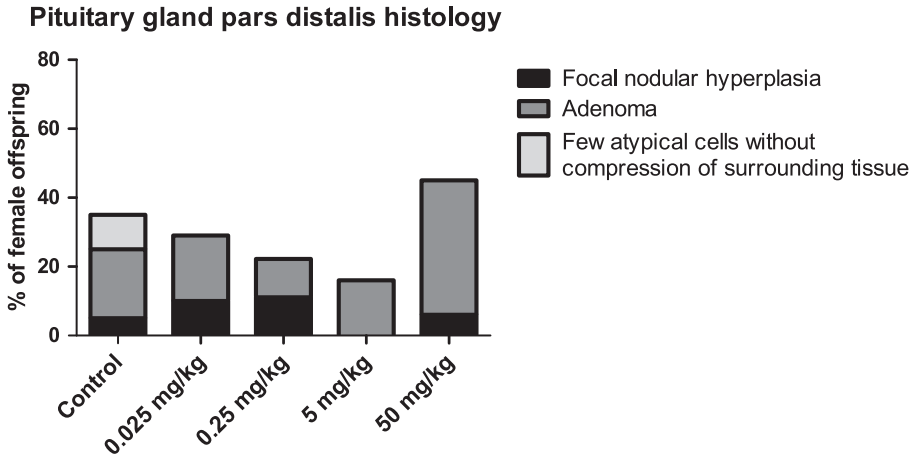


Figure 4 Pituitary gland histology in female offspring at PD 400 after perinatal exposure to 0, 0.025, 0.25, 5, or 50 mg/kg bw/day of bisphenol A (BPA). Percentages of female offspring with focal nodular hyperplasia (black), adenoma (dark gray), or with presence of few atypical cells without compression of surrounding tissue (light gray) in the *pars distalis* of the pituitary gland are shown. $N = 20, 21, 18, 19, 18$, respectively. No statistical significant differences were observed between exposed groups and controls.



BPA as reviewed by Soto *et al.* (Soto *et al.*, 2013). For example, exposure to a subcarcinogenic level of *N*-nitroso-*N*-methylurea (NMU) induced malignant tumors in females prenatally exposed to BPA (Durando *et al.*, 2007). Acevedo and co-workers found adenocarcinomas in females exposed to BPA, but not in controls, although the animals were not exposed to a carcinogen later in life

(Acevedo *et al.*, 2013). Accordingly, our observation of increased intraductal hyperplasia at PD 400 may thus indicate a higher risk for developing hyperplasia and mammary tumors in adulthood.

In general, the present findings indicate that BPA may contribute to precocious breast development and increase the risk for breast cancer in perinatally exposed individuals.

Male mammary gland effects

Interestingly, male mammary gland development also appears to be affected by BPA in a similar way as female mammary glands. Proliferative changes are found in males at different ages, as an increased outgrowth prepubertally was seen in this study and reported by Kass *et al.* (2015), and as an increased ductal area, increased number of branching points, and increased proliferation of epithelial cells was reported in adults (Vandenberg *et al.*, 2013b). Proliferative changes (hyperplasia) were not investigated in adult males in this study as evaluation of hyperplasia is hampered by the lobuloalveolar morphology of well-differentiated male mammary glands. Together, data suggest that male mammary gland development is increased by perinatal BPA exposure. In this study, mammary proliferative changes were observed already before sexual maturation of males, and already at the lowest dose of BPA (0.025 mg/kg). Based on the findings in male mammary glands, no NOAEL could be established for mammary gland effects in this study. This supports other studies showing absence of NOAEL for mammary changes in males down to the lowest dose tested, that is, 0.00025 mg/kg (corresponding to 0.25 μg/kg) (Vandenberg *et al.*, 2013b). Overall, there is a strong weight of evidence for mammary effects of perinatal BPA exposure in the μg/kg dose range.

