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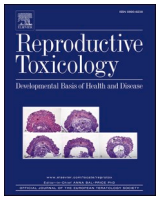

Exposure to brominated flame retardants during pregnancy and lactation increases the prevalence of breast lesions and cancer-associated pathways in sprague-dawley rats

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ABSTRACT

The mammary gland undergoes significant changes during pregnancy, lactation, and involution, making it highly susceptible to endocrine-disrupting chemicals such as brominated flame retardants (BFRs). Despite being restricted in many countries, some BFRs persist in the environment and accumulate in human tissues, including the mammary gland and human milk. This study investigates the effects of BFRs exposure during pregnancy and lactation on mammary gland development and breast cancer risk in a rat model. Dams were exposed to a mixture of polybrominated diphenyl ethers (PBDEs) and hexabromocyclododecane (HBCDD), formulated based on relative congener levels found in house dust. Post-weaning, dams were treated with 7,12-dimethylbenz[*a*] anthracene (DMBA) to initiate tumor formation. The results revealed that both low and high doses of BFRs induced lesions in mammary epithelium, with an increase in total lesion number in low dose. Molecular analysis revealed disruptions in the Wnt/β-catenin signaling pathway, leading to an increase in oncogene expression, including c-Myc and c-Jun. RNA sequencing also indicated dysregulation in calcium signaling and glucose metabolism pathways. Our findings suggest that BFR exposure during the critical window of mammary gland involution compromises the cancer-protective effects of pregnancy and lactation. These effects are particularly significant at low exposure levels, demonstrating a non-monotonic dose-response. The study underscores the potential long-term health risks associated with environmental BFR exposure and highlights the need for further research on its implications on the risks of developing breast cancer later in life.

1. Introduction

The mammary gland is a highly dynamic organ that undergoes significant structural and functional changes throughout a woman's reproductive life. These extensive changes in histomorphology and function are particularly noticeable throughout prenatal, peripubertal, pregnancy, and involution phases [1,2]. Such changes are primarily driven by hormonal signals, primarily estrogen, progesterone, prolactin, and oxytocin, and involve intricate processes like the expansion of the ductal system, differentiation of epithelial cells, alveologenesis, and apoptosis [1]. These phases of mammary gland remodeling are thus considered windows of sensitivity to endocrine-disrupting chemicals (EDCs), which can disrupt normal development and increase the risk of diseases like breast cancer [3–6].

Pregnancy promotes extensive mammary gland growth and the proliferation of mammary epithelial cells, which form milk-secreting structures named acini [7]. After parturition, new hormonal signals enhance mammary gland blood supply and drive the differentiation of mammary epithelial cells to transform acini into alveoli that synthesize and secrete milk components [8]. Finally, after weaning, the involution process begins, and it involves apoptosis of milk-producing cells, remodeling of the glandular tissue and regression of the ductal system [9]. This process is important to return to its non-lactating state.

It has been shown that a full-term pregnancy, as well as longer breast-feeding period, can reduce the risk of developing breast cancer [10,11]. It is thought that parity and breast-feeding period protect against malignant transformation by favoring the reduction of estrogen and progesterone cellular response, generation of a healthy immune

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response, completion of stem cell terminal differentiation, and elimination of harmful mutations during mammary involution [1,12,13]. Paradoxically, pregnancy is also associated with a short-term transient higher risk of breast cancer [14,15]. Pregnancy-associated breast cancer (PABC) is generally defined as breast cancer diagnosed during pregnancy or within 5 years postpartum [15,16]. PABC is an aggressive breast cancer subtype associated with poor prognosis and remains an under-studied type of breast cancer. One of the possible mechanisms associated with PABC is that the change in hormonal status associated with pregnancy favor the growth of cells, including cells containing oncogenic mutations within the breast [15,17]. Interestingly, it has been demonstrated that, compared to non-PABC, PABC have enhanced expression of genes associated with immune responses, cell cycle regulation, metabolism, and aggressive features. [18].

Brominated Flame retardants (BFRs) are molecules considered as EDCs that have been added to consumer products in recent decades to reduce flammability and flame spread during fires [19]. The most widely used brominated flame retardants additives in North America were mixtures of polybrominated diphenyl ethers (PBDEs) and hexabromocyclododecanes (HBCDD) [20,21]. Since BFRs are mainly additives, they do not form covalent bonds with the objects to which they are added, making them susceptible to release into the environment. Thus, humans are mainly exposed via inhalation and dust ingestion [22]. As well as being present in our environment, they are persistent due to their physico-chemical properties and are bioaccumulable [23]. Quantities of BFRs have been reported from diverse human tissues, including the mammary gland and human milk [24]. Although their persistence and bioaccumulative nature has led to their removal from commerce globally, they remain in many indoor environments leading to continued human exposure [25].

Our previous studies have shown that exposure during pregnancy and lactation to an environmentally-relevant mixture of HBCDD and PBDEs (DE-71, DE-79, decaBDE-209) disrupts junctional proteins in the mammary gland of rats [26–29]. Specifically, levels of phospho-β-catenin and E-cadherin were down-regulated via a mechanism probably linked to the transcription factor CREB and PKA [28]. Such dysregulations have been associated with increased cancer risk [30–32]. We thus hypothesize that an exposure to a relevant mixture of HBCDD and PBDEs during pregnancy and lactation can dysregulate the remodeling associated with pregnancy, lactation or involution, thereby reducing the cancer protective effects of parity and lactation and increase the risk of breast cancer. Although HBCDD and PBDEs are distinct chemicals, our study aimed to replicate human exposure scenarios by focusing on the mixture of these compounds as they were used and found together in house dust [33,34].

Our findings suggest that HBCDD and PBDEs exposure induces a shift of β-catenin from an adherens junction molecule to a transcription factor inducing oncogenes such as c-Myc and c-Jun following a 7,12-dimethylbenz[*a*]anthracene (DMBA) hit. Moreover, RNAseq data indicates a dysregulation in calcium and glucose metabolism following BFR exposure. Our results demonstrate that an environmentally-relevant exposure to HBCDD and PBDEs during the gestation-lactation-involution phase induces changes in the mammary epithelium suggesting a precancerous state.

2. Materials and methods

2.1. Animals

Virgin female Sprague-Dawley rats (aged 6–7 weeks) were obtained from Charles River (St-Constant, Quebec, Canada). The animals were housed individually and handled in compliance with the Canadian Council on Animal Care guidelines. Experimental protocols were approved by the Institutional Committee for Animal Protection of the Laboratoire National de Biologie Expérimentale at the Institut National de la Recherche Scientifique (protocol no. 1909–02). Rats were randomly assigned to experimental groups, with BFRs exposure beginning 1–2 weeks prior to mating. Estrous cycles were monitored using impedance, and females in the proestrus stage (impedance < 3 Kohm) were mated overnight. After mating, dams were returned to their diet supplemented with BFRs (or control) during pregnancy and lactation. The diet was formulated to deliver 0, 0.06, or 60 mg/kg/day of BFR mixture, replicating human exposure levels. Litter sizes were normalized on postnatal day 4, and weaning occurred on postnatal day 21. Following weaning, dams were put on normal diet (without BFRs) for the remaining of the experiments. General health of the pregnancies and litters (number of pups, sex ratios, body weight of pups and dams) were not affected by BFRs exposure [29]. Two days post-weaning, dams were treated with 40 mg of 7,12-dimethylbenz[*a*]anthracene (DMBA) (Sigma Aldrich; D3254) to coincide with the start of the non-reversible stage of mammary involution. Animals were monitored daily for health and the mammary glands were palpated once weekly after DMBA exposure to monitor tumour formation.

2.2. BFRs mixture exposure

BFRs mixture formulation and preparation was previously described [26–28,35]. Briefly, the mixture is composed of three technical PBDE (DE-71, DE-79 and BDE 209) and one HBCDD mixture. These PBDE were combined in a mixture to the ratio of median levels observed in Boston house dust [33,34,36]. The BFRs mixture was added into powder isoflavone-free diet (Teklad Global 2019 diet; Harlan Laboratories, Madison, Wisconsin) with 4.3 g/kg of corn oil. Diets were processed to contain 0, 0.75 and 750 mg of BFRs mixture/kg to deliver 0, 0.06 and 60 mg/kg of body weight/day respectively. Our lowest dose is an approximation for maximum human exposure calculated based on children ingestion rate of 100 mg/day, then converted to rat by scaling to the ratio of 1:6.9 (human to rat body surface area) [33,34]. Powdered diets were mixed with BFRs, pelleted and dried in a lab at Health Canada, Ottawa and transferred to color-coded containers to ensure that the animal handling team at INRS were not aware of the BFR content of the diets until all analyses were completed. Diets were stored at 4 °C for no more than one month.

