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Associations between perfluorooctanoic acid (PFOA), monobenzyl phthalate (MBzP), and trace metals and hormones and breast density among adolescent girls in Santiago, Chile

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Epidemiology

by

Claire EuiYoung Kim

2022

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ABSTRACT OF THE DISSERTATION

Associations between Perfluorooctanoic acid (PFOA), Monobenzyl phthalate (MBzP), and trace metals and hormones and breast density among adolescent girls in Santiago, Chile

by

Claire EuiYoung Kim

Doctor of Philosophy in Epidemiology

University of California, Los Angeles, 2022

Professor Karin B. Michels, Chair

Introduction: Breast cancer is the most common cancer and the leading cause of cancer-related deaths among women in the world. Yet, much of the variation of breast cancer incidence cannot be explained by known or suspected risk factors. Its etiology is comprised of several pathways, some of which point to origins during early life. Specifically, breast development and hormonal changes during puberty contribute to a 'critical period' that play an important role in affecting breast cancer risk. The heightened hormonal activity followed by rapid growth in breast tissues presents a window of susceptibility in which environmental exposures can cause significant changes that may or may not be reversible. Environmental exposures, such as endocrine disrupting chemicals (EDCs), have been purported to alter pubertal development, raising concerns about their impact on breast development and subsequently on breast cancer risk. Perfluorooctanoic acid (PFOA), monobenzyl phthalate (MBzP), and trace metals may mimic estrogen behavior and disrupt aspects of endocrine signaling. The central purpose of this research is to use epidemiologic methods to evaluate the effect of EDCs on mammary gland development and circulating hormone levels during puberty. Innovative computational methods

(e.g. G-methods) are used to identify and estimate causal effects of real-life exposures of “EDC mixtures” (i.e. accessing multiple EDCs together) and time-specific EDCs on breast density in the presence of time-varying confounders. The main objectives include: 1) to evaluate the impact of serum PFOA and urinary MBzP, individually and in combination, on breast composition during puberty; 2) to evaluate the impact of serum PFOA and urinary MBzP, individually and in combination, on hormonal levels during puberty; and 3) to evaluate the effect of trace metals on breast composition during puberty.

Methods: This dissertation uses the longitudinal Growth and Obesity Cohort Study (GOCS) of Latina girls in Santiago, Chile. GOCS prospectively followed children from ages 3-4 and obtained data annually and bi-annually on biomarker, anthropometric, demographic, and breast composition data. Urinary MBzP and trace metals concentrations and serum PFOA concentrations were assessed by liquid chromatography mass spectrometry. Breast composition was measured using dual-energy x-ray absorptiometry and evaluated as percent fibroglandular volume (%FGV) and absolute fibroglandular volume (aFGV). Chapter 2 evaluates the relation between MBzP and PFOA and breast composition across three pubertal time points (Tanner breast stage 1 (B1), Tanner breast stage 4 (B4), and 1-year post menarche (1YPM)) and breast composition (%FGV and aFGV) measured at 2-years post-menarche. Chapter 3 assesses the relation between MBzP and PFOA and hormones at 1YPM. Chapter 4 examines whether trace metals are associated with breast composition. All three chapters utilize parametric G-formula for analysis accounting for covariates that are identified a priori using directed acyclic graphs.

Results: In Chapter 2, serum PFOA concentrations corresponded to a marginal increase in absolute FGV and decreased in % FGV, while no effect was observed between MBzP and breast density measures across pubertal WOS. In Chapter 3, MBzP was associated with insulin growth factor-1 (IGF-1) and 17-OH progesterone. PFOA was associated with IGF-1 (IGF-1), 17-

OH progesterone and sex hormone binding globulin. Associations between MBzP and PFOA and hormones varied by pubertal time point, suggesting differential effects of EDC exposure by specific window of susceptibility on pubertal hormone levels. In Chapter 4, selected trace metals - barium, copper, lead, antimony, thallium, and vanadium – were associated with absolute breast density. Copper was also associated with percent FGV.

Conclusions: Overall, PFOA, MBzP and trace metals were associated with breast density and hormones at varying pubertal windows of susceptibility. By using the framework of life course epidemiology, this dissertation contributes to an understanding of the variation in breast cancer risk associated with environmental exposures in puberty. Findings help identify the most effective and appropriate time period for breast cancer prevention.

The dissertation of Claire EuiYoung Kim is approved.

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2022

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Acknowledgements

First and foremost, I would like to thank my advisor Dr. Karin Michels for her commitment to advancing my scientific career and scholarly growth. In these past four years, Dr. Michels has provided me with a wide range of research opportunities that has not only allowed me to deepen my knowledge and skills as an epidemiologist but also to develop curiosity in content areas that I've grown to love and enjoy learning about. I am also very grateful to Dr. Onyebuchi Arah who has truly been an inspiration for pursuing epidemiologic research. Dr. Arah's classes on causal inference were one of the more challenging yet the most rewarding classes I've taken at UCLA. I am honored to have had Dr. Arah on my committee for bringing challenge and rigor to my studies all the while fostering my passion for public health research. Dr. Alexandra Binder was my primary resource for getting my scientific questions answered and was instrumental in helping me complete this work. I sincerely thank Dr. Binder for being the perfect mentor I needed. I also thank Dr. Catherine Crespi who has always greeted me with warmth and support in the difficult months of dissertation work. My dissertation research was made possible by Dr. Camila Corvalan who has graciously allowed me to use the Growth and Obesity Cohort Study data. I am grateful to the other members of Dr. Corvalan's team, specifically Dr. Ana Pereira. This dissertation would not have been possible without their scientific support and technical expertise.

I would also like to express my gratitude to my mentors who are not members of the doctoral committee- Dr. Zuo-Feng Zhang and Dr. Roch Nianogo. From the start of UCLA journey, Dr. Zhang has provided me with the NIH T32 predoctoral fellowship in cancer epidemiology for which I am truly grateful for. I also thank Dr. Zhang for being a great motivator, encouraging me to work hard, study hard and not to lose focus on my academic and career goals. Dr. Roch Nianogo has taught me epidemiological methods with great patience. I will

always be appreciative of Dr. Nianogo's kindness and care towards his mentees. I also thank my UCLA sunbae, Jeewon Park who has been a tremendous help in my casual inference analyses.

I wish to acknowledge my friends who have been instrumental to my education and progress as a scientist. To Jane Lee who has been my biggest fan and supporter, I cannot express how lucky I am to have you as my big sister, confidant, and mentor. To Juhyun Kim, thank you for being the most supportive friend and always keeping me humble with your kindness. To Lara Yoon, from the UCLA welcome gift (cat lunch box) to providing constant feedback on my work, you've been the best lab mate/senior and friend I could've asked for. To Sylvia Tangney, our 'workout' dates, poke sessions, and everything in-between, they all meant the world to me during my time here in LA. To Han Sihao and Jiemin Yao, from the start till the end, you guys have always been there for me, making any bad day or week instantly better. To my childhood friends Esther Kim and Jenny An, thank you for bringing all the joy and fun into my life.

Finally, I dedicate my dissertation to my family who has made this all possible. Mom and Dad, for as long as I remember, you've always encouraged me to pursue my passion- whatever it may be. Your undying support, trust and confidence in my academic, career and overall life decisions have made me persevere and complete this achievement. Thank you for being my parents. Another huge thank you to my brother Matt and sister-in-law Tracy for motivating, supporting, encouraging, and inspiring me to be the best that I can be. To my niece Hailey, your existence has just made my life brighter. To my pups Louie and Roscoe, thank you for sticking by my side during the long late hours of work. Lastly, a special thank you to my grandparents who have started this public health, epidemiology, cancer research journey for me.

"Time-specific Impact of Mono-Benzyl Phthalate (MBzP) and Perfluorooctanoic Acid (PFOA) on Breast Density of Adolescent Latino Girls" (Chapter 2) is in preparation for journal submission. Co-authors on this manuscript include Alexandra M. Binder, Camila Corvalan, Ana Pereira, John Shepherd, Antonia M. Calafat, Julianne C. Botelho, John M. Hampton, Amy Trentham-Dietz, and Karin B. Michels. CEK, AMB, and KBM conceptualized and designed the study. AP, AMC, JCB, JS, CC, and KBM collected and analyzed data for the study. Statistical analyses were completed by CEK and supervised by AMB, JMP, ATD and KBM. CEK drafted the manuscript, and all other authors were involved in critical interpretation and revision of the manuscript.

