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Time-specific impact of mono-benzyl phthalate (MBzP) and perfluorooctanoic acid (PFOA) on breast density of a Chilean adolescent Cohort

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ABSTRACT

Introduction: High mammographic density is among the strongest and most established predictors for breast cancer risk. Puberty, the period during which breasts undergo exponential mammary growth, is considered one of the critical stages of breast development for environmental exposures. Benzylbutyl phthalate (BBP) and perfluorooctanoic acid (PFOA) are pervasive endocrine disrupting chemicals that may increase hormone-sensitive cancers. Evaluating the potential impact of BBP and PFOA exposure on pubertal breast density is important to our understanding of early-life environmental influences on breast cancer etiology.

Objective: To prospectively assess the effect of biomarker concentrations of monobenzyl phthalate (MBzP) and PFOA at specific pubertal window of susceptibility (WOS) on adolescent breast density.

Method: This study included 376 Chilean girls from the Growth and Obesity Cohort Study with data collection at four timepoints: Tanner breast stages 1 (B1) and 4 (B4), 1- year post- menarche (1YPM) and 2-years post-menarche (2YPM). Dual-energy X-ray absorptiometry was used to assess the absolute fibroglandular volume (FGV) and percent breast density (%FGV) at 2YPM. We used concentrations of PFOA in serum and MBzP in urine as an index of exposure to PFOA and BBP, respectively. Parametric G-formula was used to estimate the time-specific effects of MBzP and PFOA on breast density. The models included body fat percentage as a time-varying confounder and age, birthweight, age at menarche, and maternal education as fixed covariates.

Results: A doubling of serum PFOA concentration at B4 resulted in a non-significant increase in absolute FGV (β:11.25, 95% confidence interval (CI): −0.28, 23.49)), while a doubling of PFOA concentration at 1YPM resulted in a decrease in % FGV (β:-4.61, 95% CI: −7.45, −1.78). We observed no associations between urine MBzP and breast density measures.

Conclusion: In this cohort of Latina girls, PFOA serum concentrations corresponded to a decrease in % FGV. No effect was observed between MBzP and breast density measures across pubertal WOS.

1. Introduction

Breast cancer is the most common cancer among women, accounting for 30% of female cancers worldwide (IARC. Globcal Cancer Statistics, 2020). The volume of dense, fibrous and glandular breast tissue is one of the strongest and well-established risk factors for breast cancer. Women with higher breast density experience greater risk of breast cancer across all age groups (Wang et al., 2014). Breast density is thought to peak during puberty following menarche and represents one of the few early life predictors of breast cancer risk that may be modified by exposure

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profile (McCormack et al., 2010).

Endocrine Disrupting Chemicals (EDCs), defined as exogenous substances or mixtures that alter the endocrine system functioning, have been associated with an increased incidence of endocrine-related human diseases, including various hormone-sensitive cancers such as breast cancer (Soto and Sonnenschein, 2010). A potential mechanism between EDC and breast cancer is the interruption of the estrogen signaling pathway, which disrupts the proliferation of the stromal cells (Buoso et al., 2020). Another proposed linkage is through the creation of a tumor-favorable microenvironment, which modifies the breast matrix composition by increasing collagen fibers in the tissue stroma, contributing to a higher proportion of breast density (Ibrahim et al., 2016; Burks et al., 2017; Bodelon et al., 2021). Lastly, some EDCs are obesogenic, increasing total adiposity and reducing percent breast density (% FGV) (Gupta et al., 2020). This in effect, may misrepresent a person's risk for breast cancer, which is often screened through the BI-RADS breast density reporting system (Melnikow et al., 2016).

Phthalates and per- and polyfluoroalkyl substances (PFAS) are two classes of suspected EDCs that are of concern in relation to breast cancer development (Wang and Qian, 2021). Exposure to phthalates results from their frequent use as plasticizers in adhesives and sealants, paints and coatings, and vinyl floor tiling (Katsikantami et al., 2016). Of particular interest are benzylbutyl phthalate (BBP) and its main metabolite monobenzyl phthalate (MBzP), which are classified as endocrine disrupters for their anti-androgenic (Marcoccia et al., 2017) and pro-estrogenic effects (Silano et al., 2019; Chatterjee and Karlovsky, 2010; Wu et al., 2021; Picard et al., 2001). Several *in vitro* studies reported tumorigenic properties of BBP in increasing the proliferation of estrogen receptor (ER)-positive breast cancer cells and inducing expression of oncogenes in ER-negative breast cancer cells (Harris et al., 1997; Hsieh et al., 2012; Hsieh et al., 2012). PFAS are commonly used in the production of non-stick pans, furniture, cosmetics, and packaged food containers (Agency for Toxic Substances and disease Registry, 2022), and have been extensively studied and shown to adversely influence women's health by disrupting their reproductive system. (Rickard et al., 2022) PFAS are known to be highly persistent in the environment and some bioaccumulate, which may be attributed to their chemical and thermal stability, hydrophobic and lipophilic characteristics (Buck et al., 2011; Fu et al., 2016). Perfluorooctanoic acid (PFOA) is one of the more studied PFAS and has been purported to foster development and progression of breast cancer by disrupting the peroxisome proliferator activated receptor signaling pathways, consequently increasing hepatic aromatase and estrogens concentrations (DeWitt et al., 2009).