2.3. Tissue harvesting

All animals were euthanized via exsanguination under isoflurane anesthesia 175 days after weaning, based on latency for euthanasia seen in other studies evaluating the effects of xenobiotics during pregnancy and lactation on the development on mammary tumors [37–39]. The thoracic glands were harvested and directly fixed by immersion in 4 % paraformaldehyde for 16–24 hours at room temperature. Tissues were then embedded in paraffin, sectioned (5 μm), and stained with hematoxylin and eosin. Slides where sent to Institute of Research in Immunology and Cancer (IRIC) Histology Core Facility, University of Montreal, to be examined by a pathologist. Left inguinal mammary glands were snap frozen for protein and RNA extraction, whereas right inguinal mammary glands were processed for Whole-mount staining.

2.4. Carmine staining of Whole mammary gland

After harvesting, the right inguinal mammary gland was mounted, fixed and stained as described previously [26,40]. Briefly, the glands were placed on a large microscope slide and compressed beneath a weighted object for 5 minutes to be properly spread and adhered. Glands were then fixed for 2 days in Carnoy's fixative (ethanol, chloroform, glacial acetic acid at the ratio of 6:3:1 respectively) at room temperature. Tissues were washed with 70 % ethanol for 1 h and rehydrated in water for 30 min. A solution of carmine alum stain (2 % carmine and 5 % aluminum potassium sulfate in water) was used for 2 days to color the samples. Finally, glands were dehydrated through a series of ethanol baths (50 %, 70 %, 95 %, 100 %) and cleared in xylene for 2 days. Slides

were placed on a transilluminator (Henning Graphics LR299343), and imaged using a Canon PowerShot G9x digital camera. Lesions in the epithelial tissue were counted blindly. Images were analyzed with ImageJ (ImageJ, U.S. National Institutes of Health, Bethesda, Maryland, https://imagej.net/Downloads) and the Sholl analysis plugin for mammary gland network density [41,42]. Briefly, images were process and modify using the skeletonize tool of ImageJ. The skeletonized images were used to evaluate the branching density using the Sholl analysis plugin. This analysis identifies the total number of intersections (N) per cm^2 by applying a radius filter (step size: 0.025 cm).

2.5. Hematoxylin and Eosin staining

Paraffin embedded tissues were cut with a microtome (5 μm) and stained with hematoxylin and eosin. Briefly, sections were deparaffinized with xylene, followed by rehydration (100 %, 95 %, 70 %, 50 % ethanol baths, water). Samples were stained with Hematoxylin Vintage™ (StatLab; SL100), then acid rinse with HIGH DEF™ (SatLab; SL1003) followed by bluing with Vintage Bluing reagent (StatLab; SL102), finally staining with Eosin-Y Vintage™ (StatLab; SL101). Once stained, samples were dehydrated and cleared in xylene, then mounted using Permount (Fisher Scientific, Nepean, ON, Canada). Slides where sent to Institute of Research in Immunology and Cancer (IRIC) Histology Core Facility to be examined by a pathologist.

2.6. Western Blot

Protein was extracted from snap frozen tissues stored at −80°C. Tissues were first crushed into a powder on dry ice and 100 mg of powder were processed for protein extraction. Cold Triple-detergent (50 mM Tris, 150 mM NaCl, 0.02 % sodium azide, 0.1 % sodium dodecyl sulfate, 1 % Nonidet P40 and 0.5 % deoxycholate adjusted to a pH of 8) supplemented with 1.25 M of sodium fluoride, 1 M of sodium orthovanadate and 1x of protease and phosphatase cocktail inhibitor (Halt Protease and Phosphatase cocktail inhibitor, Fisher Scientific Canada) was use as a lysis detergent and mechanical disruption for proper lysis was done by homogenizing for 45 seconds with a tissue homogenizer (PowerGen 125, Fisher Scientific). Samples were then centrifuged at 13 000 x g for 10 min at 4 °C and supernatant was aliquoted and stored at −80°C. Quantification of protein was done using the Pierce™ bicinchoninic acid (BCA) protein assay reagent kit (ThermoFisher scientific; 23225). Semi-quantitative western blots were performed by loading total protein into TGX Stain-Free™ Acrylamide gels (Bio-Rad, Mississauga, Ontario, Canada). After electrophoresis, gels were transferred onto a PVDF membranes using the Trans-Blot Transfer System (Bio-Rad). Total proteins were visualized using the ChemiDoc MP Imaging System (Bio-Rad) and used for loading normalization. Membranes were then blocked with 0.1 % TBS-tween supplemented with 5 % dry milk or 3 % BSA. Blocked membranes were probed with primary antibodies at 4 °C overnight (Table S1). Membranes were washed with 0.1 % TBS-tween and probed with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (Table S1). The signal was revealed by using Clarity™ Western ECL Blotting Substrate (Bio-Rad) and visualized using the ChemiDoc MP Imaging System (Bio-Rad). Density of each band was normalized to total proteins in their respective lane, following comparison to a pooled sample [43].

2.7. RNA extraction and sequencing

Frozen powdered samples (~100 mg) were homogenized for 45 seconds in lysis buffer (PureZol™ RNA Isolation Reagent, Bio-Rad) followed by centrifugation at 12 000 x g (10 minutes at 4 °C). The pellets were processed for total RNA extraction using the protocol provided by the Aurum™ Total RNA Fatty and Fibrous Tissue Kit (7326830, Bio-Rad). Quantity and quality of the RNA extraction were evaluated prior to sequencing using the RNA 6000 Pico kit (5067–1513, Agilent) and the 2100 Bioanalyzer system (G2939BA, Agilent). Samples with an RNA Integrity Number (RIN) of 8 or higher were sent to the sequencing facility of the Montreal Clinical Research Institute (IRCM). IRCM proceeded to ribo-depletion sequencing using NovaSeq 6000 Sequencing System (Illumina) with a depth of 50 million reads. The quality of the raw reads was assessed with FASTQC v0.11.8 and combined with MultiQC. After examining the quality of the raw reads, trimming was performed with TRIMMOMATIC v0.36. The reads were aligned to the rat reference genome with STAR v2.7.6a with mean of 86 % of reads uniquely mapped. The raw counts were calculated with FeatureCounts v1.6.0 based on the rat reference genome (release Rnor_6.0 - RefSeq GCF_000001895.5). Ensembl genome database was used to assign reads and differential expression was performed using the DESeq2 R package [44] for each experimental group separately while controlling for the effect of the RNA extraction day. p-values were corrected for multiple testing using the Benjamini and Hochberg method. Functional enrichment analysis of DEGs (gene ontology and pathway enrichment) was performed with the gprofiler2 R package [45]. Bioinformatics analysis was performed at the Bioinformatics core facility from IRCM. The complete RNA sequencing data has been posted on the Gene Expression Omnibus Data base (Accession # PRJNA1190463).

2.8. Statistical analysis

The results are expressed as mean ± standard error of the mean (SEM). Statistical significance was evaluated using One-way ANOVA, followed by a Tukey's post-hoc test for multiple comparisons, assuming normality and equal variance. Non-parametric alternatives, such as the Kruskal-Wallis test followed by the Conover test for multiple comparisons, were used when these assumptions were not met. P value 0.05 was considered for determining significance. All statistical analyses were performed using R Studio.