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CEK was supported by T32 training grant 2T32CA009142-41 from the National Cancer Institute, National Institutes of Health. This dissertation was additionally supported by the following research grants: Public Health Service grant U01ES026130 from the National Institute of Environmental Health Sciences and the National Cancer Institute, National Institutes of Health and Public Health Service grant R01CA158313 from the National Cancer Institute, National Institutes of Health (to KBM); U2CES026561 from the National Institute of Environmental Health Sciences, National Institutes of Health; U01ES026127, P30 CA014520 National Institutes of Health (to ATD);

The content of this dissertation is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health nor the official position of the CDC. Use of trade names is for identification only and does not imply endorsement by the CDC, the Public Health Service, or the US Department of Health and Human Services.

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2. Shin, S., Lim, J., Lee, H., **Kim, CE.**, Kim, S., Lee, J., Kang, D. (2019). Association between the prevalence of metabolic syndrome and coffee consumption among Korean Adults: Results from the Health Examinees Study. *Appl Physiol Nutr Metab.* 44 (12), 1371-1378.
3. **Kim, CE.**, Shin, S., Lee, H., Lim, J., Lee, J., Shin, A., Kang, D. (2018). Association between Sleep Duration and Metabolic Syndrome: A Cross-sectional Study. *BMC Public Health.* 18 (1):720.
4. Lim, J., Lee Y., Shin, S., Lee, H., **Kim, CE.**, Lee, J., Kang, D. (2018). Association between diet quality index for Koreans (DQI-K) and total mortality in Health Examinees Gem (HEXA-G) Study. *Nutrition Research and Practice.* 12 (3), 258-264.
5. **Kim, CE.**, Shin, S., Lee, H., Lim, J., Lee, J., Kang, D. (2017). Frequency of Loud Snoring and Metabolic Syndrome among Korean Adults: Results from the Health Examinees (HEXA) Study. *International Journal of Environmental Research and Public Health.* 14 (11), 1294.
6. Shin, S., Lee, H., **Kim, CE.**, Lim, J., Lee, J., Kang, D. (2017). Association between Milk Consumption and Metabolic Syndrome among Korean Adults: Results from the Health Examinees Study. *Nutrients.* 9(10), 1102.
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SELECTED PROFESSIONAL PRESENTATIONS

1. **Kim, CE.,** Binder, AM., Corvalan, C., Pereira, A., Hampton, J., Trentham Dietz, A., Michels, KB. (August 2022). *Time-specific Effect of Perfluorooctanoic Acid (PFOA) and Monobenzyl Phthalate (MBzP) on Breast Density of Adolescent Girls in Santiago, Chile.* Poster presentation at The Developmental Origins of Health and Disease. Vancouver, Canada.
2. **Kim, CE.,** Shin, S., Lee, H., Lim, J., Lee, J., Kang, D. (October 2017). *Alcohol Consumption and Metabolic Syndrome among Korean men: Results from the Health Examinees Study*. Poster presentation at The Korean Society for Preventive Medicine. Busan, South Korea.

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1. "Breast Cancer Prevention: Before the lumps and bumps". *UCLA Grad Slam Challenge.* 14 May 2021. Available: https://grad.ucla.edu/life-at-ucla/events/2021-ucla-grad-slam-challenge/presentations/
2. "Sleep duration and metabolic syndrome". *BBC World Series radio.* 20 June 2018. Available: https://www.bbc.co.uk/programmes/w3cswjkl
3. "Snoring increases chance of adult diseases: Male 2.1 times greater risk, Female 1.5 times greater risk". *SBS News,* 03 November 2017. Available: http://news.sbs.co.kr/news/endPage.do?news_id=N1004466633&plink=ORI&cooper=NAVER

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Chapter 1. Introduction

Epidemiology of breast cancer

Breast cancer is estimated to be the most commonly diagnosed cancer (11.7% of total cancer cases) worldwide in 2020. It accounts for a quarter of cancers diagnosed among females, with approximately 2,261,419 estimated female incident breast cancer cases (hereafter simply ‘breast cancer’). Approximately 684, 996 breast cancer deaths were estimated, with an age-standardized incidence rate (aSIR) of 47.8 and age-standardized mortality (aSMR) rates of 13.6 cases per 100,000, respectively.[1]

In the United States (U.S.), breast cancer incidence rates have continued to increase about 0.5% per year, while the decline in mortality rates slowed since 2009. Approximately 281,550 incident breast cancer cases and 43,600 breast cancer deaths are estimated to occur in 2021, accounting for 30% of new cancer cases and 15% of cancer deaths among females.[2] In 2020, U.S. is among the top 10 countries with the highest breast cancer aSIR, with 93.3 cases per 100,000 females. In contrast, the aSMR was relatively low, with 12.4 cases per 100,000.[1] Based on 2015-2017 SEER data, approximately 12.9 percent of women will be diagnosed with breast cancer during their lifetime.[3]

In Chile, breast cancer is the second most diagnosed cancer (after prostate cancer), with an estimated 4,737 incident breast cancer cases and 1,256 breast cancer deaths in 2020. The estimated aSIR is 36 cases per 100,000 and aSMR 9.1 deaths per 100,000 women. Due to the aging population, the breast cancer incidence is predicted to increase by 35% and the breast cancer mortality is predicted to increase by 54.7% by 2040, respectively.[4]

Breast cancer risk factors

As with most chronic illnesses, the risk of breast cancer increases with older age. Women over the age of 55 years account for approximately 70% of all new breast cancers, with the median

age at diagnosis being 62 years.[3] Inherited genetic mutations (e.g. breast. Cancer gene 1 (BRCA1), breast cancer gene 2 (BRCA2), tumor protein (p53)) account for only 5 to 6 percent of all breast cancers.[5]

Postmenopausal breast cancer risk factors include menopausal weight gain, bone mineral density, hormone replacement therapy, and delayed menopause.[6–9] Both pre-and postmenopausal risk factors include body mass index (BMI), breast density, increased height, earlier age at menarche, nulliparity and multiparity, late age at first pregnancy, personal and family history of breast cancer, alcohol consumption, smoking, night-shift work, and ionizing radiation.[10–17] Of note, ages at menarche, first pregnancy, lactation, parity and menopause all reflect period-specific exposure to ovarian hormones, underscoring the hormone-dependent nature of breast cancer.

The importance of estrogen in breast tumorigenesis has been well-established. In post-menopausal women, treatment with exogenous estrogen increased the risk of breast cancer.[18] Similarly, the use of tamoxifen, a selective estrogen receptor modulator, was associated with lower risk of primary invasive breast cancer in both pre- and postmenopausal women.[19] On the other hand, surgical removal of ovaries in premenopausal women decreased the risk of breast cancer.[20]

Breast density and composition

Many hypothesized that both exogenous and endogenous hormones affect breast cancer risk partly mediated by breast density. Breast density is one of the strongest and well-established risk factors for breast cancer. Relative to other common risk factors, it has the highest population attributable fraction (28.9%; 95% Confidence Interval (CI) 25.3-32.5%) for breast cancer.[21] Women with higher breast density have a 4-to 5 times higher risk of breast cancer compared to women with less or no dense breast tissue, adjusted for age.[22–25]

The breast is composed of three kinds of tissue- fibrous, glandular, and fatty tissues. Fibrous tissues are connective (stroma) tissue made up of collagen fiber bundles and provide structure of the breasts. Glandular (parenchyma) tissue contains lobules and ducts that are made of epithelial cells. The lobules are glands that produce milk and ducts are small tubes that carry the milk to the nipple. Fibrous and glandular tissues collectively make up the "fibroglandular" tissue which is considered the "dense" part of the mammary gland (hereafter termed 'breast density'). Fatty tissues, composed of adipose cells, are specialized for lipid storage, act as filler tissue between the fibrous and glandular tissues, giving breasts their size and shape.[26]

Critical periods and windows of susceptibility

Developmental Origins of Health and Disease (DOHaD) emphasizes the importance of recognizing early life exposures and their influence on the presentation of disease during adulthood.[27] This concept serves as the basis for the lifecourse approach which focuses on understanding the long-term effects of exposures during gestation, childhood, adolescence, young adulthood and later adult life on future health and disease risk.[27] Epidemiologic evidence from observational studies indicates that the risk of lifelong adverse health effects is enhanced when exposure periods for disease factors coincide with the formation and differentiation of organ systems during early development.[28] In other words, organ formation and differentiation represents periods of developmental plasticity that can be considered "critical periods" where an exposure can cause significant changes that may or may not be reversible.[29]

This idea of critical periods in breast development was first alluded by Pike in 1983. Pike recognized that breast tissue does not age with chronological age, but with critical breast development and differentiation periods.[30] As such, breast development is described by four distinct, critical periods: *in utero*, pubertal, post-partum, and peri-menopause periods.[31] These early life periods represent the windows of susceptibility (WOS) for environmental exposures.[32]

Unfortunately, methodological challenges exist in evaluating the effect of exposures at each WOS on breast cancer. Principally, the long induction period inevitably decreases the reliability and accuracy of our exposure measurement and increases the possibility of misclassification and confounding by later-life exposures making it difficult to make causal inference.[33] However, evaluating environmental exposures for specific WOS will not only further our understanding of environmental influences during pubertal activity, but also help identify the most effective and appropriate time period for breast cancer prevention.