Breast tissue may be particularly sensitive to EDCs during puberty, a period of rapid growth and cellular differentiation of terminal end buds (TEBs) (Russo, 2016). As such, puberty is considered one of the critical stages of breast development as well as a 'window of susceptibility' (WOS) for environmental exposures (Russo and Russo, 2004; Terry et al., 2019; Russo and Russo, 1978). However, data on human exposure to EDCs on breast development during puberty remains scarce. The objective of our study is to evaluate the potential effects of MBzP and PFOA at specific pubertal WOS on breast density in a cohort of pubertal Chilean girls. Evaluating environmental exposures for a specific WOS is important to our understanding of environmental influences during pubertal activity and can also help identify appropriate time-periods for breast cancer prevention.

2. Methods

2.1. Study population

In 2006, the prospective Growth and Obesity Chilean Cohort Study (GOCS), recruited children ages 3–4 years in low- and middle-income families from 54 National Nursery Schools Council Program (JUNJI) located in the southeast area of Santiago, Chile. The eligibility criteria consisted of the following: 1) singletons born at term (37–42 weeks), 2) birthweight greater than or equal to 2500 g (g) and less than 4500 g, 3) healthy with no physical or psychological conditions that could severely affect growth (e.g., skin burns, brain tumor, hyperthyroidism). Girls' breast development was assessed through palpation and visual inspection by dietitians who were trained by a pediatric endocrinologist using the Tanner Staging rating scale. (Marshall and Tanner, 1969) The study prospectively followed the girls annually up to age 7.5 years, then every 6 months until they reached Tanner stage 4 (B4), and then annually thereafter including at timepoints 1-year post-menarche (1YPM) and 2-year post-menarche (2YPM). A total of 1,089 eligible children, of which 601 were girls, agreed to participate in the study (Kain et al., 2009).

Our study focused on girls who provided breast density measurement at 2YPM, urine samples at Tanner stage 1 (B1) and B4, and serum samples at B4 and 1YPM (Fig. 1). Urine and serum samples were collected at specific tanner stages to assess whether there is a differential pubertal WOS. The study protocol was approved by the Ethics Committee of the Institute of Nutrition and Food Technology (INTA), University of Chile, and the Institutional Review Board of the University of California, Los Angeles. Informed written consent was obtained from all parents or guardians of study participants. The involvement of the Centers for Disease Control and Prevention (CDC) laboratory did not constitute engagement in human subjects' research.

2.2. Urine collection

Urine samples were collected at B1 and B4. They were collected (at least 2 mL) in non-polycarbonate sterile cups between 10 AM and 12 PM during visits to the INTA from fasting girls. Once collected, urine samples were immediately vortexed and aliquoted. The processing of samples involved the homogenization and distribution of the samples into three separate aliquots to store them at −80 °C.

2.3. Urine analysis - MBzP

Urine samples collected at B1 (n = 200) and B4 (n = 200) were randomly selected and processed at the National Center for Environmental Health Laboratory at the CDC in Atlanta, GA using on-line solid phase extraction-liquid chromatography-isotope dilution-tandem mass spectrometry (Silva et al., 2007). Creatinine quantification for all urine samples was performed at Mount Sinai (Taussky, 1954). With additional funding, the remaining samples collected at B1 (n = 93) and B4 (n = 133) were analyzed at the Children's Health Exposure Analysis Resource (CHEAR) Laboratory at the Icahn School of Medicine at Mount Sinai in New York, NY using a previously described protocol (Mazzella et al., 2021). The limit of detection (LOD) for MBzP was lab-specific (CDC, 0.3 ng/ml; Mt. Sinai, 0.1 ng/ml). MBzP concentrations below LOD were imputed a value equal to the lab-specific LOD/sqrt(2) (Hornung and Reed, 1990).

A subset of 40 samples collected at B1 and B4 and initially analyzed at the CDC lab was also analyzed at the Mount Sinai lab for quality control (QC) followed by calculation of the QC intraclass correlation coefficient (ICC) using a one-way random effects model measuring absolute agreement with multiple raters/measurements to evaluate agreement between labs (Supplementary File 2) (McGraw and Wong, 1996; Shrout and Fleiss, 1979).

Prior to analysis, we standardized the distribution of EDC biomarker concentrations across assay batches. The QC samples analyzed by both labs were used to estimate the difference in the mean and relative standard deviation (SD) in biomarker concentrations between the two labs. These estimates were then used to shift the mean and scale the SD among the full sample group analyzed at CDC to that of the samples analyzed at Mount Sinai, assuming the true distribution of concentrations between the two labs was the same and there were no differences in participant characteristics for the samples analyzed at different labs.

