Title: Branching morphogenesis of the mouse mammary gland after exposure to benzophenone-3.

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Abbreviations: **AB**: alcian blue; AREG: amphiregulin; **BP3**: benzophenone-3; **BPA**: bisphenol A; **bw**: body weight; **cAMP**: cyclic adenosine monophosphate; **CSN2**: beta-casein; **CTMC**: connective tissue mast cells; **E_2**: estrogens; **ECM**: extracellular matrix; **EDCs**: endocrine-disrupting chemicals; **ESR1**: estrogen receptor alpha; **FGF2**: fibroblastic growth factor-2; **GD**: gestation day; IGF-1: insulin-like growth factor 1; **L**: lactation day; **MMC**: mucosal mast cells; **P_4**: progesterone; **PND**: postnatal day; **PR**: progesterone receptor; **q-RT-PCR**: real-time RT-PCR; **S**: safranin; **TEBs**: terminal end buds.

ABSTRACT

Pubertal mammary branching morphogenesis is a hormone-regulated process susceptible to exposure to chemicals with endocrine disruptive capacity, such as the UV-filter benzophenone-3 (BP3). Our aim was to assess whether intrauterine or *in vitro* exposure to BP3 modified the branching morphogenesis of the female mouse mammary gland. For this, pregnant mice were dermally exposed to BP3 (0.15 or 50 mg/kg/day) from gestation day (GD) 8.5 to GD18.5. Sesame oil treatment served as control. Changes of the mammary glands of the offspring were studied on postnatal day 45. Further, mammary organoids from untreated mice were cultured under branching induction conditions and exposed for 9 days to BP3 (1×10^{-6} M, 1×10^{-9} M, or 1×10^{-12} M with 0.01% ethanol as control) to evaluate the branching progression. Mice that were exposed to BP3 *in utero* showed decreased mRNA levels of progesterone receptor (PR) and WNT4. However, estradiol and progesterone serum levels, mammary histomorphology, proliferation, and protein expression of estrogen receptor alpha (ESR1) and PR were not significantly altered. Interestingly, direct exposure to BP3 *in vitro* also decreased the mRNA levels of PR, RANKL, and amphiregulin without affecting the branching progression. Most effects were found after exposure to 50 mg/kg/day or 1×10^{-6} M of BP3, both related to sunscreen application in humans. In conclusion, exposure to BP3 does not impair mammary branching morphogenesis in our models. However, BP3 affects PR transcriptional expression and its downstream mediators, suggesting that exposure to BP3 might affect other developmental stages of the mammary gland.

Keywords: BRANCHING MORPHOGENESIS; MAMMARY GLAND; BENZOPHENONE-3; 3D CULTURE; PROGESTERONE RECEPTOR; HORMONAL REGULATION.

1. Introduction

Mammary gland development begins during embryogenesis and continues throughout postnatal life, when important transformations involving proliferation, apoptosis, and tissue remodeling occur (Brisken and Scabia, 2020). In mice, the milk lines appear at embryonic stage 10.5, and by birth, they end up in five pairs of mammary glands comprising a rudimentary tree-like structure until puberty. They start to grow allometrically when the ovaries trigger the sexual steroid hormones estrogens (E2) and progesterone (P4) (Dawson and Visvader, 2021). Terminal end buds (TEBs), highly proliferative bulb-shaped structures, emerge from the tip of the ducts, invade the mammary fat pad and lead the ductal outgrowth mainly by elongation and bifurcation of the subtended ducts (Brisken and Scabia, 2020; Ferreira Slepicka et al., 2021; Paine and Lewis, 2017). Both, E2 and P4 signaling pathways induce amphiregulin (AREG) expression (Aupperlee et al., 2013) and act synergistically with insulin-like growth factor 1 (IGF-1) for TEB formation (Ruan et al., 2005; Ruan and Kleinberg, 1999). In cycling mice, P4 induces lateral-side branching, making the ductal tree a more complex structure (Brisken and Scabia, 2020). The P4 receptor (PR) expression, which is induced by E2 signaling, is extensively and homogenously expressed in the luminal epithelial cells of mammary ducts and in the body cells of TEBs during puberty (Ismail et al., 2002; Seagroves et al., 2000). In addition to influencing AREG expression, PR exerts its action in the mammary gland via the induction of its paracrine downstream effectors RANKL and WNT4 (Brisken and Scabia, 2020) and through RANKL-induction of ELF5 (Lee et al., 2013). Each of these molecules exerts a different PR-mediated response in the mammary gland. In puberty, RANKL-induced proliferation is necessary for lateral-side branching, and WNT4 is required for the self-renewal activity of mammary stem cells (Brisken and Scabia, 2020). Whereas ELF5 is essential for