Puberty and pubertal breast density

Puberty is the period in which adolescents undergo major, rapid physical and physiological changes, reaching sexual maturity and achieving fertility.[34] It is one of the critical periods for breast development during which substantial growth occurs in the epithelial, stromal and adipose tissues.[35] 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.[36] This highlights the critical period of puberty and its window of susceptibility for breast development as alterations in the normal function of the endogenous hormone levels during puberty may have permanent and irreversible effects on breast development which may subsequently affect breast cancer susceptibility later in life.[27,37]

Individual variation in pubertal timing may be influenced by genetic as well as external factors such as environmental exposures.[38] A wide range of environmental chemicals during the early development periods are now being investigated as a driving force for epigenetic disruptions that enhance disease risk in later life, including cardiovascular, metabolic, endocrine, and mental disorders and even breast cancer.[39]

Endocrine disrupting chemicals

Endocrine Disrupting Chemicals (EDCs) are defined as an "exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations".[40] The complex mechanisms of EDCs depend on specific actions at the cellular and tissue levels as well as life stage and sex.[41] Instead of focusing on a unifying mechanistic characteristic, key characteristics were developed based on the knowledge of hormone actions and EDC effects. In brief, EDCs may interfere with the endocrine system through one or more of the following mechanisms[42]: 1) Interact with or activates hormone receptors, 2) Blocks hormone receptors, 3) Modifies hormone receptor expression, 4) Changes signal transduction in hormone-response cells, 5) Induces epigenetic modifications in hormone-producing or hormone-response cells, 6) Modify hormone synthesis, 7) Changes hormone transport across cell membranes, 8) Alters hormone distribution or circulating levels of hormones, 9) Alters hormone metabolism or clearance, and/or 10) Alters the fate of hormone-producing or hormone-responsive cells. These ten key characteristics give guidance in evaluating EDCs, allowing researchers to gather evidence based on shared mechanistic information and to develop screening assays for potential EDCs as well as further targeted interventions at specific pathways of the endocrine system interference.

Perfluorooctanoic Acid (PFOA)

Perfluorooctanoic Acid (PFOA) is part of a larger chemical group called per- and polyfluoroalkyl substances (PFASs) which are highly fluorinated aliphatic molecules.[43] PFOA and perfluorooctane sulfonate (PFOS) are the most widely used and studied subset of PFAS.[44] PFOA is a man-made surfactant in the emulsion polymerization of fluoropolymers and a principal component of polymeric materials. It is known for its highly persistent, bioaccumulative, toxic, and ubiquitous nature.[45]

PFOA is most commonly found in industrial products such as carpets, floor wax, firefighting foam, sealants, and stain-resistant sprays. It is extremely resistant to biodegradation in our environment (approximately 5 to 15 years) and in humans. It has been observed that PFOA can bioaccumulate in our bodies with an estimated half-life of 4 years.[46] Due to its highly soluble property, PFOA water contamination is relatively common.

Monobenzyl Phthalate (MBzP)

Benzyl-Butyl Phthalate (BBP) is part of a larger group called phthalates which are diesters of phthalic acid. BBP is has two major monoester components, monobenzyl phthalate (MBzP) and monobutyl phthalate (MBuP). MBzP is the main metabolite measured in urine which is commonly used as a proxy to measure human BBP exposure, albeit it would be an underestimate.[47,48] The expected half-life of BBP is less-than 24 hours.[47] The toxicity of MBzP in humans has been documented, with estimated toxicity BBP by extension.

Also known as the "plasticizers", phthalates are everywhere- in consumer products and in the environment.[49] Specifically, benzyl-butyl phthalate is used for vinyl flooring, adhesives and sealants, car care products, toys, food packing, synthetic leather, industrial solvents, glues, personal care products, and automobile products.[50] The general population is exposed to phthalates through several routes: ingestion, inhalation, dermal or iatrogenic ("medical error and negligence") exposure. They are easily 'cross-contaminated', with accidental releases from one packing material to another.[49] Exposure to BBP is identified to be primarily through consumer products and indoor air.[51]

Trace metals

Approximately 20 elements are essential to the human body to maintain healthy biological processes and development. Roughly half of these essential elements are metal ("Trace

Metals"). Trace metals are present in the air, water, and food.[52] They are found in very low concentrations in the human body and are inorganic micronutrients. Some of the trace metals that are considered necessary for health include: Co, Cu, Mg, Mn, Mo, Zn.[53]

Certain metals are also classified as metalloestrogens, which are metals with the ability to produce estrogenic effect by mimicking physiological estrogens. They may modify the activity of estrogenic receptors, resulting in possible aberrant signals.[54–56] Metalloestrogens are found in numerous consumer products such as household items and cosmetics.[56] There has been growing industrial use of metalloestrogens, resulting in increasing levels in environment since the mid-20th century.[56] Epidemiologic studies reported metalloestrogens to be associated with various negative health outcomes including intellectual impairment,[57] and cancers of breast,[58–60] endometrium,[61] lung[62] and prostate.[63] While the exact mechanistic actions of metalloestrogens on health are not well-known, the growing presence of metalloestrogens as a persistent environmental contaminant is of public health concern.

Chapter 2. Time-specific Impact of Perfluorooctanoic Acid (PFOA) and Monobenzyl Phthalate (MBzP) on Breast Density of Adolescent Latino Girls

2.1 Abstract

Introduction: Breast cancer is the most common cancer among women, accounting for 30% of female cancers. 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 as well as a 'window of susceptibility' (WOS) for environmental exposures. Perfluorooctanoic acid (PFOA) and benzylbutyl phthalate (BBP) and are pervasive endocrine disrupting chemicals that may increase hormone-sensitive cancers such as breast cancer. Evaluating the potential impact of BBP (and its metabolite monobenzyl phthalate) 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 PFOA and monobenzyl phthalate (MBzP) at specific pubertal WOS on adolescent breast density.

Method: This study included 376 Chilean girls from the Growth and Obesity Cohort Study with survey data collection, physical examinations, breast density measurements, and urine and serum sample collection at four time points: Tanner breast stages 1 (B1) and 4 (B4), 1- year post menarche and 2-years post menarche. Dual-energy X-ray absorptiometry was used to assess the volume of dense breast tissue (absolute fibroglandular volume (FGV)) and percent breast density (%FGV) at 2YPM. Urine monobenzyl phthalate (MBzP) and serum PFOA concentrations were quantified using on-line solid phase extraction-liquid chromatography-isotope dilution-tandem mass

spectrometry. 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 of 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 marginal increase in absolute FGV and decreased % FGV. No effect was observed between MBzP and breast density measures across pubertal WOS.