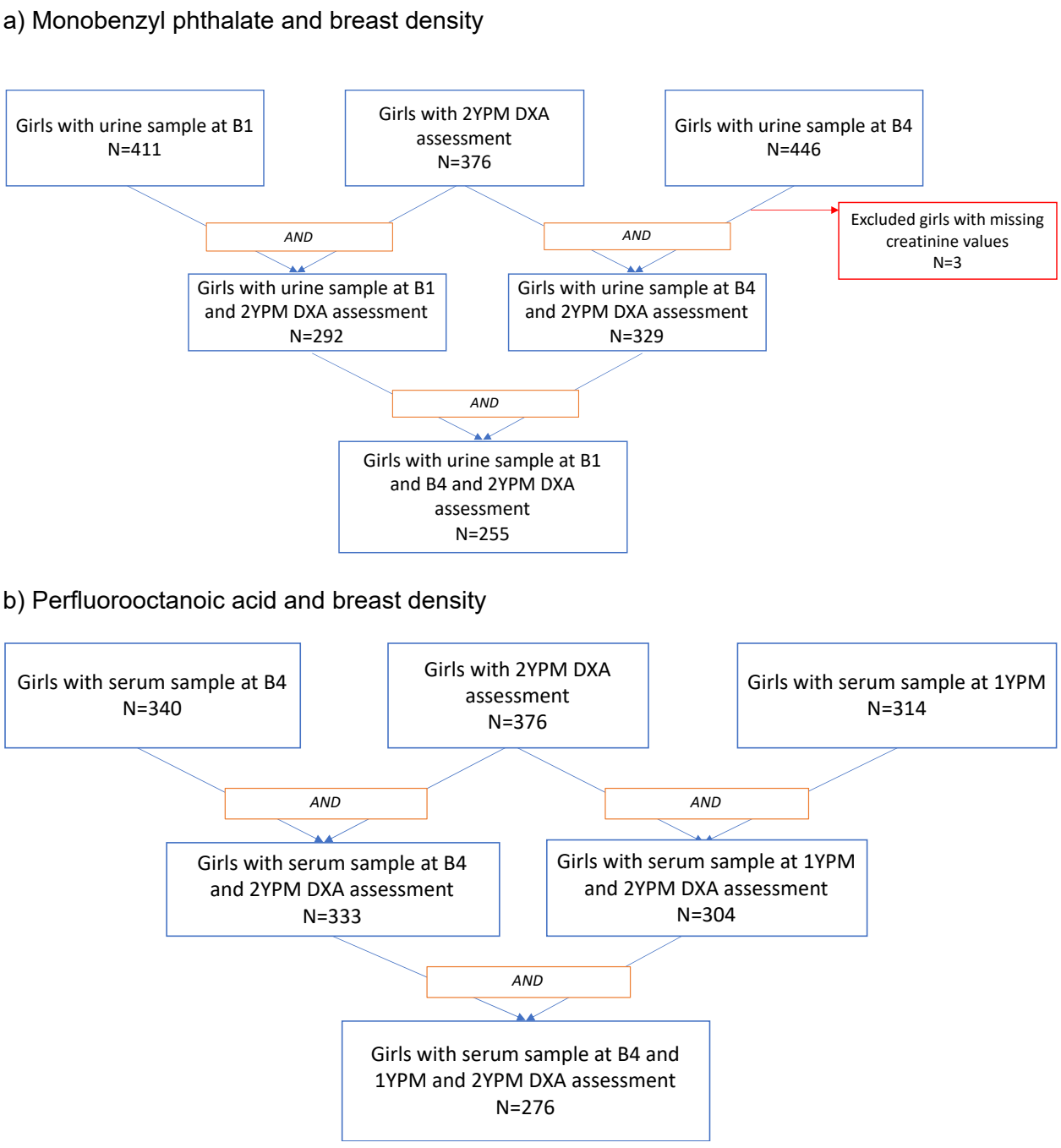

Fig. 1. Flow chart of GOCS subsamples a) Monobenzyl phthalate and breast density b) Perfluorooctanoic acid and breast density.

2.4. Blood collection

Serum samples were collected at Tanner stage B4 and 1YPM. Girls fasted (at least 8 to 12 h prior to blood collection) prior to venous samples collection before 8:30am at the INTA clinics. Study staff confirmed the state of fasting and whether the girls had a fever at the time of blood collection.

2.5. Blood analysis – PFOA

Serum samples were analyzed using on-line solid phase extraction-liquid chromatography-isotope dilution-tandem mass spectrometry (Kato et al., 2018) at the CDC National Center for Environmental Health Laboratory for the linear isomer of PFOA. The LOD was 0.1 ng/mL. PFOA concentrations below LOD were imputed a value of LOD/sqrt(2) (Hornung and Reed, 1990). PFOA concentrations were $\log_2$-transformed prior to analyses.

2.6. Assessment of breast density

Dual-energy X-ray absorptiometry (DXA) was used to assess the volume of dense breast tissue (absolute FGV) at 2YPM in a process developed by Shepherd *et al.* (version 5) (Shepherd et al., 2008). Every girl was screened for pregnancy prior to DXA assessment. In short, the left and right breast were scanned with Prodigy DXA system software (version 13.6, series 200674; GE Healthcare). Quality control and calibration was obtained using reference breast density materials. DXA assessed absolute FGV (cm^3) and total breast volume (cm^3). The percent

breast density (%FGV) represents the proportion of fibroglandular tissue volume relative to total breast volume (cm^3) multiplied by 100. Breast density studies using the DXA method reported very precise and reproducible results in adolescent girls (Shepherd et al., 2008; Kelly et al., 2009).

2.7. Covariates

Covariates were selected *a priori* based on previous knowledge regarding biological relevance. Birthweight was obtained retrospectively from health records. Anthropometric measures (e.g., weight, height) were measured every 6 to 12 months by trained dietitians. Percent body fat was measured using a bioimpedance device. Age at menarche was surveyed by study dieticians every 6 months prior to B4 and every 3 months after achieving B4. Menarche was differentiated from other potential causes of vaginal bleeding (e.g., vaginal infection, urinary infection) via questionnaire. Maternal education was collected through interview with the girls' mothers. Urine MBzP concentrations were adjusted for urinary creatinine. Urine samples with missing creatinine values were excluded (n = 3). Other missing covariate data were imputed using mean (continuous variables) or median (categorical variables) imputation.