mammary epithelial progenitor cells' differentiation towards a secretory phenotype to promote alveolar development (Lee et al., 2013).

Mammary gland development during early life is vulnerable to exposure to endocrine-disrupting chemicals (EDCs). These environmental pollutants interfere with hormonal activity or processes, which may impact lactation or increase susceptibility to subsequent diseases, including cancer (Fenton, 2006; Gore et al., 2015; Terry et al., 2019). Benzophenone-3 (BP3) is an organic UV-filter widely used in personal care products and widespread in the environment (Huang et al., 2021; Mustieles et al., 2023). BP3 has been suggested to be an EDC with estrogenic and anti-estrogenic activity (Kunz and Fent, 2006; Schlecht et al., 2004), anti-androgenic action, and anti-progestogenic effects (Schreurs et al., 2005). The rodent mammary gland has been identified as a target organ for BP3 exposure. In mice, orally exposed dams during pregnancy and lactation presented long-term alterations in the mammary gland (LaPlante et al., 2018). Also, the mammary gland of the progeny born to orally exposed dams was affected in the peripubertal period and in adulthood (Matouskova et al., 2022a, 2020). Previously, we have shown that direct exposure to BP3 modifies mammary gland hormonal regulation and, in consequence, milk proteins expression and its transcriptional regulation during mammary differentiation *in vitro* (Altamirano et al., 2020). Although several studies have addressed the effects of UV-filters after oral exposure, the main route of exposure in humans occurs through the dermal application of personal care products, mainly sunscreens and cosmetics (Krause et al., 2012). To date, there is no information on whether dermal exposure to BP3 during gestation alters the development and hormonal regulation of the mammary gland. In the present study, our aim was to evaluate whether intrauterine exposure to BP3 modifies mammary branching morphogenesis in female mice after birth. Pregnant dams were exposed

dermally to BP3, and the mammary gland of the female offspring was assessed during peripuberty. Furthermore, to identify the direct effects of BP3 on mammary ductal morphogenesis, a 3D-culture model that accurately recapitulates the *in vivo* conditions of branching morphogenesis was used (Gray et al., 2010; Lo et al., 2012).

2. Materials and methods

2.1. Animals

Female C57BL/6 or BALB/cCmedc kept in the animal facility of the Instituto de Salud y Ambiente del Litoral (ISAL; CONICET-UNL), were maintained in a controlled environment (20 ± 2°C; 14 h of light) and had free access to tap water and pellet laboratory chow (16–014,007 Rat-Mouse diet, Nutrición Animal, Santa Fe, Argentina). All the experimental protocols were approved by the Ethical Committee of the Facultad de Bioquímica y Ciencias Biológicas of the Universidad Nacional del Litoral (FBCB-UNL), Santa Fe, Argentina (Protocol #CE2019-36; 10/21/2019). All laboratory work involving animals was conducted in full compliance with the principles and procedures of the National Institutes of Health Guidelines for the use of laboratory rodents (2011). Additional exposure to compounds with endocrine activity was minimized by housing animals in stainless steel cages with sterile pine wood shavings as bedding and glass bottles with rubber stoppers to supply drinking water (Altamirano et al., 2020, 2015). For more information regarding food composition, see Altamirano et al. (2015).