2.2 Introduction

Breast cancer is the most common cancer among women, accounting for 30% of female cancers worldwide.[4] The volume of dense, fibrous and glandular breast tissue is among 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.[64] 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 profile.[65]

Endocrine Disrupting Chemicals (EDCs), defined as exogenous substances or mixtures that alter the endocrine system functioning, have been observed to be associated with an increased incidence of endocrine-related human diseases including various hormone-sensitive cancers such as breast cancer. [66] A potential mechanism between EDC and breast cancer is the interruption of the estrogen signaling pathway, which disrupts the proliferation of the stromal cells.[67] 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.[68–70] Lastly, some EDCs are obesogenic, increasing total adiposity and reducing %FGV.[71] This in effect, may misrepresent a person's risk for breast cancer which is often screened through the BI-RADS breast density reporting system.[72]

Phthalates and per- and polyfluoroalkyl substances (PFAS) are two classes of suspected EDCs that are of concern in relation to breast cancer development.[73] Exposure to phthalates results from their frequent use as plasticizers in adhesives and

sealants, paints and coatings, and vinyl floor tiling.[74] Of particular interest are benzylbutyl phthalate (BBP) and its main metabolite monobenzyl phthalate (MBzP), which are classified as endocrine disrupters for their anti-androgenic[75] and pro-estrogenic effects.[48,76–78] Several *in vitro* studies reported tumorigenic properties of BBP in increasing the proliferation of ER-positive breast cancer cells and inducing expression of oncogenes in ER-negative breast cancer cells.[79–81] PFAS are commonly used in the production of non-stick pans, furniture, cosmetics, and packaged food containers,[82] and have been extensively studied and shown to adversely influence women's health by disrupting their reproductive system.[83] 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.[84,85] 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.[86]

Breast tissue may be particularly sensitive to EDCs during puberty, a period of rapid growth and cellular differentiation of terminal end buds (TEBs).[87] As such, puberty is considered as one of the critical stages of breast development as well as a 'window of susceptibility' (WOS) for environmental exposures.[32,88,89] However, data on human exposure to EDCs on breast development during puberty remain scarce. The objective of our study is to estimate the effect 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.

2.3 Methods

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 grams (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.[90] 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.[91]

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 (**Figure 1**). The study protocol was approved by the Ethics Committee of the Institute of Nutrition and Food Technology, 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.

Urine collection

Urine samples were collected at B1 and B4. They were collected (at least 2mL) in non-polycarbonate sterile cups from fasting girls between 10 AM and 12 PM during visits to the INTA. Once collected, they 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.

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 Centers for Disease Control and Prevention (CDC) in Atlanta, GA using using on-line solid phase extraction-liquid chromatography-isotope dilution-tandem mass spectrometry.[92] Creatinine quantification for all urine samples was performed at Mount Sinai.[93] 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.[94] 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).[95]

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.[96,97]

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.

Blood collection

Serum samples were collected at Tanner stage B4 and 1YPM. Fasting (at least 8 to 12 hours prior to blood collection) venous samples were obtained 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. \

Blood analysis – PFOA

PFOA samples were analyzed using on-line solid phase extraction-liquid chromatography-isotope dilution-tandem mass spectrometry in the same manner at the CDC National Center for Environmental Health Laboratory. The LOD for PFOA

biomarker was 0.1 ng/mL. PFOA biomarker concentrations below LOD were imputed a value of LOD/sqrt(2).[95] PFOA biomarker concentrations were $\log_2$-transformed prior to analyses.

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).[98] 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) represent 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.[98,99]

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 fatness was measured using a bioimpedance device. Age of 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.

Statistical analysis

Parametric G-formula (hereafter 'G-formula') was used to investigate the causality of MBzP and PFOA's impact on breast density. G-formula, adjusting for both time-varying confounder and fixed covariates,[100] allowed estimation of time-specific effects of MBzP and PFOA on breast density at 2YPM individually and collectively. **Figure 1** 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 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**. We used SAS 9.4 software (SAS Institute Inc.) for all analyses.

2.4 Results

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 of menarche was12 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, while 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 215.5-218.8 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 PFOA exposure in doubling 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**).

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 of 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 213.7-215.9 cm^3 and %FGV ranging from 50.2-51.5%.

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 MBzP exposure at B1 only, a doubling of MBzP concentration 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 exposure 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). A sensitivity analysis of MBzP at B4 only restricting to girls with both B1

and B4 urine measurements (n=255) but without adjusting for MBzP measurement at B1 did not appreciably alter the results (data not shown).

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 percent 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).

2.5 Discussion

Our study adds to the limited research evaluating the effect of selected EDCs on breast density at specific 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.[101] The study found PFOA + zeranol exposure to induce the most phenotypic and transcriptomic changes in the 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.

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).[102] 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 exposure throughout puberty, with heightened sensitivity during assessment at B4. The breast may be more susceptible to EDC exposure 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.[88] 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.[103] 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:height ratio, and waist:hip ratio in children and adolescents. PFOA effects on adiposity measures varied by specific timepoints (e.g. 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, -18.5g).[104] 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).[105] 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 value having a strong inverse association with BMI z-score but not with waist: height nor with waist: hip ratio (per log10 unit increase of PFOA ng/ml: ß -0.264, 95%CI -0.416, -0.112).[106] 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.

MBzP and breast density

The observed MBzP concentrations were lower in our study than the MBzP concentrations reported in previous analysis of adolescent girls data from the U.S. National Health and Nutrition Examination survey (median 9.2 ng/mL and 16.0 ng/ml).[107,108] 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.[109] 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.[110] However, a separate analysis of the same BCERP cohort observed a later age for breast development (Tanner Stage B2) for girls with higher pre-pubertal MBzP concentrations.[111]

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.[112] 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.[113] Similarly, it may be the case that our null findings were reflective of the unaffected morphology of the breast tissues.

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 (e.g., hours).[114] However, studies suggest even a transient but consistent or repeated exposure to phthalates may result in adverse health outcomes.[73] 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.[115,116] 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. Regardless, it is reasonable to assume that girls will have additional PFOA exposure at 1YPM resulting from drinking water serving as constant sources of PFOA. 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 fully assess the impact of chemical mixtures of other EDCs, which may have biased our results.

Our study has several strengths including 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 serves 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 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.

2.6 Conclusion

EDCs measured at different time points throughout puberty can have varying impacts on the 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.

Figure 2. 1 Flow diagram of Growth and Obesity Cohort Study (GOCS) study population in the assessment of perfluorooctanoic acid and breast density

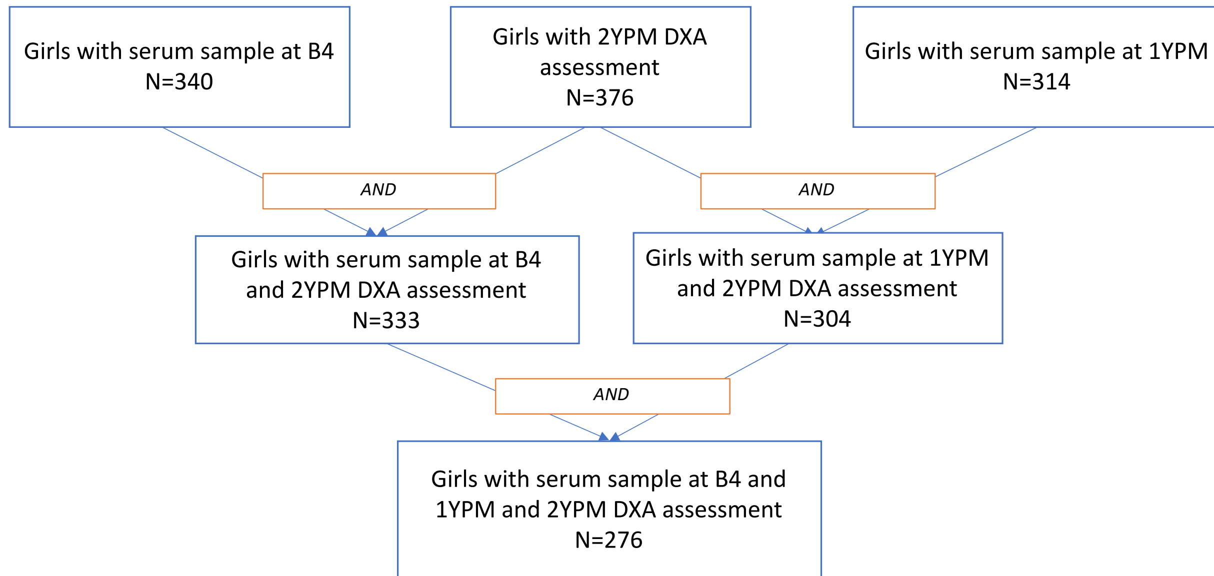


Figure 2. 2 Flow diagram of Growth and Obesity Cohort Study (GOCS) study population in the assessment of monobenzyl phthalate and breast density.