2.8. Statistical analysis

Parametric G-formula (hereafter 'G-formula') was used to investigate the causality of MBzP and PFOA's effect on breast density. G-formula, adjusting for both time-varying confounder and fixed covariates (Robins et al., 1999), allowed estimation of time-specific effects of MBzP and PFOA on breast density at 2YPM individually and collectively. Fig. 2 represents a causal directed acyclic graph (DAG) constructed based on previous knowledge regarding biological relevance of the EDC exposures, breast density and covariates. Our DAG omits potentially relevant but unmeasured covariates for simplicity. EDCs (continuous) and fat

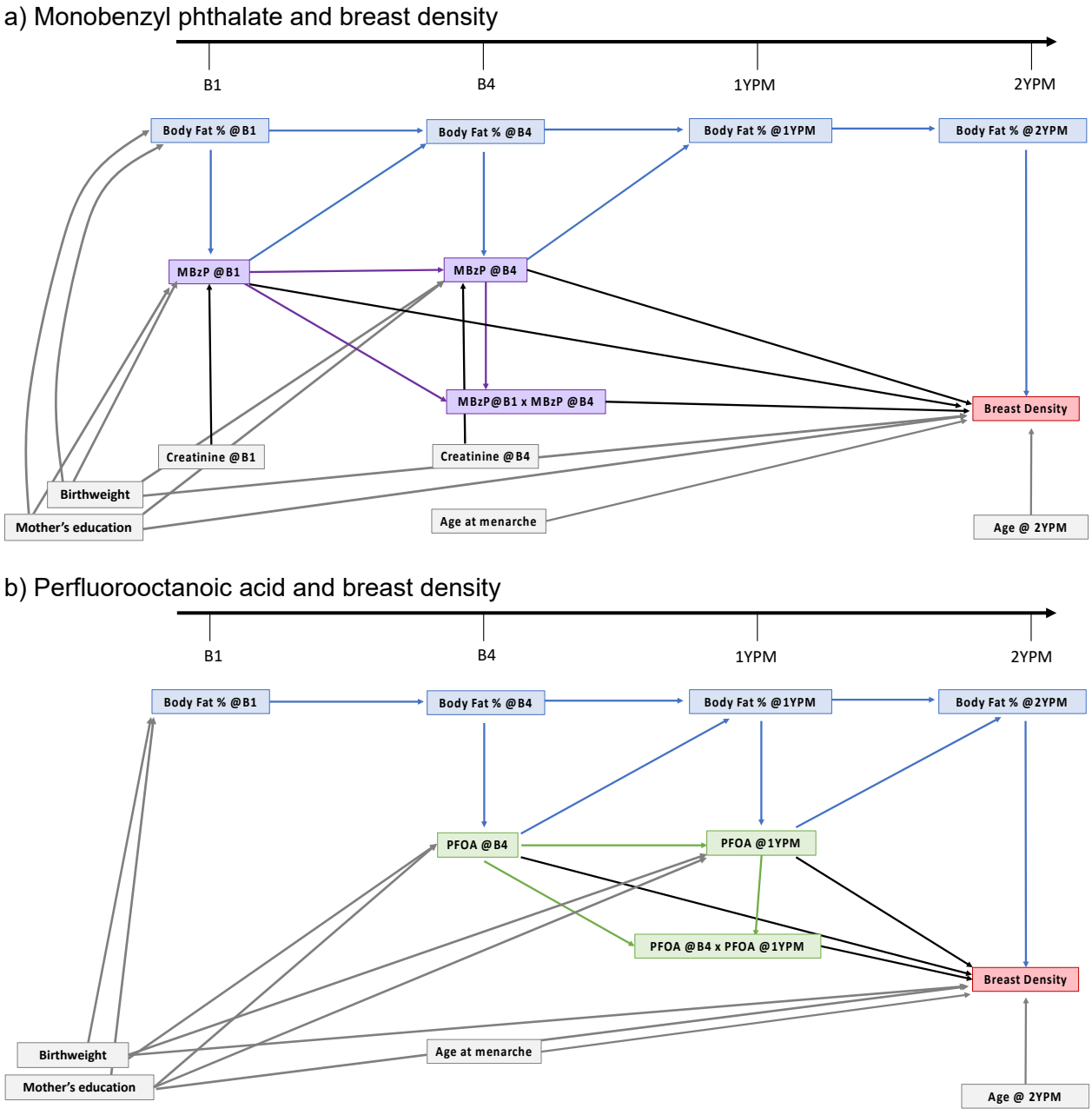

Fig. 2. Directed Acyclic Graph (DAG) a) Monobenzyl phthalate and breast density b) Perfluorooctanoic acid and breast density.

percentage (continuous) are measured longitudinally at multiple timepoints, which places fat percentage as both a confounder and a mediator in the association between EDCs and breast density. G-formula allows for the adjustment of exposure-dependent confounders by fat percentage without blocking the indirect path of EDCs to breast density through fat percentage. Time-fixed covariates include age at 2YPM (continuous), birthweight (continuous), age at menarche (continuous), maternal education (categorical: secondary education or less, greater than secondary education). More details on the application of G-formula with time-varying confounders adjustment can be found in **Supplementary File 1**. As a sensitivity analysis, we also performed a linear regression on the association between MBzP at B4 only and breast density at 2YPM without the adjustment of B1 exposure.

We used SAS 9.4 software (SAS Institute Inc.) for all analyses.