2.2. Experimental procedures and window of exposure

2.2.1 Mammary gland pubertal development after intrauterine exposure to BP3

8-week-old C57BL/6 dams (F0) were mated with males of proven fertility. The morning of the vaginal plug was assigned gestation day (GD) 0.5. Body weight (bw) gain on

GD7.5 was determined to confirm pregnancy, according to Heyne et al. (2015). Pregnant dams were housed alone, shaved on the back (Philips MG3730/15, Argentina), and randomly assigned to a treatment group. From GD8.5 to GD18.5 dams were dermally exposed to daily vehicle (control, sesame oil, Sol Azteca, Argentina) or BP3 treatment as follows; 0.15 mg BP3/kg bw/day (0.15-BP3); or 50 mg BP3/kg bw/day (50-BP3). The final volume of BP3 solutions had <10% of ethanol (BP3; 98% purity, Sigma-Aldrich, Argentina; CAS#131-57-7). A different cage per treatment group was used to let each animal rest until complete BP3 absorption. Animal bw was recorded daily to adjust the volume applied to maintain the dose constant along the experiment. The 0.15-BP3 dose was equivalent to BP3 levels detected in human breast milk samples (Molins-Delgado et al., 2018), and the 50-BP3 dose was representative of the exposure of BP3 after a whole-body sunscreen application (Janjua et al., 2008). The day of exposure initiation was chosen so as not to interfere with the implantation process and before mammary glands start to develop on embryonic day 10.5. To minimize animal stress, delivery was confirmed after cage inspection for the presence of pups on GD19.5, avoiding unnecessary manipulation. The day after birth was assigned as lactation day 1 (L1), and litter size and sex ratio were determined on L4. Litters were weighed every four days from L4 until weaning on L22. Then, females were separated from their male littermates, which were used for another experiment, and the day of vaginal opening was recorded. After CO_2 inhalation, animals were euthanized at diestrus (determined by vaginal smear) on postnatal day 45 (PND45; PND42-50). Trunk blood was collected, and serum was obtained for posterior E2 and P4 level assessment. Mastectomy was performed aseptically on both abdominal mammary gland chains. Fifth mammary gland pair was snap-frozen on liquid N_2 and stored at -80°C for real-time RT-PCR (qRT-PCR). The right fourth mammary gland was fixed in 10% (v/v)

buffered formalin for 6 h at room temperature, processed, and embedded in paraffin for histological examination and immunohistochemistry assays (Altamirano et al., 2018, 2017; Kass et al., 2015). The left contralateral gland was biopsied and whole-mounted, as described in Kass et al. (2015). Mammary gland parameters were measured on only one F1 female per litter, except for one litter in each treatment group where two F1 animals were included. Parameters measured on these two siblings were averaged to obtain a single value for their litter.

2.2.2 Mammary gland organoids directly exposed to BP3

A 3D-culture model was used to analyze the direct effect of BP3 on the branching morphogenesis of the mammary gland. Primary mammary organoids were prepared according to published procedures (Altamirano et al., 2020; Lo et al., 2012). Briefly, mammary gland pairs N° 3, 4, and 5 were aseptically removed from 8-week-old, untreated BALB/cCmedc female mice and mechanically and enzymatically digested. Organoid clusters were separated by differential centrifugation and then cultured embedded on an extracellular matrix (ECM) made of a mixture of 50:50 Geltrex® (Invitrogen, Argentina) and Collagen I (BD Biosciences, Argentina). The mixture was supplemented with 0.1% insulin-transferrin-selenium (Gibco, USA), 1% penicillin-streptomycin (Gibco), 50 μg/mL gentamicine (Northia, Argentina), and 5% charcoal-stripped fetal bovine serum (Gibco) at 37° C and 5% CO_2. After 24 h, the medium from each well was replaced with the branching medium supplemented with 2.5 nM fibroblastic growth factor-2 (FGF2; Gibco). During branching induction, mammary organoids were exposed to vehicle (0.01% ethanol); 1×10^{-6} M, 1×10^{-9} M, or 1×10^{-12} M of BP3 (Sigma-Aldrich) for 9 days, changing the medium every 48 h. Exposure was evaluated in duplicate wells. BP3 concentrations used were chosen according to the