Altogether, our data support previous studies showing that male mammary glands are susceptible to BPA exposure, and that male gland development can be more sensitive to endocrine disruption than female mammary glands (You *et al.*, 2002; Mandrup *et al.*, 2012). Male mammary glands are rarely examined when investigating chemicals suspected to be endocrine disrupting although effects may be detected earlier or at lower doses in males than in females. The implications of the findings observed in male rats are not known, but these changes may suggest that boys and men exposed to endocrine disrupters can develop more breast tissue and thus increase the susceptibility for gynecomastia.

Non-monotonic dose–response effects on mammary glands

The most marked effects observed in male and female mammary glands were seen at the lower doses tested and results at the higher doses approached control levels suggesting a non-monotonous dose–response curve for mammary gland effects. Indications of non-monotonic dose–response curves for BPA have been described by others for proliferative mammary gland changes at different ages, showing statistically significant effects at doses of 0.0025 mg/kg in 95-days old females (Murray *et al.*, 2007), 0.025 mg/kg in 110 days old females (Durando *et al.*, 2011), and 0.025 mg/kg in 7–9 months old males (Vandenberg *et al.*, 2013b) but not at higher doses. Additionally, such non-monotonic dose–response curves have also been reported for changes in gland development. Studies have shown significant effects on the number of branch points at 0.00025 and 0.0025 mg/kg BPA but not at higher doses in 3–4 months old male offspring (Vandenberg *et al.*, 2013b) and changes in ductal elongation with significant differences between 0.025 and 0.25 mg/kg BPA in 1-month old offspring (Markey *et al.*, 2001).

Interestingly, Delclos *et al.* (2014) only found significant effects (increased number of ductal structures) in mammary glands in the highest doses investigated, although they included doses of 0.025 and 0.26 mg/kg BPA and investigated a large number of animals. It should be noted, however, that endpoints showing low-dose effects in previous BPA studies and in this study (i.e. intraductal hyperplasia and epithelial proliferation) was not evaluated in adult offspring in the Delclos study (Table 1). Moreover, pups were dosed by gavage postnatally from PND1 and the exposure period in that study persisted until killed in adulthood and these differences in study design may have implications on the mammary gland development. Reasonably enough, Delclos *et al.* (2014) performed the statistical analysis in two subgroups to minimize false negative results, as they included many exposure groups (two control groups, nine BPA exposure groups and two ethinyl estradiol exposure groups) in their study. Yet, the distribution of the exposure groups in the statistical subgroups favored the high-dose groups (100 and 300 mg/kg), as all other seven BPA exposure groups were analyzed statistically together, leading to a higher risk for false negative results in all the low-dose groups compared to the two highest dose groups. If the exposure groups had been distributed equally in two subgroups (e.g. the five lowest doses in one group for statistical analysis and the four highest doses in another group), the results may have shown significant effects in other exposure groups. We have tentatively analyzed the data on duct hyperplasia presented in Delclos *et al.* (2014) in an alternative way (using Fisher's exact test) and found significantly increased frequencies of duct hyperplasia in 3-months old females down to 0.08 mg/kg bw/day (National Food Institute DTU, 2015) (Table 1).

Non-monotonic dose–response curves have been observed for other endpoints in this BPA study. Data on spatial learning and sperm count showed significant effects at the lowest dose but not at higher doses (Hass *et al.*, 2016). Data from the present and other mammary gland studies on BPA all show that effects are seen in the lower dose range up to 0.25 mg/kg and again at much higher doses (Table 1). Altogether, the available data suggest that proliferative effects of perinatal exposure to BPA on mammary glands do not follow a classical monotonic dose–response relationship.

Overall, strong evidence points to mammary effects of perinatal BPA exposure at dose levels in the μg/kg area, around 0.25 mg/kg/day or lower suggesting that the new temporary TDI for BPA set by EFSA of 4 μg/kg bw/day is not sufficiently protective with regard to endocrine disrupting effects in humans.