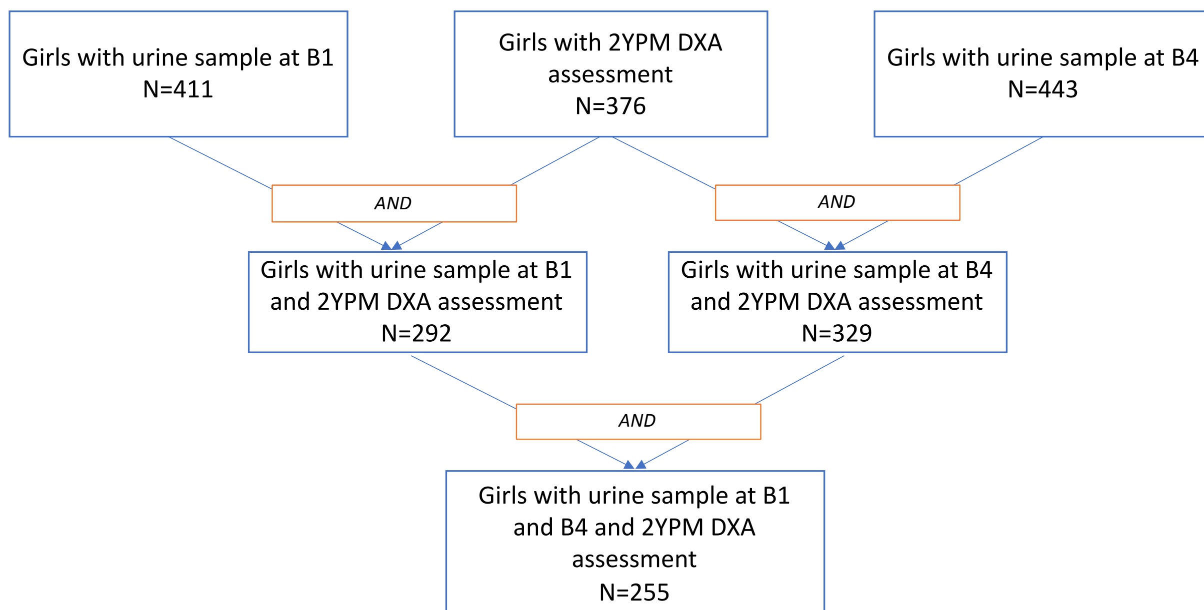


Figure 2. 3 Directed acyclic graph of the study showing the relation between perfluorooctanoic acid and breast density

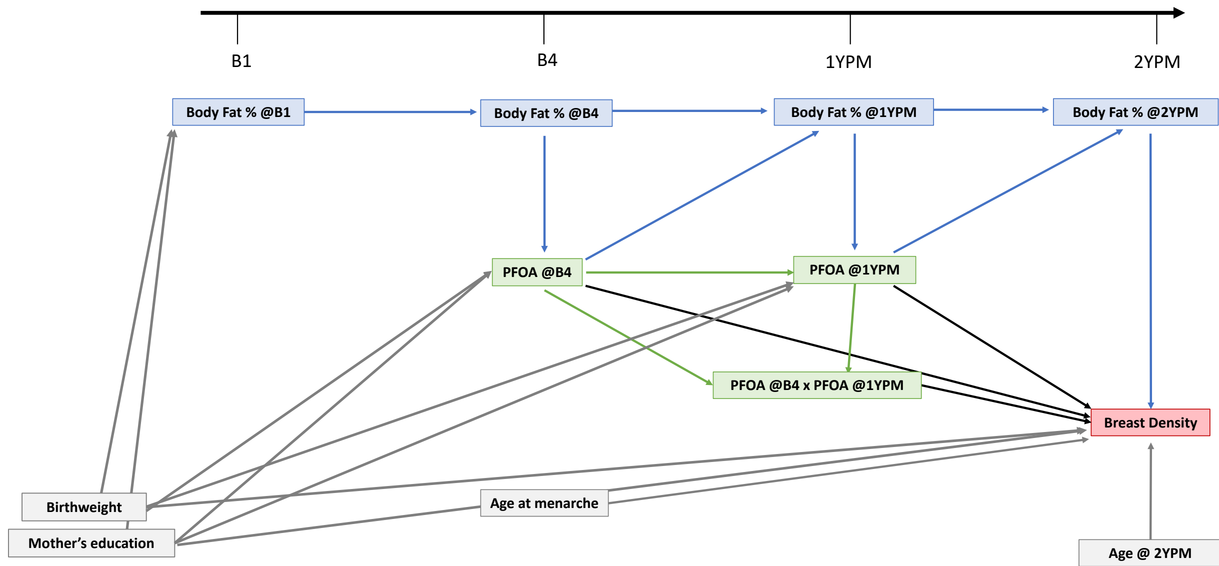


Figure 2. 4 Directed acyclic graph of the study showing the relation between monobenzyl phthalate acid and breast density

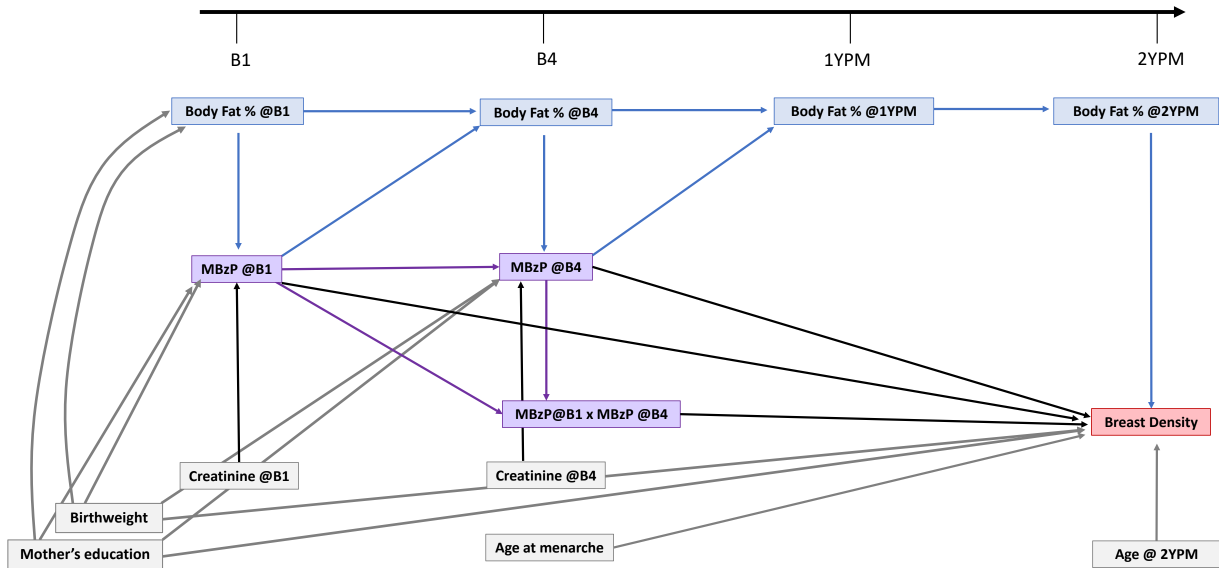


Table 2. 1 Characteristics of girls in the Growth and Obesity Cohort Study with breast density at 2-years post menarche and serum sample at Tanner breast stage B4 and 1-year post menarche

Characteristic	Window of Susceptibility	
	B4	B4 and 1YPM[a]
	(n=333)	(n=276)
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.

Table 2. 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

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=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 2. 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 and B4

Characteristic	**Window of Susceptibility**	
	B1	B1 and B4[a]
	(n=292)	(n=255)
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.

Table 2. 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

Window of Susceptibility		**Absolute Fibroglandular Volume, cm^3**			**Percent Fibroglandular Volume, %**		
		Estimate of relative change	**SE**	**95% Confidence Interval**	**Estimate of relative change**	**SE**	**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 2. 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

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.

Chapter 3. Time-specific Impact of Perfluorooctanoic Acid (PFOA) and Monobenzyl Phthalate (MBzP) on Hormone Levels of Adolescent Latino Girls

3.1 Abstract

Introduction: Endocrine disrupting chemcials (EDCs), such as monobenzyl phthalate (MBzP) and perfluorooctanoic acid (PFOA), have been observed to be associated with an increased incidence of hormone-sensitive diseases such as breast cancer. However, few epidemiologic studies have examined the effect of EDCs on hormones during puberty, an understudied critical period for breast cancer development.

Objective: To prospectively evaluate the association between MBzP and PFOA and hormones, individually and collectively, during puberty.

Method: This study included 330 Chilean girls from the Growth and Obesity Cohort Study in Santiago, Chile, with EDC biomarker collection at three timepoints: Tanner breast stages B1 and B4 and 1-year post menarche (1YPM) and serum hormone samples at 1YPM. Hormones analyzed included dehydroepiandrosterone sulphate (DHEAS), androstenedione, 17 OH progesterone (17-OHP), testosterone, estradiol, sex hormone-binding globulin (SHBG), and insulin growth factor- 1 (IGF-1). Parametric G-formula was used to estimate the time-specific association between EDCs and hormones. The analyses accounted for the covariates age, body fat percentage, birthweight, age of menarche, maternal education, insulin levels, and creatinine (MBzP only).