3. Results

3.1. BFR exposure affects mammary epithelium density and induces lesions in DMBA-exposed dams post-involution

We first evaluated the complexity of the mammary epithelium using whole mount staining of the inguinal gland (Fig. 1A). Exposure to the high dose (60 mg BFRs/Kg of body weight/Day) caused a significant reduction in the mammary density (Fig. 1A, B). Moreover, whole mounts revealed the presence of lesions/anomalies within the epithelium network (Fig. 1A, C). The number of lesions were counted by two evaluators – blinded to sample treatment group - who found a higher number of lesions in rats exposed to the low dose of BFRs (0.06 mg BFRs/Kg of body weight/Day) when compared to the control and high dose (Fig. 1C). Lesions were characterized with the help of a pathologist using random H&E-stained thoracic glands sections. Some rats exposed to BFRs, especially to the lower dose, developed ductal hyperplasia and stroma alterations and control animals only showed signs of abundant secretion (Fig S1, Table S2). High dose exposure also resulted in a significant increase in the number of the lesions when compared to control (Fig. 1C). We found that the low dose of BFRs caused lesion in approximatively 85 % of the dams, which was significantly higher than in control (≈31 %) or high dose dams (≈64 %) (Fig. 1D). Of note, discrepancies in the number of rats with anomalies detected with whole mount and with H&E are noticeable, probably due to the area of tissues examined, i.e. the entire gland for whole mount compared with sections of tissues for histology. Nevertheless, in both analyses, the number of rats with lesions was higher in animals treated with the lower dose of BFRs compared with the high dose and the controls. These results suggest that the BFRs treatment affects the formation of DMBA-induced lesions post-involution.

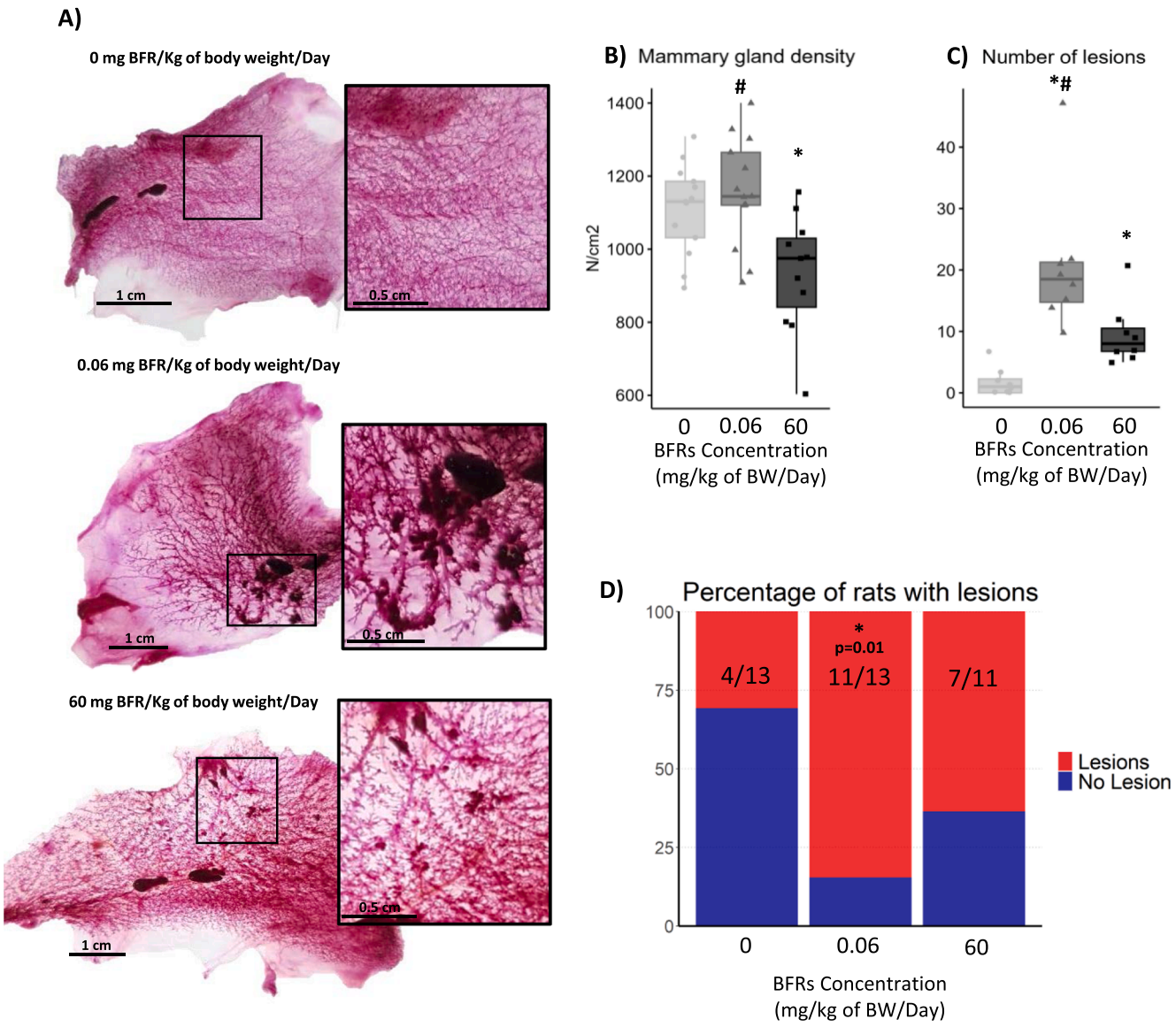

Fig. 1. Exposure to BFRs during pregnancy and lactation resulted in more lesions in the mammary gland of dams exposed to DMBA. (A) Representative images of Whole mount from each group. (B) Exposure to 60 mg BFRs/kg of BW/Day reduced the branching density of the mammary gland ($n \geq 11$);0 vs 60; $p = 0.043$; 0.06 VS 60; $p = 0.021$. (C) Lesions in Whole mounts where blindly counted. Exposure to BFRs caused an augmentation of the number of lesions; 0 VS 0.06; $p = 0.0001$; 0 VS 60; $p = 0.003$; 0.06 VS 60; $p = 0.021$. (D) Higher percentage of dams exposed to the low dose of BFRs (0.06 mg BFR/kg of BW/Day) showed lesions in the mammary gland. All samples were harvested 175 days after weaning of the pups. p-values were calculated with a Kruskal-Wallis (posthoc conover) or ANOVA (posthoc tukey). Graphs (A, B) represent means ± SEM. * = significantly different than control. # = significantly different than High exposure (60).

3.2. Low BFRs exposure resulted in transcriptomic dysregulation of calcium and glycolysis pathways in DMBA-exposed dams

To further understand mechanisms dysregulated by BFR exposure that could explain the increased number of lesions, we used transcriptomic analysis. RNA sequencing provides an untargeted approach to probe the tissue-wide molecular responses to BFR exposure and DNA damage events, such as exposure to DMBA. When comparing low dose exposure to BFRs to the control following DESeq2 analysis (adjusted p-value <0.05, log2FC <1.5), 228 and 3 genes were significantly upregulated and downregulated, respectively (Fig. 2A). Analysis of biological processes demonstrated that exposure to low dose of BFRs resulted in changes mainly related to muscle cells and muscle tissues (Fig. 2B). Further analysis revealed that BFR-associated transcriptional changes were enriched for pathways associated with cytoskeleton and binding (Fig. 2C). Moreover, KEGG pathway analysis supported that these genes are linked with calcium dynamics, glycolysis and gluconeogenesis (Fig. 2D). This suggests that BFR exposure may dysregulate calcium metabolism (Fig S2) leading to upregulation in genes associated with muscles and cytoskeleton (Fig S3). Given that many of the genes and pathways involved in cytoskeletal changes are often associated with cancer-associated fibroblasts [46,47] and the increased prevalence of stromal lesions observed in BFR-treated samples (Fig S1, Table S2), we leveraged the Data Resource of Cancer-Associated Fibroblast to identify dysregulated genes that may be linked to CAFs [48]. By processing 355 significantly dysregulated genes (padj $\leq$ 0.05 and unfiltered for the log2FoldChange) we identify 208 that can be directly linked to CAFs (Table S3).