Pituitary gland histology at PD 400

Pituitary adenomas were observed in all groups as expected because spontaneous pituitary adenomas are a common finding in aged rats (Trouillas *et al.*, 1982; Barsoum *et al.*, 1985; Carlus *et al.*, 2013). Increased incidence of pituitary adenoma has previously been shown in aged Wistar rats perinatally exposed to mixtures of endocrine disrupting chemicals, especially for a mixture of predominantly anti-androgenic chemicals (Isling *et al.*, 2014). However, in this study no statistically significant differences in the incidence of pituitary focal nodular hyperplasia and/or adenoma were observed between BPA-exposed groups and controls

and no associations between pituitary hyperplasia and/or adenomas and mammary intraductal hyperplasia were seen. Although not immunohistochemically confirmed, a high number of the pituitary adenomas seen in the available studies are likely to be prolactinomas, which commonly occur in aging rats (Trouillas *et al.*, 1982; Barsoum *et al.*, 1985). Prolactinomas can be induced in rats by prolonged administration of estrogen, as estrogen is known to stimulate prolactin release from lactotrophs and inhibit the activity of hypothalamic neuroendocrine dopaminergic neurons (Welsch *et al.*, 1971; Sarkar *et al.*, 1982; DeMaria *et al.*, 2000; Lloyd, 2015). Consequently, endocrine-disrupting chemicals with estrogenic activity could be speculated to influence the development of pituitary adenoma and the specific types of adenomas, but no relation to BPA exposure was seen in this study. Further studies on prolactin serum levels and immunohistochemistry of pituitary glands could enlighten the nature of the hyperplasias and adenomas observed in controls and exposed animals.

CONCLUSION

Our study confirmed the findings reported by others, showing intraductal hyperplasia in the μg/kg bw dose area but not at higher doses of BPA. Moreover, male mammary development was increased in the lowest dose investigated, that is, 25 μg/kg bw per day but not at higher doses and no NOAEL was determined for mammary gland effects. This effect observed in rats at 25 μg/kg bw per day indicate that the new temporary TDI of 4 μg/kg bw per day proposed by EFSA is not sufficiently protective with regard to effects on mammary gland development.

In this study, effects of BPA on mammary gland development was found at 0.025 or 0.25 mg/kg BPA depending on the endpoint, method, and age of investigation. The effects observed in this low-dose range are considered likely to be treatment-related effects although no significant effects are observed at higher doses and a classical monotonic dose–response curve is not observed. The available data on BPA effects on mammary glands support that non-monotonic dose–response relationships for these effects occurs. As these effects are expected to be related to the endocrine-disrupting mode of action of BPA, the results indicate that also other endocrine disruptors may show non-monotonic dose–response relationships for mammary gland effects with effects occurring only at the lower doses. In future studies on endocrine disrupters, it is therefore recommended to consider inclusion of low doses. Also, in risk assessment of endocrine disrupters there is a need for more awareness that non-monotonic dose–response relationships can occur.

ACKNOWLEDGEMENTS

The present research was performed with the excellent contribution of laboratory technicians Vibeke Kjær, Sarah Simonsen, Ulla El-Baroudy, Heidi Letting, Dorte Korsbech, Lillian Sztuk, Bo Herbst, and Birgitte Plesning together with Anne-Marie Ørngreen and the animal technicians. They are all thanked for their outstanding work in this project.

DECLARATION OF INTERESTS

The authors declare to have no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

FUNDING

The work presented was supported by grants from the Danish Environmental Protection Agency.

MEETING COMMENTS

Martine Applanat (Paris, France)

There is controversy concerning the lower doses of bisphenol A (BPA) and their effects on the mammary gland. I was surprised that you saw hyperplasia of the intraduct epithelium at 400 postnatal days but not before following a dose of 250 μg/kg/day. Some technical details must be considered in order to interpret the results. Were the housekeeping cages controlled and BPA free? Was the diet phytoestrogen-free? Did you measure blood BPA concentration and what was the lowest detectable level? At what stage of the estrous cycle were the rats sacrificed? Measurement of these parameters is fundamental for the evaluation of the statistical effect of the results.