predicted no effect concentration (1.32 μg/L or 5.8×10^{-9} M) (Kim and Choi, 2014) and plasma concentration of BP3 after repeated whole-body topical application of sunscreens in humans (200 μg/L or 0.9×10^{-6} M) (Janjua et al., 2008). Furthermore, we have previously demonstrated that both of these BP3 concentrations impair milk protein expression and their transcriptional regulation *in vitro* (Altamirano et al., 2020). Concentrations of stock solutions of BP3 in absolute ethanol were diluted in branching medium. The final ethanol concentration in the medium was less than 0.01% (v/v). Representative images of treatment groups were acquired each experimental day with a Spot Insight V3.5 color video camera attached to an Olympus CK40 inverted phase contrast microscopy (Olympus Optical Co., Ltd., Japan) for posterior branching morphology analysis.

2.3 Mammary organoids branching morphological score

A semiquantitative score was utilized to rank the mammary organoids morphology, considering their branching status progression along the experiment. Considering the sequential steps of branching induction and progression of mammary organoids in 3D-cultures (Ewald et al., 2008; Gray et al., 2010; Nguyen-Ngoc et al., 2015), the criteria defined were as follows: grade 1 was assigned to cyst-shaped organoids (a simple layer of epithelial cells enclosing a central lumen); grade 2 organoids were compact bundles of epithelial cells, with or without budding initiation (buds sprouting from the center of the organoid); grade 3 organoids had elongating, rounded buds; and grade 4 organoids presented epithelial repolarization in the central lumen or branches. Three evaluators assessed each organoid blinded to the treatment group and experimental day.

2.4. Estradiol and Progesterone serum levels

Given the small blood volume of mice and, therefore, the amount of serum obtained per animal, sera from 4-6 animals from the same experimental group were pooled. Hormone analysis was performed by electrochemiluminescence assay (ECLIA-Roche, Argentina) after diethyl ether extraction, as described by Portelinha et al. (2015). The lower limits of detection were 5 pg/mL for E2 and 0.05 ng/mL for P4.

2.4. Mammary gland whole-mount analysis

As previously described (Altamirano et al., 2018, 2017), images of mammary gland whole-mounts stained with carmine alum were recorded using a Spot Insight V3.5 color video camera attached to a Stemi 305 stereomicroscope (ZEISS, Argentina). All images were analyzed using ImageJ software (NIH, USA; imagej.net/ij/index.html), and evaluations were carried out without knowing the treatment group. Mammary gland parameters measured were glandular area and perimeter, ductal elongation, epithelial fraction area, and number and size of TEBs (bulbous structures located at the tips of the ducts and with a surface area of at least 0.03 mm^2) (Altamirano et al., 2018, 2017; Kass et al., 2015; Matouskova et al., 2022b).

2.5. Histologic examination and immune cells quantification

Mammary gland paraffin sections (5 µm) were stained with Mayer's Hematoxylin & Eosin for histoarchitecture inspection (Kass et al., 2001). Mast cells and eosinophils associated with mammary ducts were identified with Alcian-Blue/Safranin (AB/S) (Purnell et al., 1974) or alkalyne Sirius red stain (Luque et al., 1996), respectively. For degranulation activity identification, mast cells with stained granules outside of the cells or with cytoplasmic "holes" (blank spaces) were taken into account (Varayoud et al., 2004). Mast cells in the connective tissue of the stroma or associated with vessels were

not taken into account (Lilla and Werb, 2010). Results were expressed as the number of mast cell types or eosinophils per duct. Slide evaluation was performed using Olympus BH2 light microscopy (Olympus Optical Co., Ltd., Japan).