Results: A doubling of MBzP concentration at B1 only resulted in a statistically significant decrease in IGF-1 (β:-5.84, 95% Confidence Interval (CI): -10.44, -1.58) and a slight increase in 17-OHP (β:0.04, 95%CI: 0.01, 0.06). We also found a joint total effect of a doubling of MBzP at B1 and B4 with a significant decrease in IGF-1 (β:-8.42, 95% CI: -16.23, -0.21). A doubling of PFOA concentration resulted in a statistically significant increase in IGF-1 (β: 25.22, 95%CI: 14.62, 35.91), 17-OHP (β: 0.13, 95%CI: 0.06, 0.19), and SHBG (β: 3.52, 95%CI: 0.38, 6.74).

Conclusion: In this cohort of Latino girls, MBzP was associated with IGF-1 and 17-OHP. PFOA was associated with IGF-1, 17-OHP and SHBG, Associations between MBzP and PFOA and hormones varied by pubertal time point, suggesting differential effects of EDC exposure by specific window of susceptibility on pubertal hormone levels.

3.2 Introduction

Breast cancer etiology involves the synergistic interaction between hormonal influences and traditional carcinogenesis pathways involving random genetic mutation and DNA damage.[117] The role of estrogen in breast tumorigenesis has been well established among adult women. For example, in post-menopausal women, exogenous estrogen treatment significantly increased the risk of breast cancer.[18] Similarly, the use of tamoxifen, a selective estrogen-receptor modulator, has been associated with lower risk of primary invasive breast cancer in both pre- and postmenopausal women.[19] In reverse, surgical removal of ovaries, a major source of estrogen, production decreased the risk of breast cancer in premenopausal women.[20] While a substantial number of breast cancer studies have focused on hormonal influences among adults, few studies have examined this during puberty, which is another distinct and critical period of breast development characterized by heightened hormonal activity and stromal-epithelium modifications.[31,32]

Specifically, the female sex hormones estradiol and progesterone directly, and androgens (e.g., testosterone, dehydroepiandrosterone sulfate (DHEAS), androstenedione) indirectly play pivotal roles in the female reproductive system and for secondary female sex characteristics including the growth of mammary glands. Other important hormone and protein structures include insulin-like growth factor 1 (IGF-1), known to be implicated in numerous processes with growth-promoting actions regulating cell proliferation, differentiation, apoptosis and angiogenesis in different tissues in an endocrine, paracrine and autocrine fashion[118] and sex hormone-binding globulin (SHBG), the blood transport protein for estrogens and androgens.[119] The concentration of sex hormones and related proteins during puberty are associated with several pubertal clinical milestones, including age at menarche and thelarche (i.e., breast budding).[120,121]

Growing evidence suggests that the age at thelarche worldwide have become increasingly earlier in recent years.[122,123] Premature onset of thelarche has been linked to increased risk of breast cancer, raising concerns related to this trend.[122,124,125] While the precise mechanisms underlying earlier pubertal onset is not fully known, it has been postulated that exposure to environmental chemicals during the early developmental periods may contribute to hormonal and homeostatic disruptions, suggesting puberty to be a window of susceptibility (WOS) for breast cancer.[32,125,126] Further, epidemiologic studies have found exposure to environmental toxins to be associated with increased risk of metabolic and endocrine disorders and breast cancer, highlighting the potential long-term consequences of hormonal disruptions.[39,127,128]

Among the environmental exposures that may increase breast cancer risk are endocrine disrupting chemicals (EDCs). EDCs are hormonally active exogenous substances that alter or interfere with the endocrine system.[129] They mimic natural hormones by binding to endocrine nuclear receptors and disrupt the synthesis, transport, metabolism, and elimination of hypothalamic, pituitary, and peripheral hormones.[42] Butyl-benzyl phthalate (BBP) and its main metabolite, mono-benzyl phthalate (MBzP), are EDCs of particular interest due to their potential role in altering pubertal outcomes and fostering cancer development in rats.[81,130,131] BBP is a substance commonly used as a plasticizer and is not chemically bound to products, which allows it to be easily found in foods, dust, and air.[50] Another EDC that may be relevant for understanding breast cancer risk is perfluorooctanoic acid (PFOA), which is found in household items such as carpets, clothes, frying pans, and cardboard packaging among others.[45,46] Animal studies suggest that it can disrupt the peroxisome-proliferator-activated-receptor-signaling pathways, consequently increasing hepatic aromatase activity and estrogen concentrations.[86,132] Given, BBP and PFOA's anti-androgenic and pro-estrogenic properties, their pervasiveness is

an important concern for breast cancer risk, specifically during critical periods of breast development.[48,75–78,133]

While both MBzP and PFOA are commonly detected in humans, few epidemiologic studies have examined the effect of these EDCs on hormones related to the growth and development of girls during puberty, an understudied WOS for breast cancer development. Evaluation of the impact of MBzP and PFOA on hormones in this population may help clarify a potential molecular effect of EDCs on breast cancer etiology, The current study presents the first step towards understanding the effects of MBzP and PFOA exposure on hormone concentrations at specific pubertal WOS among Chilean adolescent girls.

3.3 Methods

Study population

This study uses data from the prospective Growth and Obesity Chilean Cohort Study (GOCS), which was established in 2006 and recruited children ages 3-4 from low- and middle-income families in Santiago, Chile. Eligibility criteria included: 1) singletons born at 37-42 weeks, 2) birthweight greater than or equal to 2500 grams and less than 4500g, and 3) healthy with no physical or psychological conditions. Pubertal stages were evaluated by clinical examination based on Marshall and Tanner guidelines (Tanner Staging).[90] Breast development was assessed through palpation and visual inspection by dietitians who were trained by a pediatric endocrinologist (VM). The study prospectively followed children annually up to age 7.5 years, then every 6 months until they reached Tanner stage 4 (B4) and then annually thereafter including 1 year post-menarche (1YPM). A total of 1,195 eligible children agreed to participate in the study, of which 601 were girls.[91]

We focused our analyses on girls who provided urine samples at Tanner stage B1 and B4 and serum samples at B4 and 1YPM. For our urine and serum samples at B4, we excluded samples from girls who had already experienced menarche by B4 to account for the potential confounding by the menstrual cycle on the association between EDCs and hormones (**Figure 1**). The study protocol was approved by the Ethics Committee of the Institute of Nutrition and Food Technology, University of Chile (INTA), and the Institutional Review Board of the University of California, Los Angeles. Participating girls gave assent and informed written consent was obtained from all parents or guardians of study participants.

Urine collection

Urine samples were collected at B1 and B4. They were collected (at least 2mL) in non-polycarbonate sterile cups from fasting study participants between 10 AM and 12 PM during visits to the INTA clinics. The samples were immediately vortexed, aliquoted, and temporarily stored at -80°C before being shipped to a laboratory for MBzP and creatinine quantification.

Urine analysis - MBzP

Initial samples collected at B1 (n=200) and B4 (n=200) were analyzed at the National Center for Environmental Health Laboratory at the Centers for Disease Control and Prevention (CDC) in Atlanta, GA using on-line solid phase extraction-liquid chromatography-isotope dilution-tandem mass spectrometry.[134] With additional funding, the remaining samples collected at B1(n=211) and B4 (n=242) 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.[94] The limit of detection (LOD) for MBzP biomarker was lab-specific; the value at CDC was 0.3 ng/ml and Mount Sinai the value was 0.1ng/ml. MBzP concentrations below LOD were imputed a value of the lab-specific LOD/sqrt(2).[95]

A total of 40 MBzP urine samples analyzed at the CDC were also analyzed at Mount Sinai as duplicates to perform quality control and between-lab standardization. Before statistical analyses, CDC-analyzed MBzP biomarker data were normalized to the Mount Sinai-analyzed MBzP biomarker data using scaling parameters calculated with the 40 duplicate samples. We assumed no significant differences in study participant characteristics in the normalization step as samples were randomly chosen to be analyzed at the CDC. All urine creatinine concentrations were quantified at Mount Sinai.[93] MBzP biomarker and creatinine concentrations were $\log_2$-transformed prior to analyses.