Karen Riiber Mandrup (Søborg, Denmark)

We did not identify intraductal hyperplasia before the age of 14 months, although this has been described in other studies. In other studies, hyperplasia at an earlier age was detected using more quantitative methods including taking several serial histological sections of each gland and counting the cells. We only scored one section per gland and were less likely to detect minor changes at an earlier age. Our cages and drinking bottles were BPA free. The diet was selected to be soy and alphaalpha free. Our conditions were controlled to minimize contamination from the environment.

Pete Myers (Charlottesville, USA)

It is good to see that you have replicated Ana Soto's work especially as you work for a Government agency. You cited the study of Delclos *et al.* (2014) which differed from other studies in that low-dose levels were not significantly different from their controls. That paper should not be considered because the controls were contaminated with BPA and the results are not reliable.

Karen Riiber Mandrup

We can discount their apparent lack of low-dose effect. However, their higher doses were greater than the control levels and the effects seen in those animals are valid.

David Kristiansen (Copenhagen, Denmark)

Have you studied the mechanism of the intraduct hyperplasia to see if there is signal transduction, and have you examined the adipocytes after exposure to BPA? It is known that growth of alveolar cells comes from fat cells by a process of transdifferentiation because of signaling from the gonad.

Karen Riiber Mandrup

We have not investigated the mechanisms behind the changes observed.

REFERENCES

Acevedo N, Davis B, Schaeberle CM, Sonnenschein C & Soto AM. (2013) Perinatally administered bisphenol A acts as a mammary gland carcinogen in rats. *Environ Health Perspect* 121, 1040–1046.

Amara JF & Dannies PS. (1983) 17β-estradiol has a biphasic effect on GH cell growth. *Endocrinology* 112, 1141–1143.

Ayyanan A, Laribi O, Schuepbach-Mallepell S, Schrick C, Gutierrez M, Tanos T, Lefebvre G, Rougemont J, Yalcin-Ozuysal Ö & Brisken C. (2011) Perinatal exposure to bisphenol A increases adult mammary gland progesterone response and cell number. *Mol Endocrinol* 25, 1915–1923.

Barsoum NJ, Moore JD, Gough AW, Sturgess JM & De LaIglesia FA. (1985) Morphofunctional investigations on spontaneous pituitary tumors in Wistar rats. *Toxicol Pathol* 13, 200–208.

Betancourt AM, Eltoum IA, Desmond RA, Russo J & Lamartiniere CA. (2010) In utero exposure to bisphenol A shifts the window of susceptibility for mammary carcinogenesis in the rat. *Environ Health Perspect* 118, 1614–1619.

Boberg J, Mandrup KR, Jacobsen PR, Isling LK, Hadrup N, Berthelsen LO, Elleby A, Kiersgaard M, Vinggaard AM, Hass U & Nellemann C. (2013) Endocrine disrupting effects in rats perinatally exposed to a dietary relevant mixture of phytoestrogens. *Reprod Toxicol* 40, 41–51.

Bonefeld-Jørgensen EC, Long M, Hofmeister MV & Vinggaard AM. (2007) Endocrine-disrupting potential of bisphenol A, bisphenol A dimethacrylate, 4-n-nonylphenol, and 4-n-octylphenol in vitro: new data and a brief review. *Environ Health Perspect* 115(Suppl 1), 69–76.

Cardy RH. (1991) Sexual dimorphism of the normal rat mammary gland. *Vet Pathol* 28, 139–145.

Carlus M, Elies L, Fouque MC, Maliver P & Schorsch F. (2013) Historical control data of neoplastic lesions in the Wistar Hannover Rat among eight 2-year carcinogenicity studies. *Exp Toxicol Pathol* 65, 243–253.