2.6. Cell proliferation and steroid hormone receptors expression

The proliferative index (Ki67) and the expression of estrogen receptor alpha (ESR1) and PR were evaluated in paraffin sections by immunohistochemistry as previously described (Altamirano et al., 2018; Kass et al., 2015). Sections were incubated overnight at 4 °C with primary antibodies against Ki67 (1:1500) (Gomez et al., 2020), ESR1 (1:50; clone 6-F11, Novocastra Laboratories Ltd., UK), and total PR (PR-A and PR-B isoforms; 1:200; clone A0098, Dako Corp., Carpinteria, CA, USA). Anti-rabbit or anti-mouse secondary antibodies (biotin-conjugated) were used. Ki67 and secondary antibodies were from ISAL (UNL-CONICET, Argentina). Reactions were developed using an avidin-biotin peroxidase method with diaminobenzidine (Sigma-Aldrich) as a chromogen substrate. Ki67-, ESR1-, and PR-positive cells were counted on epithelial structures with Olympus BH2 light microscopy (Olympus Optical Co., Ltd., Japan) and expressed as percentages of total cells. All mammary structures per tissue section were analyzed.

2.7. qRT-PCR

The qRT-PCR assay was carried out as previously described (Altamirano et al., 2020). Briefly, mammary glands or organoids from each experimental group were individually homogenized in TRIzol reagent (Invitrogen), and RNA was prepared according to the manufacturer's protocol. Then, equal quantities of total RNA were reverse-transcribed into cDNA. The primer sequences and reaction conditions used for PCR are shown in

Table 1. cDNA levels were detected using a real-time PCR system StepOne Cycler (Applied Biosystems Inc., Life Technologies, USA). Product purity was confirmed by dissociation curves, and random samples were subjected to agarose gel electrophoresis. Calculation of the relative expression level of each target was conducted using REST-MCS© software (gene-quantification.de/rest-mcs.html; (Pfaffl et al., 2002; Pfaffl, 2001). L19 and the control group were used to normalize the CT values. No significant differences in CT values were observed in L19 between the different experimental groups.

2.8 Statistical analysis

Normal distribution of values was evaluated by the Shapiro-Wilk test. Results were expressed as the mean ± SEM. One-way ANOVA, followed by Dunn's post-test, was used for parametric analysis. Conversely, for non-parametric analysis, Kruskal-Wallis, followed by Dunn's post-test, was used. Analysis of the relative expression of target genes was performed by the Pair Wise Fixed Reallocation Randomization Test©, incorporated in the REST-MCS© software (Pfaffl et al., 2002), setting 9000 randomizations per analysis. BP3 treatment groups were compared against the control group or vehicle. In all cases, $p<0.05$ was considered significant.

3. Results

3.1 Reproductive parameters and steroid hormone levels are not affected by exposure to BP3

As observed in Table 2, exposure to BP3 did not affect: a) the biometric and reproductive parameters of the dams (mean body weight during gestation and lactation, and length of gestation); b) the number of pups per litter and their sex ratio; c) the

timing of vaginal opening of female pups; and d) their body weight on L4, at weaning, on vaginal opening day, or at PND45. Furthermore, the serum E2 and P4 levels at PND45 in the female F1 offspring were not different between experimental groups (Table 2).

3.2 Mammary gland ductal outgrowth and histoarchitecture at PND45 are not modified by intrauterine exposure to BP3

Regarding the mammary gland ductal outgrowth in the control group, mammary ducts elongated beyond the central lymph node, and TEBs were still present (Fig. 1A). There were no differences in ductal outgrowth and morphometric parameters evaluated of BP3-intrauterine-exposed females compared to that of controls (Fig. 1A and Table 3). The histoarchitecture was also conserved among experimental groups, presenting both bilayered ducts and multilayered TEBs immersed in the stromal compartment (Fig. 1B). Regarding the immune cells, the population of mast cells and eosinophils associated with ducts and TEBs was analyzed. Connective tissue mast cells (CTMC, S-positive), mucosal mast cells (MMC, AB-positive) (Fig. 1C), and degranulated mast cells, were identified in the mammary gland, but there were no differences in the amount of them associated with epithelial structures (Fig. 1D and E). Similarly, there were no differences in the number of eosinophils between experimental groups (Figure 1F, G and H).

3.3 Intrauterine exposure to BP3 modifies PR and WNT4 mRNA expression ratio without altering the epithelial proliferation index and ESR1 expression

Most mammary ducts had a low proliferative index in all experimental groups (Fig. 2A). There were no differences in the percentage of epithelial cells positive for Ki67

(Fig. 2B), ESR1 (Fig. 2A and B), or its mRNA expression ratio (Fig. 2C) between groups.