Fewer genes were significantly dysregulated upon exposure to the higher dose, with 29 upregulated and 0 downregulated (Fig. 2E). The dysregulation of biological process (Fig. 2F), molecular function (Fig. 2G) and KEGG pathway analysis (Fig. 2H), shows little relationship between affected pathways. To note, most of the perceived effects may be linked to oxidative stress. Finally, no genes were significantly

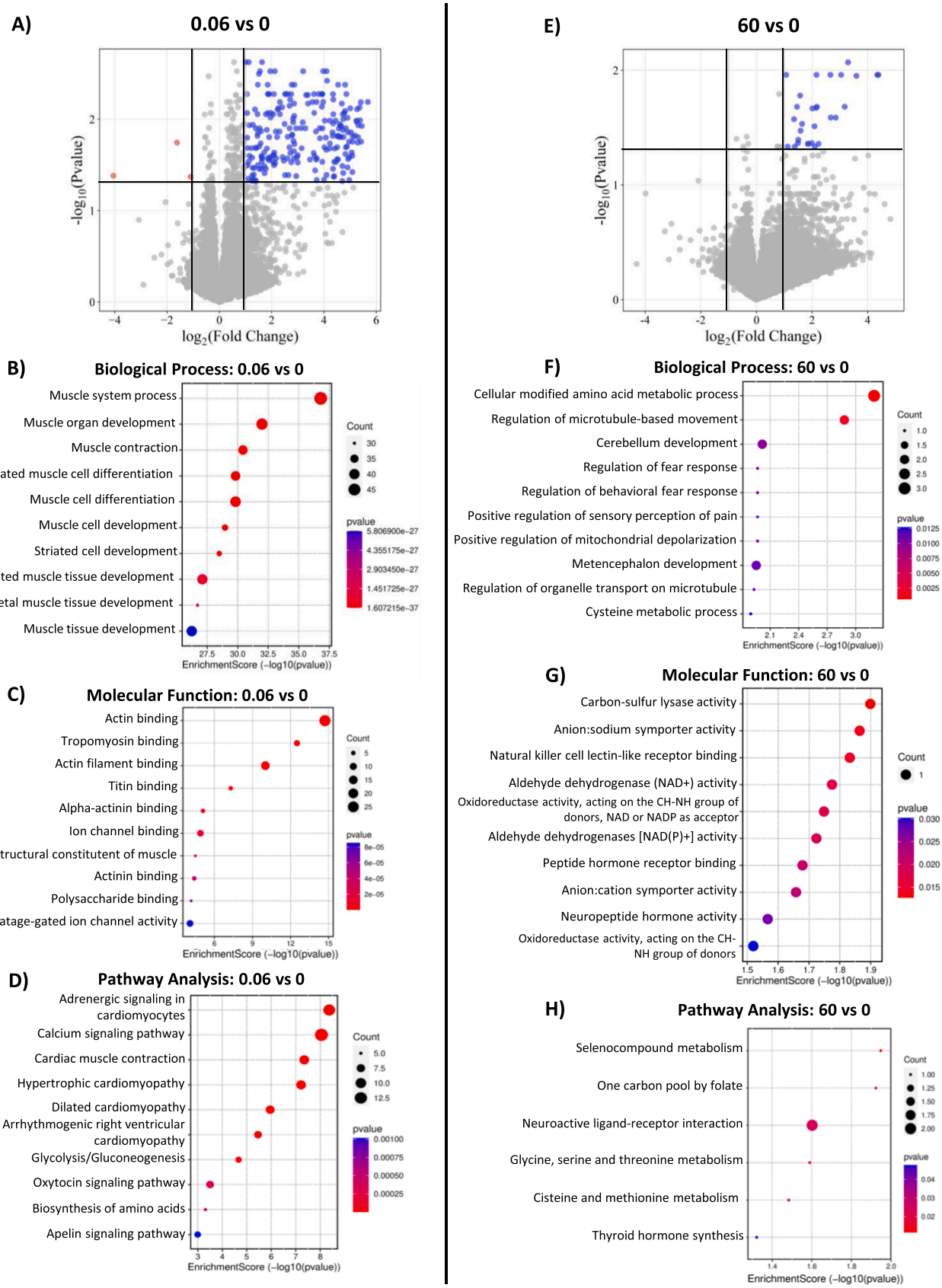

Fig. 2. Exposure to low dose of BFRs induced transcriptomic dysregulation in the mammary gland of dams exposed to DMBA. (A, E) Volcano plot of RNA seq data, all genes are plotted and significant upregulated (blue) and downregulated (red) genes are shown. Exposure to low dose of BFRs showed higher number of dysregulated genes. (B, F) Significant Biological Process according to up- or down-regulated genes. (C, G) Significant dysregulated Molecular Function according to up- or down-regulated genes. (D, H) KEGG Pathway analysis according to up- or down-regulated genes. All samples were harvested 175 days after weaning of the pups. Data was considered significant when the adjusted p-value ≤ 0.05 and $-1.5 \leq \text{Log2FC} \geq 1.5$.

dysregulated when comparing low exposure to high exposure. When comparing the significantly dysregulated genes by BFR exposure, only one (Contactin1) is in common (Fig S4), suggesting different effects depending on the dose.

3.3. BFR exposure during pregnancy and lactation in DMBA-exposed dams disrupts the phosphorylation dynamics of β-catenin

Changes in pathways associated with the cytoskeleton, calcium influx and muscle contraction lead us to believe that cell-cell interactions were affected by BFRs, as observed in our previous study [26–28], most particularly affecting β-catenin phosphorylation. Accordingly, when we examined more carefully the genes that were dysregulated by the treatment, low dose exposure resulted in the up-regulation of the Kelch-like (Klhl) protein family (Fig. 2A, Table S4), which has been linked to cancer progression via Wnt/β-catenin pathway [49]. Tripartie motif (TRIM) family proteins transcripts were also up-regulated in the low dose exposure (Fig. 2A, Table S4). The TRIM protein family can mediate several signaling pathways including the Wnt/β-catenin [50,51]. Moreover, a semi-supervised analysis of RNAseq data focused on the Wnt/β-catenin pathway also suggests a dysregulation of this pathway (Fig S5) when low-BFR exposure is compared to control. Specifically, we observed an up-regulation in different pathways, such as Pathways in cancer, Adherens junction, and Cytoplasmic microtubule organization (Fig S5). To evaluate the dynamics of β-catenin, which is mainly controlled by phosphorylation, we thus measured the levels of its different phosphorylated forms using western blot. BFR exposure did not affect total levels of β-catenin (Fig. 3A) or the β-catenin phosphorylated at Ser33/37/Thr41 (Fig. 3B). However, BFRs exposure significantly reduced the levels of β-catenin phosphorylated at position Ser675, and more importantly in rats exposed to the low dose (Fig. 3C). In contrast, low dose exposure to BFR resulted in an increase of the phosphor-(Tyr654)-β-catenin (Fig. 3D) when compared to control but not to the high dose (Fig. 3D). Finally, BFR exposure (low and high) resulted in a significant reduction in-phosphorylation of Ser45 of β-catenin (Fig. 3E). Altogether, these results indicate that low and high dose exposures to BFR induce major changes in the phosphorylation dynamics of the β-catenin signaling pathway.

3.4. Low dose exposure to BFRs alters protein levels of downstream targets of Wnt/β-catenin in DMBA-exposed dams

To gain better insight on the mechanistic effects of the dysregulation of the Wnt/β-catenin pathway, levels of downstream proteins of the pathway were evaluated. Nuclear factor LEF1 is a co-factor that binds to β-catenin to regulate transcription upon activation of the Wnt/β-catenin pathway [52]. We observed a trending but non-significant increase in the levels of LEF1 protein expression following the low dose exposure to BFRs ($p = 0.06$) (Fig. 4A). While the BFRs treatments did not affect levels of CyclinD1 and CD44 (Fig. 4B, C), the low dose exposure to BFRs induces an increase in c-Jun and c-Myc protein (Fig. 4D, E). The high dose exposure also significantly increased the levels of c-Myc, but not c-Jun protein (Fig. 4D, C). These results suggested an activation of the Wnt/β-catenin pathway in the mammary gland of rats treated with the low dose of BFRs.

3.5. Exposure to BFRs during pregnancy and lactation causes a shift in levels and patterns of phosphorylation of Stat3 and Stat5 in DMBA-exposed dams

It has been documented that Wnt/β-catenin interacts with the Jak/Stat pathway [53], an important player in mammary gland biology [54,55]. More specifically, Stat3 and Stat5 are key mediators of alveologenesis, lactation and involution [54,55], showing the dynamics of activation through their phosphorylation status during those stages. BFR exposure did not significantly affect total Stat3 protein expression (Fig. 5A), but significantly increased the phosphorylated form of Stat3 at Tyr705 (Fig. 5B). When comparing the effects of BFR exposure on Stat5, low dose resulted in a significant reduction of Stat5 expression compared to control and high dose (Fig. 5C), without significantly affecting phosphorylated form of Stat5 at Tyr694 (Fig. 5D). This suggests a shift in the Stat5 phosphorylation dynamics, where low dose exposure significantly decrease the overall levels of Stat5, but most remaining proteins appear to be phosphorylated, resulting in an increased ratio of phospho-(Tyr694)-Stat5 on Stat5 (Fig. 5E). Overall, our results suggest that the BFRs treatments, specifically the low dose, affect the regulation of Wnt/β-catenin and JAK/STAT pathways, resulting in an environment that favors the formation of lesions many weeks after weaning and DMBA exposure.