Christiansen S, Axelstad M, Boberg J, Vinggaard AM, Pedersen GA & Hass U. (2014) Low-dose effects of bisphenol A on early sexual development in male and female rats. *Reproduction* 147, 477–487.

Cotroneo MS, Wang J, Fritz WA, Eltoum IE & Lamartiniere CA. (2002) Genistein action in the prepubertal mammary gland in a chemoprevention model. *Carcinogenesis* 23, 1467–1474.

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. (2014) Toxicity evaluation of bisphenol A administered by gavage to Sprague Dawley rats from gestation day 6 through postnatal day 90. *Toxicol Sci* 139, 174–197.

DeMaria JE, Livingstone JD & Freeman ME. (2000) Ovarian steroids influence the activity of neuroendocrine dopaminergic neurons. *Brain Res* 879, 139–147.

Durando M, Kass L, Piva J, Sonnenschein C, Soto AM, Luque EH & Muñoz-de-Toro M. (2007) Prenatal bisphenol A exposure induces preneoplastic lesions in the mammary gland in Wistar rats. *Environ Health Perspect* 115, 80–86.

Durando M, Kass L, Perdomo V, Bosquiazzo VL, Luque EH & Muñoz-de-Toro M. (2011) Prenatal exposure to bisphenol A promotes angiogenesis and alters steroid-mediated responses in the mammary glands of cycling rats. *J Steroid Biochem Mol Biol* 127, 35–43.

EFSA (2015) Scientific Opinion on the risks to public health related to the presence of bisphenol A (BPA) in foodstuffs: part II – toxicological assessment and risk characterisation. *EFSA J* 13, 1–621.

Hass U, Christiansen S, Boberg J, Rasmussen MG, Mandrup K & Axelstad M. (2016) Low-dose effect of developmental bisphenol A exposure on sperm count and behaviour in rats. *Andrology* 4, 594–607.

Hovey RC, Asai-Sato M, Warri A, Terry-Koroma B, Colyn N, Ginsburg E & Vonderhaar BK. (2005) Effects of neonatal exposure to diethylstilbestrol, tamoxifen, and toremifene on the BALB/c mouse mammary gland. *Biol Reprod* 72, 423–435.

Hvid H, Thorup I, Oleksiewicz MB, Sjogren I & Jensen HE. (2011) An alternative method for preparation of tissue sections from the rat mammary gland. *Exp Toxicol Pathol* 63, 317–324.

Isling LK, Boberg J, Jacobsen PR, Mandrup KR, Axelstad M, Christiansen S, Vinggaard AM, Taxvig C, Kortenkamp A & Hass U. (2014) Late-life effects on rat reproductive system after developmental exposure to mixtures of endocrine disrupters. *Reproduction* 147, 465–476.

Kass L, Durando M, Altamirano GA, Manfroni-Ghibaudo GE, Luque EH & Muñoz-de-Toro M. (2015) Prenatal bisphenol A exposure delays the development of the male rat mammary gland. *Reprod Toxicol* 54, 37–46.

Lloyd RV. (1990) Tumors of the pituitary gland. In: *Pathology of Tumours in Laboratory Animals, Vol 1 – Tumours of the Rat*, (eds V Turusov & U Mohr), pp. 499–518. International Agency for Research on Cancer, Lyon.

MacKenzie WF & Boorman GA. (1990) Pituitary gland. In: *Pathology of the Fischer Rat Reference and Atlas*, (eds GA Boorman, SL Eustis, MR Elwell, CA Montgomery & WF Mackenzie), pp. 485 –500. Academic Press, Inc., San Diego.

Mandrup KR, Hass U, Christiansen S & Boberg J. (2012) Perinatal ethinyl oestradiol alters mammary gland development in male and female Wistar rats. *Int J Androl* 35, 385–396.

Mandrup KR, Johansson HKL, Boberg J, Pedersen AS, Mortensen MS, Jørgensen JS, Vinggaard AM & Hass U. (2015) Mixtures of environmentally relevant endocrine disrupting chemicals affect mammary gland development in female and male rats. *Reprod Toxicol* 54, 47–57.