Gestational exposure to BP3 produced no changes in the pattern of PR protein expression in epithelial cells (Fig. 3A) or in the proportion of PR-positive cells (Fig. 3B). However, PR mRNA expression was decreased in both BP3-exposed groups (Fig. 3C; $p<0.05$). Furthermore, the mRNA expression of WNT4 was also decreased in the 50-BP3 group in comparison to the control group ($p<0.05$). Yet, there were no differences in the mRNA expression of RANKL, ELF5, IGF-1 and AREG between experimental groups (Fig. 3C).

3.4 Direct exposure to BP3 does not affect the branching morphogenesis progression of mammary organoids but impairs PR signaling pathway

A 3D-culture model was used to distinguish the direct effects of BP3 on the mammary gland during branching progression and whether they resemble the effects of the intrauterine exposure seen *in vivo*. No cyst-shaped organoids (grade 1) were seen by day 2 in any treatment condition. This demonstrated a successful branching induction by FGF2 in our 3D-culture model with a mixture of ECM components.

Mammary organoids exposed to the vehicle progressed from simple clusters of epithelial cells with budding initiation on day 2, to more complex, multiple-branched structures by days 5 and 7, and with repolarization of the epithelial layers on day 9 (Fig. 4A). The branching induction in BP3-directly exposed organoids progressed similarly to the vehicle-exposed ones, and no statistical difference in the branching score was seen between treatment groups (Fig. 4B).

At the end of the experiment on day 9, the mRNA expression of steroid hormone receptors (ESR1 and PR), PR signaling molecules (WNT4, RANKL, ELF5, and AREG)

and IGF-1 was evaluated (Fig. 5). ESR1 mRNA expression was similar between the experimental groups, whereas PR mRNA expression was reduced in the three BP3 concentrations tested ($p<0.05$). In addition, RANKL and AREG mRNA expression was also similarly decreased by BP3 at 1×10^{-9} M and 1×10^{-6} M concentrations ($p<0.05$). In contrast, mRNA expression of IGF-1 was increased only in 1×10^{-12} M of BP3 ($p<0.05$), and there were no changes in the expression of WNT4 and ELF5.

4. Discussion

During the pubertal development of the female mouse mammary gland, the gestational exposure to BP3 decreased the mRNA expression of PR and of its downstream effector, WNT4. Moreover, direct exposure to BP3 during the branching induction of mammary organoids also decreased the mRNA expression of PR and of its downstream target genes RANKL and AREG. However, mammary gland branching morphogenesis was unaffected by either gestational or direct exposure to BP3. Therefore, our results showed that exposure to BP3 induced subtle modifications in the hormonal regulation of the mammary gland without affecting its histoarchitecture, tissue organization, or outgrowth at the analyzed endpoints.

In this study, dams were dermally exposed to BP3 during pregnancy to analyze the mammary gland of their offspring during peripubertal development. This approach resembles the human use of sunscreens and cosmetics (Krause et al., 2012). It has been previously demonstrated that after its dermal application, BP3 is detected in plasma and amniotic fluid in pregnant dams (Santamaria et al., 2020). Using the same BP3 dose of 50 mg/kg bw/day as Santamaria et al. (2020), which elicites a BP3 concentration of 22.4±2.3 ng/mL in serum and 22.6±10.8ng/mL in amniotic fluid, the mRNA expression of PR and WNT4 was affected in the mammary gland of peripubertal mice. This dose is