4. Discussion

The mammary gland is a highly dynamic organ that undergoes significant structural and functional transformation throughout a woman's reproductive life. These structural and functional transformations are mainly regulated by hormones, which render the mammary gland particularly sensitive to EDCs. We evaluated the potential effects of exposure to an environmentally relevant mixture of BFRs during pregnancy and lactation and its link to cancer. Our study adds to the growing body of evidence that exposure to EDCs, particularly BFRs like PBDEs and HBCDD, during pregnancy and lactation can disrupt normal mammary gland development and potentially compromise the anticancer protective effects associated with parity and breastfeeding, and/or render the gland more prone to PABC. Notably, BFRs disrupt calcium dynamics and may directly modulate the cytoskeleton, leading to increased energy demands, as evidenced by the upregulation of genes associated with glycolysis and gluconeogenesis pathways. Moreover, changes in Wnt/β-catenin and Jak-Stats pathways may lead to a precancerous state as suggested by the higher number of mammary gland lesions in BFR-treated animals.

4.1. BFRs exposure caused mammary epithelium anomalies

Our results demonstrate that exposure to a relevant mixture of BFRs combined with DMBA induces notable changes in the mammary epithelium of Sprague-Dawley rats. Specifically, we observed the appearance of anomalies/lesions within the exposed rat population. More importantly, these lesions are characterised as hyperplasia and fibrous stroma in the exposed groups, compared to the presence of abundant secretion in the control group. A cohort study showed that the risks of developing an invasive carcinoma is three times greater when there is hyperplasia within the breast [56]. The presence of fibrous stroma is not directly linked with an increased risk of developing breast lesions. Fibrous stroma is generally considered benign but is the leading cause of missed breast cancer diagnosis [57]. Nevertheless, stroma fibrosis may lead to cancer invasion and aggressiveness by altering the ECM stiffness [58,59]. Furthermore, mammary gland density strongly correlates with an increase presence of stromal collagen [60], leading to an increased risk of developing breast cancer [61].

Changes in the epithelium of the mammary gland following exposure to the same mixture of BFRs has previously been reported. Our team showed that offspring from dams exposed throughout pregnancy and lactation to the same treatments develop abnormalities within the mammary epithelium at post-natal day (PND) 21 and 46 [26,27]. In addition, when these exposed rats are given DMBA at PND46, they tend to develop more aggressive tumors [62]. In mice, BPA causes a stimulation of mammary epithelium growth [63,64]. Effects on the mammary epithelium are also seen when pups are exposed, transplacentally, to zearalenone (xenoestrogen) [65]. Direct effects of EDCs on the mammary epithelium of pups have been reported many times. Of great concern, few studies focus on dams directly exposed through gestation and lactation, where the gland is highly remodeled and susceptible to

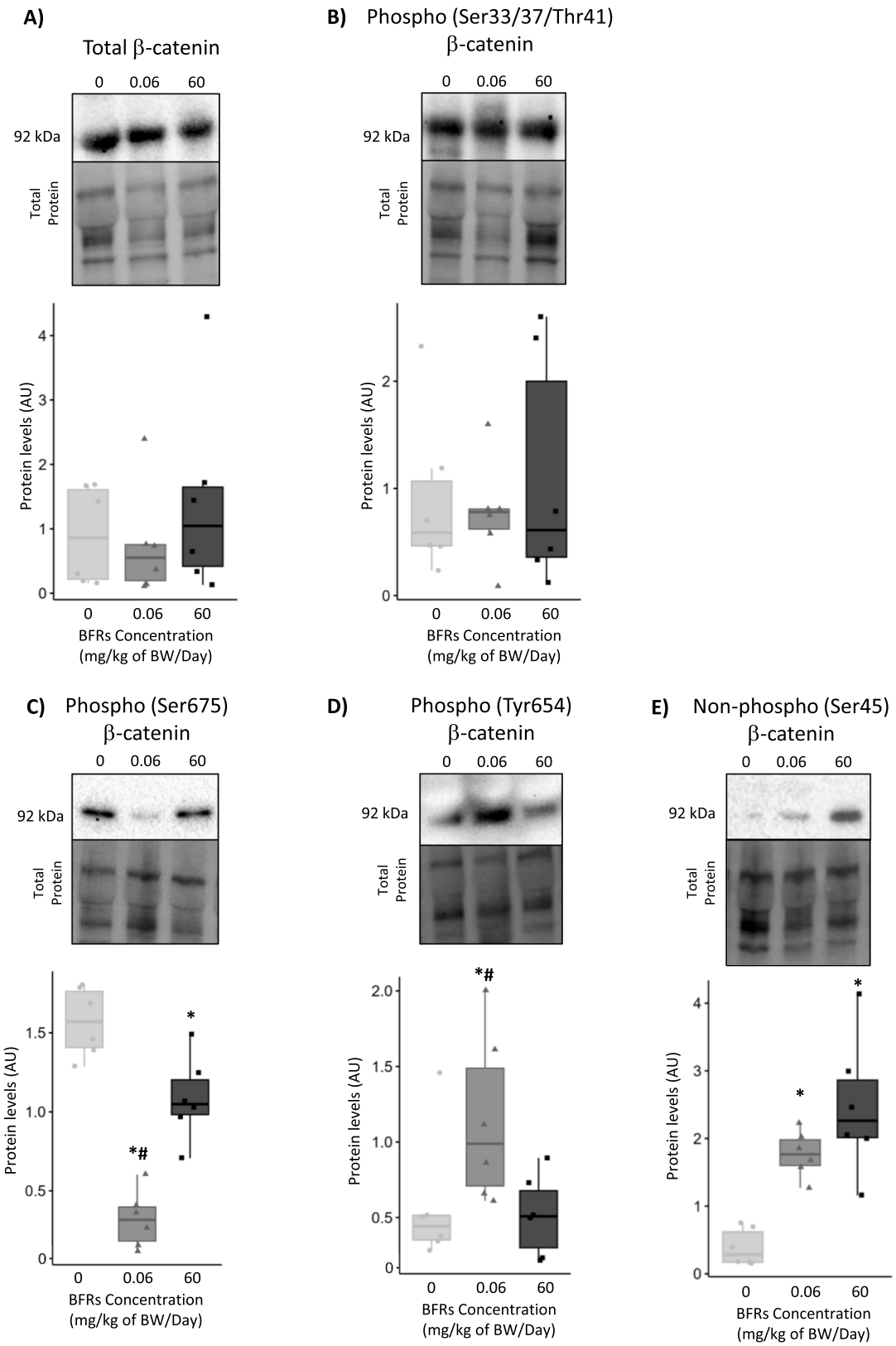

Fig. 3. Exposure to the low dose of BFRs during pregnancy and lactation changes the phosphorylation dynamics of β-catenin. Semi-quantitative western blot analysis of total proteins extracted from the mammary glands after exposure to 0, 0.06 or 60 mg BFRs/Kg of body weight/Day and DMBA. Graphs show protein levels of (A) total β-catenin, (B) phospho-β-catenin (Ser33/37/Thr41), (C) phospho-β-catenin (ser675); 0 VS 0.06; $p = 0.00001$; 0 VS 60; $p = 0.0037$; 0.06 VS 60; $p = 0.0004$, (D) phospho-β-catenin (Tyr654); 0 VS 0.06; $p = 0.0280$; 0.06 VS 60; $p = 0.0318$ and (E) non-phospho-β-catenin (Ser45); 0 VS 0.06; $p = 0.0008$; 0 VS 60; $p = 0.00001$. Histograms represent protein band normalized to the total protein level. All samples were harvested 175 days after weaning of the pups. p-values were calculated with a Kruskal-Wallis (posthoc conover) or ANOVA (posthoc tukey) ($n = 6$). Graphs represent means ± SEM. * = significantly different than control. # = significantly different than High exposure (60).