3. Results

3.1. PFOA and breast density

The current analysis of PFOA and breast density included two subsamples, 333 girls with serum samples in B4 and 276 girls with serum samples with both B4 and 1YPM (Table 1**)**. In both subsamples, the mean age at B4 was 10.9 years, body fat percentage at B4 was 27.1%, age at menarche was 12 years, and birthweight 3.3 kg. The proportion of maternal education was nearly the same in both subsamples, 78% with secondary education or less and 22% with greater than secondary education. In the B4 only subsample, the geometric mean concentration of PFOA at B4 was approximately 1.3 ng/ml. In the sample with both B4 and 1YPM, the geometric mean PFOA concentration at B4 was 1.2 ng/ml and at 1YPM was 0.98 ng/ml, mean age at 1YPM of 12.5 years, and body fat percentage at 1YPM of 30%. Both subsamples had similar age at 2YPM of 13.5 years, and body fat percentage at 2YPM of 32.4%. Breast density measurements were similar in both subsamples, absolute FGV ranging from 215.5 to 218.8 cm^3 and %FGV approximately 51%.

Under the hypothetical intervention of PFOA exposure at B4 only, a doubling of PFOA concentration at B4 resulted in a non-significant increase in absolute FGV (β: 11.25, 95% confidence interval (CI): −0.28, 23.49). The joint total effect of doubling in PFOA concentrations at both B4 and 1YPM resulted in non-significant effect of PFOA on absolute FGV (β: 20.64, 95% CI: −10.77, 49.35). On the other hand, a doubling of PFOA concentration at 1YPM resulted in a decrease in % FGV (β: −4.61, 95% CI: −7.45, −1.78) (Table 2).

Table 1
Characteristics of girls in the Growth and Obesity Cohort Study with breast density at 2-years post-menarche and serum sample at Tanner breast stage B4 (n = 333) and serum samples at both Tanner breast stage B4 and 1-year post-menarche (n = 276).

Characteristic	Window of Susceptibility	
	B4	B4 and 1YPM[a]
	(n = 333)	(n = 276)
Serum Perfluorooctanoic Acid (PFOA), ng/ml (mean (SD))		
Tanner Stage B4	1.27 (0.79)	1.24 (0.76)
1-Year Post-Menarche	–	0.98 (0.57)
Age, years (mean (SD))		
Tanner Stage B4	10.89 (0.93)	10.93 (0.91)
1-Year Post-Menarche	–	12.54 (0.98)
Body fat percentage (mean (SD))		
Tanner Stage B4	27.06 (5.21)	27.11 (5.18)
1-Year Post-Menarche	–	30.16 (5.14)
Age at menarche, years (mean (SD))	12.01 (0.93)	12.09 (0.88)
Birthweight, kg (mean (SD))	3.36(0.42)	3.34 (0.43)
Maternal education (n (%))		
Secondary education or less	262 (78.68)	215 (77.90)
Greater than secondary education	71 (21.32)	61 (22.10)
2-Year Post- Menarche (2YPM)		
Age at 2YPM	13.50 (1.06)	13.57 (1.03)
Body fat percentage at 2YPM (mean (SD))	32.46 (5.82)	32.42 (5.67)
Absolute Fibroglandular Volume, cm^3 (mean (SD))	218.83 (77.23)	215.51 (78.17)
Percent Fibroglandular Volume, % (mean (SD))	50.76 (15.14)	50.70 (15.00)

[a] Subsample of girls with serum samples at B4 and 1YPM.

3.2. MBzP and breast density

The current analysis of MBzP and breast density included two subsamples, 292 girls with urine samples in B1 and 255 girls with samples in both B1 and B4 (Table 3). In both subsamples, the mean age at B1 was 7.4 years, body fat percentage at B1 of 25%, age at menarche approximately 12 years old, birthweight 3.34 kg. The proportion of maternal education was nearly the same in both subsamples, 77% with secondary education or less and 23% with greater than secondary education. The geometric mean concentration of MBzP at B1 was 6.8 ng/ml. In the sample with both B1 and B4, the mean MBzP concentration at B1 was 6.83 ng/ml and at B4 was 3.54 ng/ml, mean age at B4 of 11 years, and body fat percentage at B4 of 27%. Both subsamples had similar age at 2YPM of 13.7 years, and body fat percentage at 2YPM of 32%. Breast density measurements were similar in both subsamples, absolute FGV ranging from 213.7 to 215.9 cm^3 and %FGV ranging from 50.2 to 51.5%.

Overall, our results do not support a relation between MBzP and % FGV and absolute FGV, under all hypothetical interventions of specific WOS (Table 4). Under the hypothetical intervention of BBP exposure at B1 only, a doubling of MBzP concentration present at B1 resulted in minimal evidence of 0.84 cm^3 decrease in absolute FGV at 2YPM (β: −0.84, 95%CI: −6.19, 4.78). On the other hand, hypothetical interventions of MBzP at B4 only (β: 3.39, 95% CI: −1.89, 8.88) and joint total effect of B1 and B4 resulted in minimal evidence of increase in absolute FGV at 2YPM (β: 2.88, 95% CI: −3.58, 9.99).

3.3. Associations between both MBzP and PFOA and breast density

In a subset of girls who provided both urine and serum samples at B4 (n = 241), we analyzed the joint total effect of MBzP and PFOA at B4 on breast density at 2YPM **(**Table 5**).** Doubling both MBzP and PFOA concentrations at B4 resulted in a non-significant increase in absolute FGV (β: 4.29, 95% CI: −4.14, 12.83), with no evidence of an interaction between MBzP and PFOA at B4 (p-value: 0.71), and a null effect on % FGV (β: 0.11, 95% CI: −1.65, 1.77), with no evidence of an interaction between MBzP and PFOA at B4 (p-value: 0.32).