Blood collection

Serum samples were collected at Tanner stage B4 and 1YPM. The girls were asked to fast for at least 8 to 12 hours prior to blood collection. Venous samples were obtained 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. Serum samples at 1YPM were taken on the 2nd to 7th day of the follicular phase of the menstrual cycle.

Blood analysis – PFOA and hormones

PFOA levels were assessed at the CDC National Center for Environmental Health Laboratory. Standard serum solutions containing PFOA were prepared in water/methanol (50/50) by serial dilutions from commercial solutions (50 or 2 μg/mL in methanol) of PFOA. All prepared solutions were frozen (-70 °C) in 2.0mL polypropylene vials until use. Serum samples (50 μL) were dispensed into autosampler vials, added 50 μL of stable isotope-labeled internal and 0.1 M formic acid and vortexed before being analyzed using the on-line solid phase extraction-high performance liquid chromatography-isotope dilution-tandem mass spectrometry serum method. General approach used for this analysis is described previously.[92] The LOD for

PFOA biomarker was 0.1 ng/mL and concentrations below LOD were imputed a value of LOD/sqrt(2).[95] PFOA biomarker concentrations were $\log_2$-transformed prior to analyses.

Six hormone concentrations were analyzed at the Institute of Maternal and Child Research, University of Chile: dehydroepiandrosterone sulphate (DHEAS), androstenedione, 17 OH progesterone (17-OHP), testosterone, estradiol, sex hormone-binding globulin (SHBG), and insulin growth factor- 1 (IGF-1). Concentrations of DHEAS, androstenedione, 17-OH progesterone (17-OHP), and testosterone were analyzed by liquid chromatography-mass spectrometry in a high-performance liquid chromatography (HPLC) Agilent system (Santa Clara, CA, USA) 1260 coupled to an AB Sciex 3200 Quantum ultratriple quadrupole mass spectrometer (Foster City, CA, USA). Further details on liquid chromatography separation are described elsewhere.[135] The sensitivities for DHEAS, androstenedione, 17-OHP, and testosterone were 75, 0.03, 0.05, and 0.01 ng/mL, respectively. Serum estradiol was measured by RIA (Pantex, Santa Mónica, CA, USA). The sensitivity of this assay was 5.0 pg/mL. Sex hormone-binding globulin (SHBG) (sensitivity: 0.5 nmol/L) were performed using an immunoradiometric assay (Izotop Laboratories, Budapest, Hungary). IGF-1 levels were measured by RIA (sensitivity 5 ng/ml).

Covariates

Covariates were identified based on *a priori* review of published evidence and using directed acyclic graphs (DAGs) of the causal relation between EDCs and hormones. Anthropometric measures (e.g. weight, height) were collected bi-annually by study personnel, birthweight was obtained from health records and percent body fatness was measured using a bioimpedance device. Age of menarche was surveyed every 6 months prior to Tanner stage B4 and every 3 months after achieving B4. Maternal education was collected through interviews with the girls' mothers. Serum insulin concentration was measured by RIA (sensitivity 0.5

mUI/ml). All missing covariate data were imputed using mean imputation, except for maternal education which was imputed using median imputation.

Statistical analysis

Parametric G-formula (hereafter 'G-formula') was used for the statistical analysis of MBzP and PFOA and hormone levels. Adjusting for both time-varying confounder and fixed covariates,[100] G-formula estimated the time-specific effects of MBzP and PFOA on hormone levels at 1YPM individually and collectively. A directed acyclic diagram (DAG) representing the hypothesized causal relation between EDC exposures, hormones and covariates is represented in **Figure 1.** MBzP and PFOA concentrations (continuous) and fat percentage (continuous) are collected at multiple timepoints, which treats fat percentage as a confounder that is affected by the prior measurement of the EDC. G-formula allows for the adjustment of these exposure-dependent confounders by fat percentage without blocking the indirect path of EDCs to breast density through fat percentage. Our models adjusted for time-fixed covariates include age at 1YPM (continuous), insulin levels at 1YPM (continuous), birthweight (continuous), age at menarche (continuous), and 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**. We used SAS 9.4 software (SAS Institute Inc.) for all analyses.

3.4 Results

MBzP and hormones

In both subsamples, the mean age of our girl participants at B1 was 7.4 years, body fat percentage at B4 was 25%, age of menarche was 12.5 years and the mean birthweight was 3.33 kg (**Table 1**). In both subsamples, majority of the girls' mothers received a secondary

education or less (76.31% and. 77.70%). The mean concentration of MBzP at B1 was 5.73 ng/mL and in the subsample of girls with both B1 and B4 without menarche, MBzP concentration was 7.03 ng/mL at B1 and 3.65 ng/mL at B4.

Relative change in hormone levels at 1YPM associated with a doubling of MBzP concentrations at specific WOS is presented in **Table 2**. A doubling of MBzP concentration at B1 only resulted in a statistically significant decrease in IGF-1 (β:-5.84, 95% Confidence Interval (CI): -10.44, -1.58) and a slight increase in 17-OHP (β:0.04, 95%CI: 0.01, 0.06). We also found a joint total effect of a doubling of MBzP at B1 and B4 with a significant decrease in IGF-1 (β:-8.42, 95% CI: -16.23, -0.21). We did not observe any effect in a doubling of MBzP concentration at B4 only. A sensitivity analysis of MBzP at B4 only restricting to girls with both B1 and B4 urine measurements (n=148) but without adjusting for MBzP measurement at B1 did not appreciably alter the results (data not shown).

PFOA and hormones

The analysis of PFOA and hormone levels included two subsamples, girls who had not yet reached menarche at B4 and with serum samples in B4 (n=226), and girls with serum samples from both B4 and 1YPM (n=264) (**Table 3**). In the analysis using B4 values only, the mean age at B4 was 10.8 years, body fat percentage at

B4 was 26.8%. In the subsample of both B4 and 1YPM, the mean age at B4 was 10.9 years and body fat percentage at B4 was 27%. In both subsamples, age of menarche approximately 12 years, and the mean birthweight was 3.3 kg. In both subsamples, majority of the girls' mothers received a secondary education or less (76.44% and. 78.03%). The mean concentration of PFOA at B4 among girls who had not yet reached menarche at B4 was 1.30 ng/mL; in subsample of girls with both B4 and 1YPM serum samples, PFOA concentration was 1.26 ng/mL at B4 and 1.00 ng/mL at 1YPM.

Relative change in hormone levels at 1YPM associated with a doubling of PFOA concentrations at specific WOS is presented in **Table 4**. Under the hypothetical intervention of PFOA exposure at 1YPM only, a doubling of PFOA concentration resulted in a statistically significant increase in IGF-1 (β: 25.22, 95%CI: 14.62, 35.91), 17-OH progesterone (β: 0.13, 95%CI: 0.06, 0.19), and SHBG (β: 3.52, 95%CI: 0.38, 6.74). We did not observe any association between PFOA and hormone levels under hypothetical interventions of PFOA exposure at B4 only and joint total effect of B4 and 1YPM.

Exposure to both MBzP and PFOA and hormones

In a subset of girls who provided both urine and serum samples at B4 (n=225), we analyzed the joint total effect of MBzP and PFOA exposure at B4 prior to menarche on hormone levels at 1YPM **(Table 5).** We did not find significant change in hormone levels when doubling both MBzP and PFOA at B4 prior to menarche regardless of other WOS exposures.

3.5 Discussion

In our cohort of Chilean pubertal girls, we evaluated the impact of PFOA and MBzP, individually and in combination, on sex hormones, SHBG and IGF-1. We found IGF-1 and progesterone to be associated with both PFOA and MBzP and SHBG to be associated with PFOA only. These results contribute to the growing evidence evaluating the impact of environmental factors during puberty, by highlighting the differential effect of PFOA and MBzP on specific hormones.

MBzP and hormones

In our study, we found MBzP exposure to be associated with IGF-1 and progesterone but varied by exposure WOS. A decrease in IGF-1 at 1YPM was observed when doubling MBzP

at B1 only and at B1 and B4 jointly, while an increase in progesterone at 1YPM was observed for doubling MBzP at B1 only. Previous epidemiological studies report mixed results regarding phthalate exposure and IGF-1. A cross-sectional study assessed phthalate exposure in relation to thyroid and growth hormones among Taiwanese children aged 2-18 years old. They found mono-ethyl phthalate (MEP) to be negatively associated with IGF-1 (ß: -0.027; p-value: 0.029), but a null effect with MBzP.[136] In another cross-sectional study, urinary monocarboxyisoocctyl phthalate (MCiOP) and di(2-ethylhexyl)phthalate (DEHP) metabolites were inversely associated with serum IGF-1 concentrations among Danish boys aged 4-9 years old, while no association was found with MBzP. In the same study, no association was observed between phthalates and IGF-1 among girls.[137] One explanation for this difference may be that the effect of MBzP on IGF-1 is age- and sex-specific. The Taiwan study included both sexes with ages ranging from 2-18 years old while the Dutch study included prepubertal girls with a lower baseline IGF-1 concentration than that of the girls in our study. Another explanation might be the cross-sectional study designs used in previous studies compared to the present cohort study which prospectively collected MBzP concentrations prior to the girls' hormone measurements.