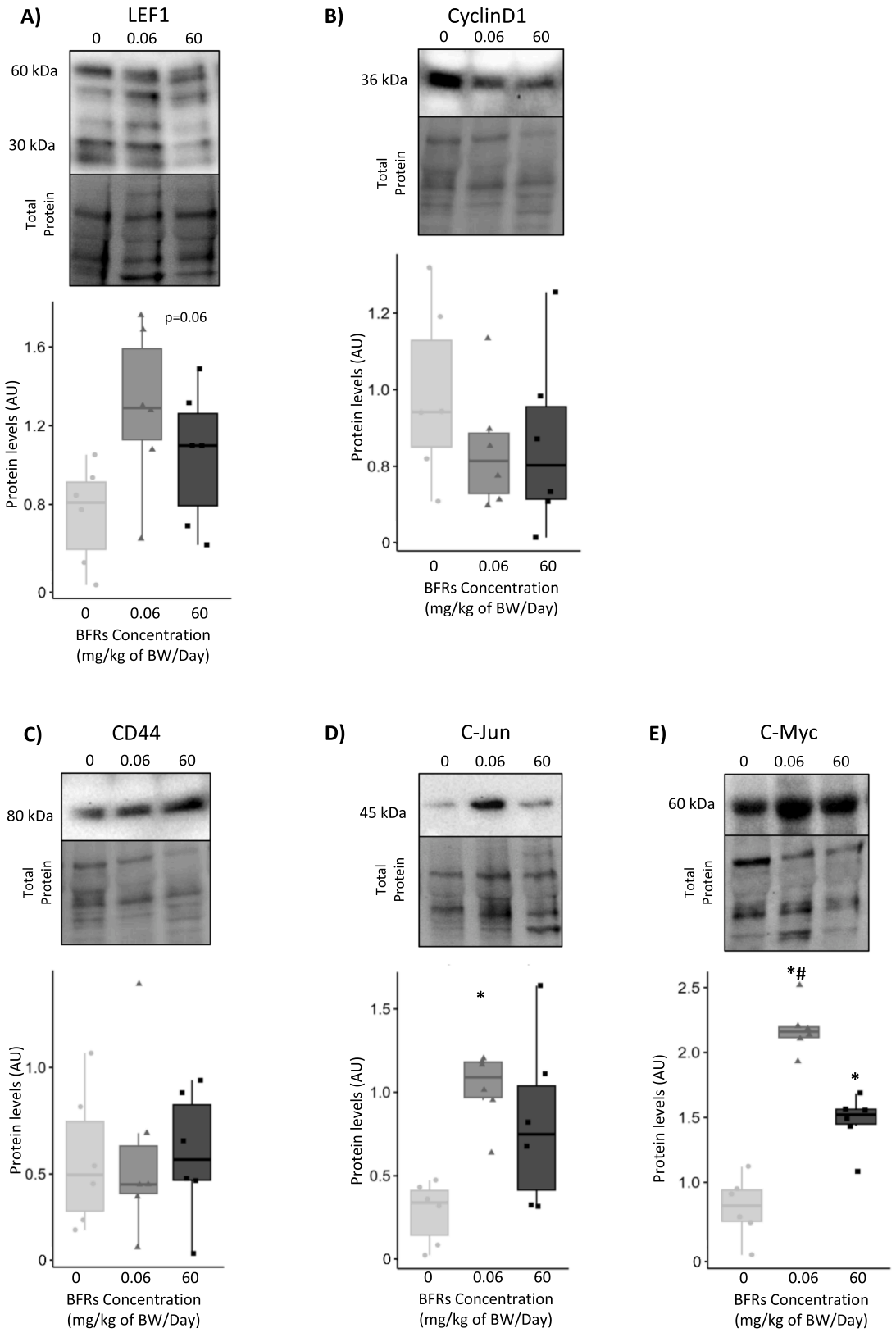

Fig. 4. Exposure to BFRs induces oncogenes expression of the Wnt/β-catenin pathway in dams exposed to DMBA. Semi-quantitative western blot analysis of total proteins extracted from the mammary glands after exposure to 0, 0.06 or 60 mg BFR/Kg of body weight/Day. Graphs show protein levels of (A) LEF1, average intensity of all bands between 25 and 58 kDa were utilized for quantification, (B) Cyclin D1, (C) c-MYC; 0 VS 0.06; $p = 0.0001$; 0 VS 60; $p = 0.0004$; 0.06 VS 60; $p = 0.0002$, (D) c-Jun; 0 VS 0.06; $p = 0.0021$ and (E) CD44. Histograms represent protein band normalized to the total protein level. All samples were harvested 175 days after weaning of the pups. p-values were calculated with a Kruskal-Wallis (posthoc conover) or ANOVA (posthoc tukey) ($n = 6$). Graphs represent means ± SEM. * = significantly different than control. # = significantly different than High exposure (60).

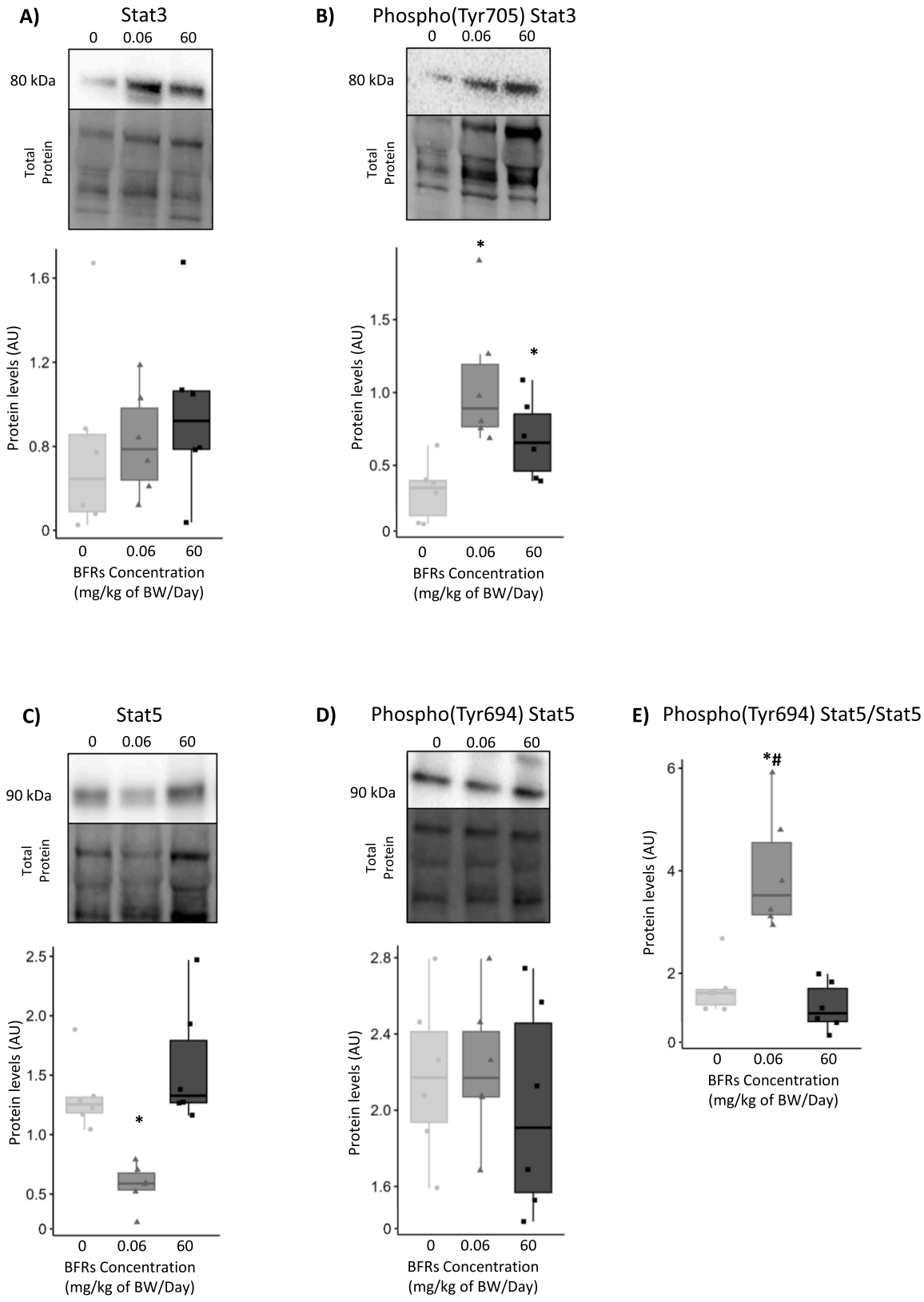

Fig. 5. Exposure to BFRs affects the phosphorylation patterns of Stat3 and Stat5 in DMBA-exposed dams. Semi-quantitative western blot analysis of total proteins extracted from the mammary glands after exposure to 0, 0.06 or 60 mg BFR/Kg of body weight/Day. Graphs show protein levels of (A) Total Stat-3, (B) Phopho-Stat-3 (Tyr705); 0 VS 0.06; $p = 0.0002$; 0 VS 60; $p = 0.0136$, (C) Total Stat-5, (D) Phospho-Stat-5 (Tyr694) and (E) fold difference of phospho-Stat-5 (Tyr694)/Total Stat-5; 0 VS 0.06; $p = 0.0008$; 0.06 VS 60; $p = 0.0001$. Histograms represent protein band normalized to the total protein level. All samples were harvested 175 days after weaning of the pups. p-values were calculated with a Kruskal-Wallis (posthoc conover) or ANOVA (posthoc tukey) ($n = 6$). Graphs represent means ± SEM. * = significantly different than control.