4. Discussion

Puberty is one of the critical periods for breast development, during which substantial growth occurs in the epithelial, stromal, and adipose tissues. After puberty, there is minimal development in the epithelial and stromal tissue, suggesting that much of the absolute dense volume observed during puberty is carried over to young women before their first pregnancy. (Ghadge et al., 2020) Therefore, the significant changes in pubertal breast density, both absolute and percent, may be predictive of adult breast density, a major risk factor for breast cancer. Our study adds to the limited research evaluating the effect of selected EDCs on breast density at specific pubertal WOS. Of note, the current study is the first to use G-formula to assess effect estimates under hypothetical interventions of specific WOS timepoints as well as the joint total effect of these timepoints.

The current study is part of the Breast Cancer and the Environmental Research Program (BCERP), a consortium evaluating the role of WOS and exposure to environmental chemicals in breast cancer etiology. In parallel to our study, a mice study in BCERP examined the effect of pubertal exposure to BBP, PFOA, and zeranol on mammary gland development. (Su et al., 2022) The study found PFOA + zeranol exposure to induce the most phenotypic and transcriptomic changes in the

Table 2
Simulated relative change[a] in breast density associated with log2 (ng/ml) increase in serum perfluorooctanoic acid (PFOA) among Growth and Obesity Cohort Study participants with breast density at 2-years post-menarche and serum sample at Tanner breast stage B4 (n = 333) and serum samples at both Tanner breast stage B4 and 1-year post-menarche (n = 276).

Window of Susceptibility		Absolute Fibroglandular Volume			Percent Fibroglandular Volume		
		Estimate of relative change	Standard Error	95% Confidence Interval	Estimate of relative change	Standard Error	95% Confidence Interval
Tanner Stage B4[b]	N = 333	11.25	6.03	(-0.28, 23.49)	1.20	1.14	(-1.03, 3.52)
1-Year Post-Menarche (1YPM)[c]	N = 276	−13.12	7.39	(-27.76, 2.16)	−4.61	1.39	(-7.45, −1.78)
Joint total effect of B4 and 1YPM[d]	N = 276	20.64	14.82	(-10.77, 49.35)	−0.11	2.64	(-5.26, 4.93)

[a] Estimates of relative change accounted for covariates mother's education, birthweight, body fat%, age at 2YPM, age at menarche. 1YPM only analysis additionally accounted for B4 PFOA concentrations.
[b] relative change in breast density when doubling PFOA concentration at Tanner Stage B4 only, regardless of subsequent concentration at 1YPM.
[c] relative change in breast density when doubling PFOA concentration at 1YPM only, regardless of prior concentration at Tanner Stage B4.
[d] relative change in breast density when doubling PFOA concentration at both Tanner Stage B4 and 1YPM.

Table 3
Characteristics of girls in the Growth and Obesity Cohort Study with breast density at 2-years post-menarche and urine sample at Tanner breast stage B1 (n = 292) and urine samples at both Tanner breast stage B1 and B4 (n = 255).

Characteristic	Window of Susceptibility	
	B1	B1 and B4[a]
	(n = 292)	(n = 255)
Urine Monobenzyl Phthalate (MBzP), ng/ml (mean (SD))		
Tanner Stage B1	6.75 (15.72)	6.83 (16.49)
Tanner Stage B4	–	3.54 (6.76)
Age, years (mean (SD))		
Tanner Stage B1	7.40 (0.56)	7.40 (0.56)
Tanner Stage B4	–	11.02 (0.94)
Body fat percentage (mean (SD))		
Tanner Stage B1	25.60 (4.49)	25.27 (4.38)
Tanner Stage B4	–	26.71 (5.39)
Age at menarche, years (mean (SD))	12.15 (0.95)	12.18 (0.90)
Birthweight, kg (mean (SD))	3.34 (0.42)	3.34 (0.41)
Maternal education (n (%))		
Secondary education or less	225 (77.05)	197 (77.25)
Greater than secondary education	67 (22.95)	58 (22.75)
2-Year Post- Menarche (2YPM)		
Age at 2YPM	13.65 (1.09)	13.69 (1.05)
Body fat percentage at 2YPM (mean (SD))	32.52 (6.12)	32.08 (6.03)
Absolute Fibroglandular Volume, cm^3 (mean (SD))	213.69 (81.07)	215.87 (78.07)
Percent Fibroglandular Volume, % (mean (SD))	50.24 (15.43)	51.47 (15.46)

[a] Subsample of girls with urine samples at B1 and B4.

mammary gland, while no effect was observed in the BBP + PFOA exposure. As EDCs are more often present in mixtures than single mixtures, it is possible that certain mixtures induce synergistic effects.

4.1. PFOA and breast density

The observed PFOA concentrations in our study were comparable to PFOA concentrations in the U.S. general population of girls aged 12–19 years old (median 1.17 ng/ml). (Centers for Disease Control and Prevention. Perfluoroalkyl and Polyfluoroalkyl Substances: Surfactants, 2023) For PFOA, we observed a non-significant increase in absolute FGV when doubling the PFOA concentration at B4 only and at B4 and 1YPM together (i.e. joint total effect). These findings suggest that breast development may have WOS to PFOA throughout puberty, with heightened sensitivity during assessment at B4. The breast may be more susceptible to EDCs during the B4 stage, in which the breast tissue experiences exponential growth with rapid differentiation and proliferation of TEBs, compared to 1YPM when the breast is relatively mature (Russo and Russo, 2004). On the other hand, a doubling of PFOA concentration at 1YPM resulted in a decrease in % FGV (β:-5.63, 95% CI: −8.29, −3.04). This may be due to residual confounding by fatty tissues in the breast that is not entirely captured by our fat percentage covariate. Overall, our results indicate a potential differential WOS of PFOA on breast density, though we cannot rule out significant findings by chance.