similar to the lowest value detected in human serum (28–392 ng/mL) after a whole-body application of a sunscreen lotion containing 10% (w/w) of BP3 (Janjua et al., 2008). Recently, the European Union (EU) reduced the permitted BP3 content in sunscreens to 2.2% for products applied to the body (EU, 2022). Therefore, applying sunscreen to the whole body after this regulation may result in even lower BP3 plasma levels than previously reported (Janjua et al., 2008; Krause et al., 2018; Matta et al. 2020). Dermal exposure to BP3 during pregnancy did not cause any alteration in dams' weight gain, litter size and sex ratio or nursing. Furthermore, the growth and development of female offspring exposed *in utero* to BP3 were similar to controls, with no change in the day of vaginal opening or serum E2 and P4 levels at the endpoint evaluated. In addition, immune cell infiltration, epithelial proliferative status, ESR1 and PR protein expression, and ESR1 mRNA expression in the mammary gland were comparable to the control animals. Finally, mammary ductal outgrowth during branching morphogenesis was also unaffected. In contrast to our results, other groups reported increased body weight and modest alterations in the morphology of the mammary gland in female mice perinatally exposed to BP3. More concretely, they observed increased ductal extension and number of TEBs, decreased epithelial PR-positive cells, and a transient increment in the infiltration of mast cells near mammary ducts (Matouskova et al., 2022a, 2020). The discrepancies between these results and ours may be explained by the different experimental approaches used: mouse strains (Balb/C vs C57BL/6), exposure period (gestation and lactation vs gestation), BP3 doses and administration route in dams (µg/kg bw/day vs mg/kg bw/day, oral vs dermally), and endpoint period evaluated (PND32-35 vs PND45). In F1 offspring, the impact of gestational exposure to BP3 on mammary gland development during pregnancy or on

mammary gland function and milk quality during lactation was not addressed in our or in the mentioned study.

In mice, PR promoter activity and protein expression during puberty, which is induced by E2 signaling, are restricted to the body cells of TEBs and most epithelial cells in the inner layer of ducts, whereas in adulthood, PR expression is attenuated by the combined action of both E2 and P4 (Ismail et al., 2002; Seagroves et al., 2000). Although in this study the steroid hormone serum levels, the pattern of epithelial PR expression, and the proportion of PR-positive cells were unchanged in BP3-exposed mice, the lower levels of PR mRNA expression would suggest that PR transcription in the mammary gland could be a target of gestational exposure to BP3. It is possible that BP3 would be acting on PR transcriptional regulation through other molecular mechanisms that are not directly driven by P4 or E2 levels. Post-translational modifications regulate PR transcriptional activity and degradation (Abdel-Hafiz and Horwitz, 2014). Cho et al. (1994) reported increased levels of PR mRNA but not PR protein in MCF7 cells treated with insulin or IGF-1, and increased levels of both PR mRNA and protein by stimulation with cyclic adenosine monophosphate (cAMP). In addition, BP3 is reported to induce expression of the phosphodiesterase 4B, which in turn decreases the activation of cAMP-dependent transcription factors like cAMP-response element binding protein in normal human keratinocytes (Kim et al., 2018). Therefore, BP3 could impair the cAMP cascade modifying the balance between PR transcript expression and translation in mammary epithelial cells. To better understand how BP3 affects the mammary gland, further studies should investigate the interplay between protein kinases and phosphodiesterases, as well as the activity of these enzymes. Additionally, the cAMP status and phosphorylation cascade in response to growth factors in mammary epithelial cells should also be evaluated.

After gestational exposure to BP3, changes in PR expression were accompanied only by a decreased expression of WNT4. Gestational exposure to bisphenol A (BPA, another EDC) has also been shown to modify PR and WNT4 expression in the mammary gland of peri-pubertal mice (Markey et al., 2001; Muñoz-de-Toro et al., 2005). However, in contrast to the results found herein, BPA induces epithelial protein expression of PR and mRNA expression of WNT4, with alterations in adulthood like enhanced lateral-side branching and alveolar budding in virgin animals (Markey et al., 2001; Muñoz-de-Toro et al., 2005). Our results suggest that gestational exposure to BP3, besides impairing PR transcripts, may differentially affect PR target genes as well. Pubertal expression of WNT4 is involved in controlling mammary stem cell niches and the regeneration potential of the mammary epithelium through canonical WNT signaling in the myoepithelium, which induces protease-driven ECM changes important in mammary tissue remodeling (Rajaram et al., 2015). Although the pubertal branching morphogenesis of the mammary gland was not affected by BP3 in this study, the decreased mRNA expression of WNT4 could be an early sign of major mammary developmental alterations during pregnancy and lactation. This calls for follow-up studies analyzing mammary gland functionality in females that have been exposed intrauterine to BP3.