Markey CM, Luque EH, Munoz de Toro M, Sonnenschein C & Soto AM. (2001) In utero exposure to bisphenol A alters the development and tissue organization of the mouse mammary gland. *Biol Reprod* 65, 1215–1223.

Markey CM, Coombs MA, Sonnenschein C & Soto AM. (2003) Mammalian development in a changing environment: exposure to endocrine disruptors reveals the developmental plasticity of steroid-hormone target organs. *Evol Dev* 5, 67–75.

Moral R, Wang R, Russo IH, Lamartiniere CA, Pereira J & Russo J. (2008) Effect of prenatal exposure to the endocrine disruptor bisphenol A on mammary gland morphology and gene expression signature. *J Endocrinol* 196, 101–112.

Muñoz-de-Toro M, Markey CM, Wadia PR, Luque EH, Rubin BS, Sonnenschein C & Soto AM. (2005) Perinatal exposure to bisphenol-A alters peripubertal mammary gland development in mice. *Endocrinology* 146, 4138–4147.

Murray TJ, Maffini MV, Ucci AA, Sonnenschein C & Soto AM. (2007) Induction of mammary gland ductal hyperplasias and carcinoma in situ following fetal bisphenol A exposure. *Reprod Toxicol* 23, 383–390.

Murrill WB, Brown NM, Zhang JX, Manzolillo PA, Barnes S & Lamartiniere CA. (1996) Molecular epidemiology and cancer prevention: prepubertal genistein exposure suppresses mammary cancer and enhances gland differentiation in rats. *Carcinogenesis* 17, 1451–1457.

Myers JP, Zoeller RT & vom Saal FS. (2009) A clash of old and new scientific concepts in toxicity, with important implications for public health. *Environ Health Perspect* 117, 1652–1655.

National Food Institute DTU (2015) DTU Evaluation of EFSA's New Scientific Opinion on Bisphenol A.

OECD (2011a) Guidance Document for histologic evaluation of endocrine and reproductive tests in rodents (106) part 4: Mammary Gland, pp. 103–115. Available at: http://www.oecd.org/chemicalsafety/testing/43754898.pdf.

OECD (2011b) Guidance Document for histologic evaluation of endocrine and reproductive tests in rodents (106) part 5: Preparation, Reading and Reporting of Vaginal Smears, pp. 116–125. Available at: http://www.oecd.org/chemicalsafety/testing/43804816.pdf.

Sarkar DK, Gottschall PE & Meites J. (1982) Damage to hypothalamic dopaminergic neurons is associated with development of prolactin-secreting pituitary tumors. *Science* 218, 684–686.

Schedin P, Mitrenga T & Kaeck M. (2000) Estrous cycle regulation of mammary epithelial cell proliferation, differentiation, and death in the Sprague–Dawley rat: a model for investigating the role of estrous cycling in mammary carcinogenesis. *J Mammary Gland Biol Neoplasia* 5, 211–225.

Singh M, McGinley JN & Thompson HJ. (2000) A comparison of the histopathology of premalignant and malignant mammary gland lesions induced in sexually immature rats with those occurring in the human. *Lab Invest* 80, 221–231.

Soto AM & Sonnenschein C. (1987) Cell proliferation of estrogen-sensitive cells: the case for negative control. *Endocr Rev* 8, 44–52.

Soto A, Brisken C, Schaeberle C & Sonnenschein C. (2013) Does cancer start in the womb? Altered mammary gland development and predisposition to breast cancer due to in utero exposure to endocrine disruptors. *J Mammary Gland Biol Neoplasia* 18, 199–208.

Takagi H, Shibutani M, Lee KY, Lee HC, Nishihara M, Uneyama C, Takigami S, Mitsumori K & Hirose M. (2004) Lack of modifying effects of genistein on disruption of the reproductive system by perinatal dietary exposure to ethinylestradiol in rats. *Reprod Toxicol* 18, 687–700.