A mice study by Tucker et al. focused on prenatal PFOA exposure observed significant delays in mammary gland development, which persisted into young adulthood but had no effect on pubertal timing onset (Tucker et al., 2015). These experimental studies underscore the importance of exposure WOS, both prenatal and pubertal, as critical periods in which PFOA may alter breast development.

Most observational studies examining the effect of early life PFOA exposure focused on anthropometric indicators of adiposity, including birthweight and BMI, waist-to-height ratio, and waist-to-hip ratio in children and adolescents. PFOA effects on adiposity measures varied by specific exposure assessment timepoints (i.e. prenatal, 2-week postpartum maternal, and adolescent), suggesting differential WOS for the relation with adiposity. The Healthy Start Study of 628 U.S. mother-infant pairs in Colorado assessed prenatal exposure to PFOA on birthweight. PFOA concentration from maternal serum collected at 27 weeks gestation was inversely associated with birthweight (PFOA highest tercile vs. lowest tercile: ß −92.4 g, 95%CI −166.2 g, −18.5 g). (Starling et al., 2017) A prospective study with 490 mother–child pairs from the Faroe Islands found that 2-week postpartum maternal PFOA concentrations were significantly associated with increased risk of the child being overweight at 5 years old, adjusting for child sex, duration of breastfeeding, and maternal pre-pregnancy BMI (per log10 unit increase of PFOA ng/ml: ß 1.50, 95%CI 1.01, 2.24). (Karlsen et al., 2017) On the other hand, a cross-sectional study including girls 12–19 years old in Cincinnati and San Francisco Bay area found higher median PFOA concentrations in the sample compared to the average U.S. population PFOA concentrations (5.8–7.3 ng/mL vs. 3.8 ng/mL), with log-transformed PFOA concentrations having a strong inverse association with BMI z-score but not with waist-to-height nor with waist-to-hip ratio (per log10 unit increase of PFOA ng/ml: ß −0.264, 95%CI −0.416, −0.112). (Pinney et al., 2019) Collectively, these observational studies suggest an effect of PFOA on adiposity measures that varies with age at exposure assessment, which further highlights the importance of using G-formula to avoid adjusting for a potentially important mediator, fat percentage, in the association between PFOA and breast density.

4.2. MBzP and breast density

The observed MBzP concentrations were lower in our study than the MBzP concentrations reported in previous analysis of adolescent girls

Table 4
Simulated relative change[a] in breast density associated with log2 (ng/ml) increase in urine monobenzyl phthalate (MBzP) among Growth and Obesity Cohort Study participants with breast density at 2-years post-menarche and urine sample at Tanner breast stage B1 (n = 292) and urines samples at both Tanner breast stages B1 and B4 (n = 255).

Window of Susceptibility		Absolute Fibroglandular Volume, cm^3			Percent Fibroglandular Volume, %		
		Estimate of relative change	Standard Error	95% Confidence Interval	Estimate of relative change	Standard Error	95% Confidence Interval
Tanner Stage B1[b]	N = 292	−0.84	2.74	(-6.19, 4.78)	0.09	0.45	(-0.78, 1.03)
Tanner Stage B4[c]	N = 255	3.39	2.73	(-1.89, 8.88)	0.22	0.50	(-0.80, 1.18)
Joint total effect of B1 and B4[d]	N = 255	2.88	3.52	(-3.58, 9.99)	0.27	0.69	(-1.03, 1.65)

[a] Estimates of relative change accounted for covariates mother's education, birthweight, body fat%, age at 2YPM, age at menarche, creatinine. Tanner Stage B4 only analysis additionally accounted for B1 MBzP concentrations.
[b] relative change in breast density when doubling MBzP concentration at Tanner Stage B1 only, regardless of subsequent concentration at Tanner Stage B4.
[c] relative change in breast density when doubling MBzP concentration at Tanner Stage B4 only, regardless of prior concentration at Tanner Stage B1.
[d] relative change in breast density when doubling MBzP concentration at both Tanner Stage B1 and B4.

Table 5
Simulated relative change[a] in breast density associated with log2 (ng/ml) increase in urinary monobenzyl phthalate (MBzP) and serum perfluorooctanoic acid (PFOA) among Growth and Obesity Cohort Study participants with breast density at 2-years post-menarche and urine and serum samples at Tanner breast stage B4 (n = 241).