In 3D cultures of mammary organoids embedded in a commercial ECM, growth factors like FGF2 promote the initiation and elongation of new ducts (Ewald et al., 2008; Huebner et al., 2016). Mammary organoids progressively branched to form complex structures presenting elongating, rounded buds, and exposure to BP3 resulted in a morphological pattern similar to that of the vehicle. As in the intrauterine exposure to BP3, PR mRNA expression was directly affected by BP3, with a lower expression than the vehicle. Under the hormone-deprived conditions assessed in the *in vitro* experiment,

BP3 action on PR expression could be either a direct effect on PR or through ESR1-induced PR expression. In this regard, it has been shown in a gene reporter assay that BP3 repressed PR transcription at a similar concentration used in this study (5.2×10^{-6} M) (Schreurs et al., 2005). However, when evaluating the direct effects of BP3 on mammary organoids during lactational differentiation, BP3 (1×10^{-12} M) increased both the mRNA expression of PR and ESR1 (Altamirano et al., 2020). In this experiment, no tested BP3 concentration modified ESR1 mRNA expression during branching morphogenesis *in vitro*. Thus, the specific actions of direct BP3 exposure would depend not only on the concentrations used but also on the experimental conditions and the mammary gland developmental endpoints evaluated. Hence, the exact mechanism of action of BP3 on the steroid hormone receptors expression of mammary epithelial cells remains unclear. In addition, it has been shown that 3D cultures of mice mammary epithelial cells retain P4 induction of target genes in a PR-dependent manner (Jardé et al., 2016; Obr et al., 2013; Santos et al., 2009), whereas E2 treatment downregulates RANKL and WNT4 (Jardé et al., 2016). Here, exposure to BP3 increased the mRNA expression of IGF-1 only at the lowest concentration tested. However, BP3 exposure decreased the mRNA expression of RANKL and AREG, but not WNT4 or ELF5. PR-induced RANKL expression is predominantly controlled at the mRNA maturation/stability level rather than at the transcriptional level (Tanos et al., 2013). Therefore, it is tempting to hypothesize that BP3 impairs PR transcriptional activity during branching morphogenesis. Compared to other mouse strains, such as BALB/c, C57BL/6 mice have higher basal expression of WNT4 and are less responsive to P4 than to E2 stimulation (Aupperlee et al., 2009). In addition, P4 stimulation alone is not sufficient to induce RANKL expression (Aupperlee et al., 2013, 2009). However, E2, P4 or a combination of both can induce AREG expression in either C57BL/6 or

BALB/c mice (Aupperlee et al., 2013). The difference in the effect of BP3 on RANKL and WNT4 expression between our *in vitro* and *in vivo* models may be due to differences in mouse strains.

5. Conclusion

Exposure to BP3 subtly impaired the hormonal regulation of key molecular pathways that are involved in the development of the mammary gland both *in vivo* and *in vitro*. Particularly, PR transcriptional expression and activity would be targets of BP3 exposure on the mammary gland during branching morphogenesis. However, these changes did not temporarily correlate with modifications in mammary ductal outgrowth or branching morphogenesis.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Gonzalo Schierano-Marotti: Investigation, Formal analysis, Data curation, Validation, Visualization, Writing – original draft, Writing – review & editing. **Gabriela A. Altamirano:** Conceptualization, Funding acquisition, Project administration, Resources, Methodology, Investigation, Validation, Writing – original draft, Writing – review & editing. **Sofia Oddi:** Investigation, Validation, Visualization. **Ayelen L. Gomez:** Investigation, Validation, Visualization. **Nicole Meyer:** Writing – review & editing. **Mónica Muñoz-de-Toro:** Visualization. **Ana C. Zenclussen:** Writing – review & editing. **Horacio A. Rodríguez:** Resources, Writing – review & editing. **Laura Kass:** Conceptualization, Funding acquisition, Project administration, Resources, Methodology, Investigation, Validation, Writing – original draft, Writing – review & editing.

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Data Availability

Data will be made available upon request.

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