EDCs. Our results suggest that, in both offspring and dams, exposure to BFRs during sensitive windows of exposure influence the risk of breast cancer.

4.2. Exposure to BFRs upregulated genes associated with muscles, calcium and glucose metabolism pathways

RNA sequencing revealed that BFR treatment caused significant increase in expression of genes associated with muscle cell function and cytoskeletal organization suggesting that BFRs may disrupt normal epithelial-stromal interactions and cellular homeostasis in the mammary gland. The upregulation in genes associated with calcium influx may directly affect the remodeling of the cytoskeleton potentially leading to higher traction forces between cells. One possibility, although we did not directly test it, is that BFRs caused an increased presence of CAFs within the mammary gland. CAFs have higher energy metabolism and are phenotypically related to myofibroblasts [66], have a more defined cytoskeleton compared to normal fibroblasts [67] and produce higher traction forces [68]. These changes in the stromal composition may lead to a stiffer and denser extracellular matrix (ECM) [69,70]. This aligns closely with our findings suggesting an increased glycolysis and gluconeogenesis, and of fibroadenomas in the H&E-stained samples. Genes identify by our CAFs analysis are strongly link to increased energy metabolism (Aco2, Eno3, Gapdh, Idh3a, Ky, Lhda, Mt-cyb, Mt-nd1, Mt-nd5, Pgam2, Pfkm, Suclg1, Uqrb) [71–75]. Other up-regulated genes identified are associated with an increase secretion of subunits of laminin (Lamb1, Lamb2) and may increase ECM stiffness [76]. Stiffness of the stroma is linked with increase cell motility and tumor metastasis [77,78]. Furthermore, it has been reviewed that mechanical forces, such as ECM stiffness, can modulate the Wnt/β-catenin pathway, although specific mechanism are still unknown [79]. Additional analyses are required to evaluate this possibility.

4.3. Molecular mechanisms: β-catenin phosphorylation dynamics is disrupted by BFRs and seems to increase its function as a transcription factor

β-Catenin is a multifunctional protein that plays a crucial role in both cell adhesion and signal transduction. In the context of adherens junctions, β-catenin binds to E-cadherins, facilitating cell-cell adhesion and maintaining tissue integrity [80]. However, β-catenin is also a key component of the Wnt signaling pathway, where it acts as a transcription factor in the nucleus, regulating the expression of genes involved in cell proliferation, differentiation, and survival. These two functions of β-catenin are tightly regulated through a phosphorylation-dependent degradation mechanism. When β-catenin is free in the cytoplasm, not linked to adherens junctions, it gets phosphorylated at specific residues (Ser33/Ser37/Thr41), targeting it for ubiquitin-mediated proteolysis, leading to its degradation [81]. This phosphorylation thus serves as a signal for the destruction of β-catenin in the cytoplasm, preventing its accumulation and subsequent activation of Wnt target genes [82]. In contrast, the phosphorylation of β-catenin at Tyr654 has been linked to enhanced Wnt/β-catenin signaling [83,84]. When β-catenin is stabilized in the cytoplasm through this phosphorylation, it translocates to the nucleus and binds to LEF1 and other TCF family member [85]. This complex activates the target genes in cell proliferation, differentiation, and survival [52,86].

We found that exposure to BFRs, particularly at low doses, significantly altered the phosphorylation dynamics of β-catenin, which suggests a potential shift in its functional role. Specifically, the exposure to BFRs appears to disrupt the normal degradation process of β-catenin. While we noted no changes in the phosphorylation at Ser33/37/Thr41, which typically marks β-catenin for degradation, there was a significant decrease in the expression of the Ser675 phosphorylated form. This reduction in Ser675 phosphorylation may facilitate the transition of β-catenin from its role as an adherens junction molecule to a cytoplasmic location [85]. No adverse effects of BFRs exposure on total β-catenin and the phosphorylated form at Ser33/37/Thr41 suggests that the degradation of cytoplasmic β-catenin is not compromised. Phosphorylation at Ser45 is known to create a priming site necessary for initiating the phosphorylation-degradation cascade of β-catenin [87]. Therefore, the increase in non-phosphorylated β-catenin may indicate a disruption in the normal regulatory mechanisms that control β-catenin levels and increased the cytoplasmic stability of β-catenin. Low dose exposure to BFRs also increased, but not significantly ($p = 0.06$), LEF1 protein expression, a co-factor of β-catenin that promotes the transcription of Wnt-responsive genes [88]. The observed changes in phosphorylation dynamics following low-dose BFRs exposure may thus lead to increased levels of nuclear β-catenin, thereby activating the canonical Wnt/β-catenin signaling pathway.

Consequently, we then evaluated downstream proteins of the Wnt/β-catenin pathway to determine whether BFRs exposure during pregnancy and lactation can activate this pathway, thus contributing to a potential increased risk of PABC. Interestingly, c-Jun and c-Myc protein expression was upregulated upon exposure to the lower dose of BFRs. Yet, both c-Jun and c-Myc are upregulated in PABC compared to non-PABC [89]. Authors even hypothesise that c-Myc may be a biomarker for PABC [89]. c-Myc is a well-known oncogene that acts as a key regulator of cell metabolism and growth. It is activated by numerous oncogenic pathways and promotes various metabolic alterations that can lead to malignant transformation [90]. In breast cancer, c-Myc has been shown to modulate genes related to glucose metabolism, particularly in estrogen receptor-negative (ER-negative) tumors [91].

Similarly, c-Jun is frequently overexpressed in breast cancer tissues compared to normal breast tissue [92,93]. Accordingly, the overexpression of c-Jun in poorly metastatic MCF7 cell line resulted in increased migration and metastasis to the liver when injected into the tail vein of immunocompromised mice [94]. This overexpression was also associated with various oncogenic processes, including cell proliferation, migration and survival [94,95]. Notably, c-Jun has been implicated in regulating glucose metabolism in breast cancer cells through its interaction with glucose transporter 1 (GLUT1), facilitating increased glucose uptake and metabolism [96]. In our study, RNA sequencing analysis also suggested a potential link between Wnt/β-catenin pathway activation and the observed disruptions in glycolysis and gluconeogenesis upon exposure to the low dose of BFRs.

Interestingly, we observed no significant effects on CD44 expression. CD44 is known to enhance Wnt activity in a concentration-dependent manner [97], and its lack of upregulation may indicate an alternative regulatory mechanism of the Wnt/β-catenin pathway activation in the context of BFR exposure, further studies are needed to confirm this. Additionally, no effects were observed on Cyclin D1. Cyclin D1 is critical for the development of mammary cancers driven by certain oncoproteins; however, its role is less pronounced in cancers induced by oncogenic factors, such as c-Myc or Wnt-1 [98]. The absence of changes in these proteins suggests that the activation of the Wnt/β-catenin pathway in response to low BFRs exposure may not rely on the typical regulatory mechanisms associated with CD44 and Cyclin D1.

Together, those results suggest that exposure to the low dose of BFRs activate the Wnt pathway, leading to change in the β-catenin phosphorylation dynamics, and promoting its transcriptional activity. This activation could promote the transcription of c-Jun and c-Myc, and dysregulation of pathways associated with glycolysis and gluconeogenesis. Interestingly, β-catenin phosphorylation dynamics dysregulation following BFRs exposure has also been seen in dams, at the end of lactation, and in the pups exposed in utero and through lactation [26–28]. This finding underscores the sensitivity of the β-catenin to BFRs exposure during critical mammary gland developmental stages.