Window of Susceptibility		Absolute Fibroglandular Volume			Percent Fibroglandular Volume		
		Estimate of relative change	SE	95% Confidence Interval	Estimate of relative change	SE	95% Confidence Interval
Tanner Stage B4[b]	N = 241	4.29	4.31	(-4.14, 12.83)	0.11	0.86	(-1.65, 1.77)

[a] Estimates of relative change accounted for covariates mother's education, birthweight, body fat%, age at 2YPM, age at menarche, creatinine (MBzP only).
[b] joint total effect of MBzP and PFOA concentrations at Tanner Stage B4. Relative change in breast density when doubling both MBzP and PFOA at Tanner Stage B4 regardless of other WOS exposures.

data from the U.S. National Health and Nutrition Examination Survey with median 9.2 ng/mL and 16.0 ng/ml (Silva et al., 2004; Zeng et al., 2022). This may have contributed to our null finding of MBzP on both absolute FGV and %FGV. This result is consistent with the finding in our previous GOCS study, which analyzed the association between MBzP urinary concentrations at B1 and B4 and breast density at B4 among a smaller subset of GOCS participants (Binder et al., 2018). Similarly, a longitudinal cohort study of girls aged 6–8 years from BCERP reported null findings of pubertal exposure to phthalates of high molecular weight (including BBP, the parent compound of MBzP) with breast development (Wolff et al., 2010). However, a separate analysis of the same BCERP cohort observed a later age for breast development (Tanner Stage B2) for girls with higher prepubertal MBzP concentrations (Wolff et al., 2014).

While the associations between MBzP and human adolescent breast development remain unclear, animal studies provide further evidence of the potential detrimental effect of BBP/MBzP on overall growth and pubertal development. A mice study found that a high dose of BBP and its monoester metabolite MBzP can produce developmental and reproductive toxicity in rodents (Program and Monograph, 2003). Another found neonatal and prepubertal exposure of BBP to affect gene expression profile in mammary gland tissue of mice. The study also reported, while there were no significant morphological changes of the mammary gland, there was an increase in proliferative index in TEBs and in lobule I (Moral et al., 2007). Similarly, it may be the case that our null findings were reflective of the unaffected morphology of the breast tissues.

4.3. Strengths and limitations

A limitation of our study is the potential of exposure misclassification resulting from a single spot urine and serum collection at each timepoint, especially for MBzP which has a relatively short half-life (Hoppin et al., 2002). However, studies suggest that even a transient but consistent or repeated exposure to phthalates may result in adverse health outcomes (Wang and Qian, 2021). Given the ubiquitous nature of most phthalates, metabolite concentrations of phthalates were found to have moderate to good correlation over weeks or months in children (Watkins et al., 2014; Teitelbaum et al., 2008). Conversely, PFOA has a much longer half-life of approximately 3.5 years, meaning the PFOA measurement at 1YPM may also reflect PFOA exposure that happened at or before B4 (Olsen et al., 2007). It is important to note that our study included biomarker concentrations at two different WOS timepoints across puberty, compared to a single time period in other cohorts, allowing for examination of EDCs and breast density across specific pubertal stages. Lastly, while we assessed MBzP and PFOA individually and together, we cannot assess the effect of mixtures of other EDCs, which may have biased our results. However, analyzing mixtures can result in complex interactions and confounding factors. Focusing on single analytes allow for a more specific, practical intervention for chemicals of known concern (i.e. underlying mechanisms and pathways involved) rather than entire mixtures.

Our study is the first to investigate how PFOA and MBzP affect pubertal breast density, a breast cancer predictor. Results show inconsistent associations through pubertal timepoints, suggesting that breast development may have differential window of susceptibility for environmental factors. Our research contributes to an understanding of the variation in breast cancer risk associated with environmental exposures early in life helping to identify the most effective and appropriate time period for breast cancer prevention. Specific strengths of our study include the prospective data collection and the ability to assess the critical periods of EDC exposure on pubertal breast density. The lack of randomization in observational studies limits casual interpretation of the results. However, the use of G-formula in conjunction with the identifiability assumptions, we assessed the effect of MBzP and PFOA on breast density as opposed to cross-sectional associations. Additionally, the method permits the estimation of a single, marginal effect estimate

averaged across the observed distribution of the covariates. This way, we avoid overadjustment of the models by including covariates like fat percentage that serve as both a confounder and a mediator between the EDCs and breast density. Lastly, under the numerous hypothetical interventions developed based the casual DAG, we are able to estimate the effect of EDCs from a specific WOS (e.g. B1 only, B4 only for MBzP; B4 only, 1YPM only for PFOA) as well as the joint total effect of EDCs (e.g. both B1 and B4 for MBzP; both B4 and 1YPM for PFOA; both MBzP and PFOA at B4). Secondary analyses using standard linear regression were performed to explore whether our current results in differed direction and strength (results not shown). While there were overlapping confidence intervals between our current results and standard analyses, all the secondary analyses resulted in null effects of MBzP and PFOA on breast density.

5. Conclusion

EDCs concentrations measured at different timepoints throughout puberty can have varying impacts on pubertal breast density. In our study PFOA exposure evaluated at B4 resulted in a non-significant increase in absolute FGV, while PFOA exposure assessed at 1YPM resulted in a decreased pubertal % FGV. We did not find an effect of MBzP on either absolute FGV and %FGV during puberty.

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Claire E. Kim: Conceptualization, Formal analysis, Writing – original draft. **Alexandra M. Binder:** Conceptualization, Writing – review & editing. **Camila Corvalan:** Conceptualization, Resources, Writing – review & editing. **Ana Pereira:** Writing – review & editing. **John Shepherd:** Methodology, Writing – review & editing. **Antonia M. Calafat:** Investigation, Writing – review & editing. **Julianne C. Botelho:** Investigation, Writing – review & editing. **John M. Hampton:** Formal analysis, Writing – review & editing. **Amy Trentham-Dietz:** Writing – review & editing. **Karin B. Michels:** Conceptualization, Resources, Writing – review & editing, Supervision, Funding acquisition.

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.

Data availability

The data that has been used is confidential.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2023.108241.

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