Tharp AP, Maffini MV, Hunt PA, VandeVoort CA, Sonnenschein C & Soto AM. (2012) Bisphenol A alters the development of the rhesus monkey mammary gland. *Proc Natl Acad Sci USA* 109, 8190–8195.

Thompson HJ, McGinley JN, Wolfe P, Singh M, Steele VE & Kelloff GJ. (1998) Temporal sequence of mammary intraductal proliferations, ductal carcinomas in situ and adenocarcinomas induced by 1-methyl-1-nitrosourea in rats. *Carcinogenesis* 19, 2181–2185.

Thompson HJ, Singh M & McGinley J. (2000) Classification of premalignant and malignant lesions developing in the rat mammary gland after injection of sexually immature rats with 1-methyl-1-nitrosourea. *J Mammary Gland Biol Neoplasia* 5, 201–210.

Thomsen AR, Almstrup K, Nielsen JE, Sørensen IK, Petersen OW, Leffers H & Breinholt VM. (2006) Estrogenic effect of soy isoflavones on mammary gland morphogenesis and gene expression profile. *Toxicol Sci* 93, 357–368.

Trouillas J, Girod C, Claustrat B, Curé M & Dubois MP. (1982) Spontaneous pituitary tumors in the Wistar/Furth/Ico rat strain. An animal model of human prolactin adenoma. *Am J Pathol* 109, 57–70.

Vandenberg LN, Maffini MV, Schaeberle CM, Ucci AA, Sonnenschein C, Rubin BS & Soto AM. (2008) Perinatal exposure to the xenoestrogen bisphenol-A induces mammary intraductal hyperplasias in adult CD-1 mice. *Reprod Toxicol* 26, 210–219.

Vandenberg LN, Maffini MV, Sonnenschein C, Rubin BS & Soto AM. (2009) Bisphenol-A and the great divide: a review of controversies in the field of endocrine disruption. *Endocr Rev* 30, 75–95.

Vandenberg LN, Colborn T, Hayes TB, Heindel JJ, Jacobs DR, Lee DH, Shioda T, Soto AM, vom Saal FS, Welshons WV, Zoeller RT & Myers JP. (2012) Hormones and endocrine-disrupting chemicals: low-dose effects and nonmonotonic dose responses. *Endocr Rev* 33, 378–455.

Vandenberg LN, Colborn T, Hayes TB, Heindel JJ, Jacobs J, Lee DH, Myers JP, Shioda T, Soto AM, vom Saal FS, Welshons WV & Zoeller RT. (2013a) Regulatory decisions on endocrine disrupting chemicals should be based on the principles of endocrinology. *Reprod Toxicol* 38, 1–15.

Vandenberg LN, Schaeberle CM, Rubin BS, Sonnenschein C & Soto AM. (2013b) The male mammary gland: a target for the xenoestrogen bisphenol A. *Reprod Toxicol* 37, 15–23.

Wang XJ, Bartolucci-Page E, Fenton SE & You L. (2006) Altered mammary gland development in male rats exposed to genistein and methoxychlor. *Toxicol Sci* 91, 93–103.

Welsch CW, Jenkins T, Amenomori Y & Meites J. (1971) Tumorous development of in situ and grafted anterior pituitaries in female rats treated with diethylstilbesterol. *Experientia* 27, 1350–1352.

You L, Sar M, Bartolucci EJ, McIntyre BS & Sriperumbudur R. (2002) Modulation of mammary gland development in prepubertal male rats exposed to genistein and methoxychlor. *Toxicol Sci* 66, 216–225.

Zhang X, Chang H, Wiseman S, He Y, Higley E, Jones P, Wong CKC, Al-Khedhairy A, Giesy JP & Hecker M. (2011) Bisphenol A disrupts steroidogenesis in human H295R cells. *Toxicol Sci* 121, 320–327.