4.4. Stat3 and Stat5 disruption in BFRs exposed animals

The transcription factors Stat3 and Stat5 play crucial, yet

contrasting, roles during pregnancy, lactation, and involution. Stat5 is primarily activated during late pregnancy and lactation, promoting alveologenesis, which is essential for milk production, and maintaining cell viability within the mammary epithelium [99,100]. This activation is critical for the differentiation of mammary epithelial cells, ensuring that the gland develops properly to support lactation. Conversely, during the involution phase, there is a concurrent deactivation of Stat5 and activation of Stat3. This shift facilitates the apoptosis of excess alveolar cells, allowing the mammary gland to return to its pre-pregnant state [54,55]. The dysregulation of these pathways can have significant implications for breast cancer development, particularly in the context of PABC.

In addition, some breast cancers exhibit constitutive activation of Stat3, which is associated with increased cell proliferation and resistance to apoptosis, thereby promoting tumor growth [101]. The interplay between Stat3 and Stat5 is particularly relevant in this context. While Stat5 generally supports a more differentiated and less aggressive tumor phenotype, the activation of Stat3 can lead to a more malignant behavior in breast cancer cells [102,103]. Studies have shown that a subset of breast tumors displays dual activation of both Stat3 and Stat5, which correlates with a more aggressive phenotype [104,105]. In the context of our findings, the observed shifts in the phosphorylation dynamics of Stat3 and Stat5 suggest that BFRs can disrupt the normal regulatory mechanisms governing these pathways. The increase in phosphorylated Stat3 and the decrease in total Stat5 levels following BFR exposure may create a pro-tumorigenic environment, favoring the development of lesions in the mammary gland post-involution [106,107]. Furthermore, the activation of Stat3 in response to BFR exposure may support a tumor-promoting microenvironment by enhancing expression of oncogenic factors, while the concurrent reduction in Stat5 could diminish the protective effects typically conferred by this transcription factor [108,109]. The balance between these two signaling pathways is critical. A shift towards Stat3 activation at the expense of Stat5 may predispose the mammary gland to neoplastic transformation, particularly in the context of additional carcinogenic exposures such as DMBA treatment [109,110]. Finally, it was shown that increase in intracellular Ca^{2+} triggers Stat3 signaling leading to breast cancer stem cell enrichment [111]. Interestingly, our KEGG pathway analysis showed a dysregulation in genes linked with calcium dynamics, supporting a relationship with increased Stat3 phosphorylation. Our results thus suggest that exposure to the low dose of BFRs can dysregulate Stat3 and Stat5 dynamics, thus favouring a pro-cancerous stage.

4.5. DMBA and breast cancer

While our study provides evidence of the adverse effects of BFR exposure on mammary gland health suggesting an increased risk of developing breast cancer, it is not without limitations. The absence of palpable tumors within the experimental timeframe necessitates caution in extrapolating these findings to long-term cancer risk. DMBA has been extensively used to induce mammary tumors in peripubertal animals [112,113]. This model is especially efficient because, at puberty, the mammary epithelium is mainly made up off highly proliferative and undifferentiated cells making the gland particularly susceptible to chemical carcinogens [114]. In our study, the administration of DMBA shortly after weaning may have coincided with a period of heightened apoptosis and tissue remodeling, potentially limiting the carcinogenic effects of DMBA. Major decrease in tumor yield has been shown when DMBA is given after 55 days of age due to a sharp decrease in proliferation of epithelial cells [115]. Another study indicates that pregnancy and childbirth decrease sensitivity of Sprague-Dawley rats to DMBA, leading to a decrease in mammary tumor incidence and latency in breast carcinogenesis [37]. This could explain the absence of palpable tumors in the BFRs-exposed dams, as the physiological changes associated with involution may have counteracted the expected tumorigenic response to DMBA [116,117]. In our study, non-exposed animals treated with DMBA showed minimal changes in their mammary gland structure. In contrast, BFR-exposed animals exhibited significant alterations in mammary gland architecture including lesions, hyperplasia, and fibrous stroma, gene expression, and protein profiles. Given that DMBA is known to induce mammary tumors in Sprague Dawley rats, the minimal alterations in the control group suggest that pregnancy, parity, and lactation may have conferred protective effects. However, BFR-exposed animals developed a phenotype associated with an increased risk of mammary tumors later in life, indicating that BFR exposure may disrupt these protective mechanisms.

Nevertheless, the choice to give DMBA after weaning in our study was guided by several factors. First, we did not want to chemically induce breast cancer during pregnancy to avoid exposure to the fetuses, which were also analyzed in this project [112]. Second, while DMBA is primary known to promote carcinogenesis by creating DNA adducts in proliferative cells, other mechanisms of action have been associated with increased cancer risks in non-proliferating cell [112]. Thus, we were hypothesizing that the BFR exposure will create an environment that will exacerbate or promote tumor formation through those other mechanisms. Finally, DMBA was successfully used to induce breast cancer in Sprague-Dawley involuting rats, supporting the fact that this period could be sensitive to DMBA-induced breast cancer [37,118]. Importantly, the doses of DMBA given to the rats were significantly higher in these studies (10 mg/100 g body weight at a dose interval of 2 weeks [37], and 15 mg/rat [118]) and may contribute to the observed tumorigenicity. Future studies should focus on longitudinal assessments to determine the effects of BFRs exposure over time and their direct correlation with tumorigenesis.

4.6. Discrepancies in whole mount and H&E staining in mammary gland lesion detections

Interestingly, the number of lesions/anomalies per rats were not as numerous in the H&E-stained samples compared to whole mount. In addition to the differences in the area of tissue covered by the two analyses, the difference may also be due to the fact that the same gland was not taken for both set of experiments, from thoracic and inguinal glands, respectively. Indeed, mammary gland tumor development is characterized by significant heterogeneity, with tumor incidence and biological behavior varying based on their anatomical location along the anterior-posterior and left-right axes [119]. Moreover, differences can be observed in the staining used. For example, whole mount preparations of the mammary gland provide insights into its three-dimensional structure and histological changes. Carmine Alum, a nuclear stain, highlights epithelial structures within the mammary stroma, allowing for assessment of tissue changes, disease progression, and treatment effects [120]. On the other hand, H&E will stain the nuclei, cytoplasm and ECM. This staining provides detailed images of thin tissue sections, making it ideal for examining cellular and subcellular features helping for proper diagnosis [121]. In summary, whole-mount preparations offer a three-dimensional, intact view of tissue, while H&E staining provides detailed cellular-level information on thin tissue sections. While the number of lesions/anomalies were not as numerous in the H&E-stained samples compared to whole mount, the complementary use of both assessments in our study allowed for sensitive quantification of epithelial lesions across the entire gland in whole mounts, while review of H&E sections facilitated histologic characteristic of lesion type across the microenvironment, including the stroma.

5. Conclusion

Taken together, our study shows that low dose exposure to BFRs during pregnancy and lactation disrupts the protective effect of parity and full-term lactation against breast cancer and increases the risk of breast cancer later in life. We suggest that the exposure alters the Wnt/β-catenin phosphorylation dynamics, directly increasing levels of

oncogenes (c-Myc and c-Jun). Moreover, we show an increased number of lesions within mammary tissues by histology and a disruption of the cytoskeleton dynamics and glucose metabolism mainly linked to the Ca^{2+} pathways by RNAseq. An important observation from our study is the pronounced effects at levels of BFR exposure approaching realistic human exposure levels but not at much higher doses. This non-monotonic dose response is not uncommon for endocrine disruptors and highlights the complexity of BFRs toxicity. The low dose effect of BFRs toxicity was also seen in our previous work [26–28]. It underscores the need for careful consideration of low-dose effects in risk assessment. In conclusions, our findings underscore the potential for BFRs to disrupt mammary gland development and function during sensitive periods, which could compromise the natural protective effects of pregnancy and lactation against breast cancer. As these chemicals remain in our environment, continued research into their effects on mammary gland biology and breast cancer risk is crucial.

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CRediT authorship contribution statement

Plante Isabelle: Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Patten Shunmoogum A.:** Writing – review & editing, Supervision. **Wade Michael Gordon:** Writing – review & editing, Supervision, Conceptualization. **Juárez Melany:** Writing – review & editing, Data curation, Conceptualization. **McDermott Alec:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Formal analysis, Data curation, Conceptualization.

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.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.reprotox.2025.108928.

Data availability

Data will be made available on request.

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