To date, longitudinal assessment of pubertal phthalate exposure and sex hormones relation remains scarce. A pilot study of the Taiwan Maternal and Infant Cohort Study followed children from ages 2 to 11 years old to examine pre- and post-natal phthalate exposure on sex hormones.[138] At ages 8 and 11 years, the girls in the Taiwanese cohort shared similar MBzP concentration levels to our study (median: 7.74 mg/dL and 2.92 mg/dL, respectively) but found no association between MBzP and progesterone. The study also reported null associations between MBzP and testosterone and estradiol, similar to the results of our study. Conversely, an Australian study of adolescent girls age 14-16 years old did not observe any associations between MBzP (median: 1.26 ng/ml) and androgens.[139] The mixed results between our study and these cohort studies may be attributable to the different analyses methods used (e.g., G-

formula vs. generalized estimating equation linear regression analysis) and the varying covariate adjustments.

PFOA and hormones

In our study, we observed statistically significant increases in 17OHP, SHBG, and IGF-1 with a doubling of PFOA at 1YPM. PFOA's anti-androgenic and pro-estrogenic properties may have resulted in an uptake of SHBG while decreasing the estradiol levels.[133] A cross-sectional study using the National Health and Nutrition Examination Survey (NHANES) among 12-19 year old girls in the US reported similar PFOA concentration levels as our GOCS cohort (mean: 1.00 ng/mL, 95%CI: 0.70, 1.40). The study reported a null association between PFOA and testosterone and SHBG levels but found a linear negative association with estradiol (p-trend: 0.027).[140] Conversely, in a cohort study of Taiwan females aged 12-17 years old with a higher serum PFOA levels (geometric mean: 2.73 ng/mL; SD: 2.97) than our population. observed an inverse association between PFOA and serum levels of SHBG (p-trend: <0.05).[141] Previous studies also addressed the impact of prenatal exposure of PFOA on hormones and related proteins, another sensitive WOS.[128] A UK cohort study observed a null effect between prenatal PFOA exposure (maternal 3rd tercile: >4.1 ng/ml) and SHBG but a positive association with testosterone among girls with an mean age of 15 (ß: 0.24; 95%CI: 0.05, 0.43).[142] Jensen et al. found among girls with a mean age 3.8 years old, a doubling of prenatal PFOA exposure resulted in null effect of androstenedione and DHEAS (% change: 4.8; 95%CI: -7.3, 18.5 and % change: 0.4; 95%CI: -15.5, 19.3).[143] Several factors may contribute to the differences in results between our study and previous studies including different exposure window period, age of the girls at the time of hormone measurement, variation in covariate adjustments, use of different log transformations of PFOA and the differences in the hormones studied.

Strengths and limitations

An important limitation of our study is the single measurement of both EDCs and hormones at a given pubertal timepoint. Given the dynamic nature of hormones, there is difficulty in fully interpreting the latent effect of prior EDC exposures at B1 and B4 and subsequent hormone levels at 1YPM. Hormone levels are constantly changing, and we cannot rule out the possibility of natural pubertal growth and its associated hormonal changes affecting EDC dilution and excretion rates. This may attribute to the non-significant findings of PFOA exposure at B4 with hormones at 1YPM, while a cross-sectional assessment of PFOA and hormones measured at 1YPM resulted in significant findings. To account for this potential growth effect, we accounted for body fat percentage and insulin (both markers of growth) to the models. Additionally, PFOA's long half-life of 3.5 years suggest a relatively stable and consistent PFOA exposure between B4 and 1YPM.[144] Conversely, the short half-life of MBzP biomarker may be subject to a greater fluctuation and may not represent the long-term pattern of MBzP exposure.[114] However, studies have found even consistent, low-dose phthalate exposures to be associated with adverse health outcomes.[73]

Despite these limitations, our study has several strengths. Our study is the first to prospectively collect EDC exposure and subsequent hormone levels during pubertal timepoints. Puberty is an established WOS for EDCs to interfere with hormone regulation that may potentially lead to long-term adverse health outcomes. We also assessed the effect of EDCs on several important sex hormones, SHBG and IGF-1 which highlighted the specific impact of PFOA and MBzP on IGF-1 and progesterone at varying pubertal WOS. To date, there are no other studies evaluating PFOA and MBzP in combination on hormones. Moreover, with the use of G-formula, we were able to estimate the effect of EDCs from specific pubertal WOS (e.g. B1 only, B4 only for MBzP; B4 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) on hormones at

1YPM. Based on the causal DAG, we considered fat percentage as both a mediator and a confounder in our models, which limited bias from covariate overadjustment.

3.6 Conclusion

A doubling of MBzP exposure at B1 only was associated with changes in IGF-1 and 17-OHP while a doubling of MBzP exposure at both B1 and B4 was associated with a decrease in IGF-1 only. Similarly, PFOA exposure at 1YPM resulted in an increase in IGF-1, 17-OHP and SHBG, while no effect was observed in doubling PFOA concentrations at B4 only and at both B4 and 1YPM. These results suggest differential effects of EDC exposure by specific WOS on pubertal hormone levels.

Figure 3A. 1 Flow diagram of Growth and Obesity Cohort Study (GOCS) study population in the assessment of monobenzyl phthalate and hormones

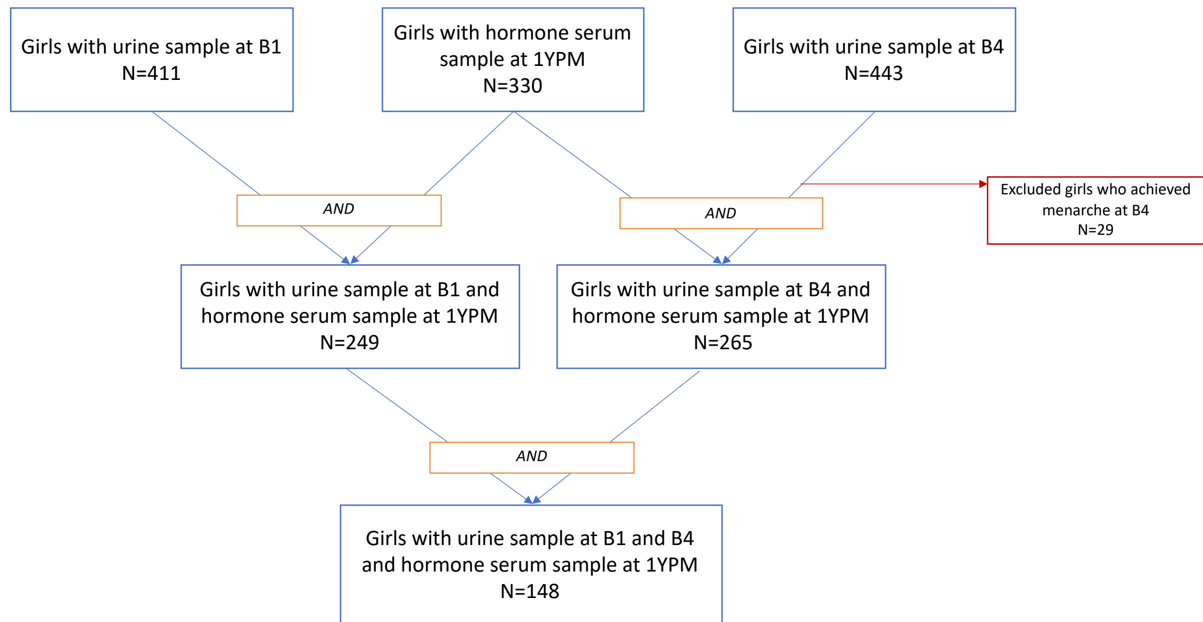


Figure 3A. 2 Flow diagram of Growth and Obesity Cohort Study (GOCS) study population in the assessment of perfluorooctanoic acid at B4 and hormones

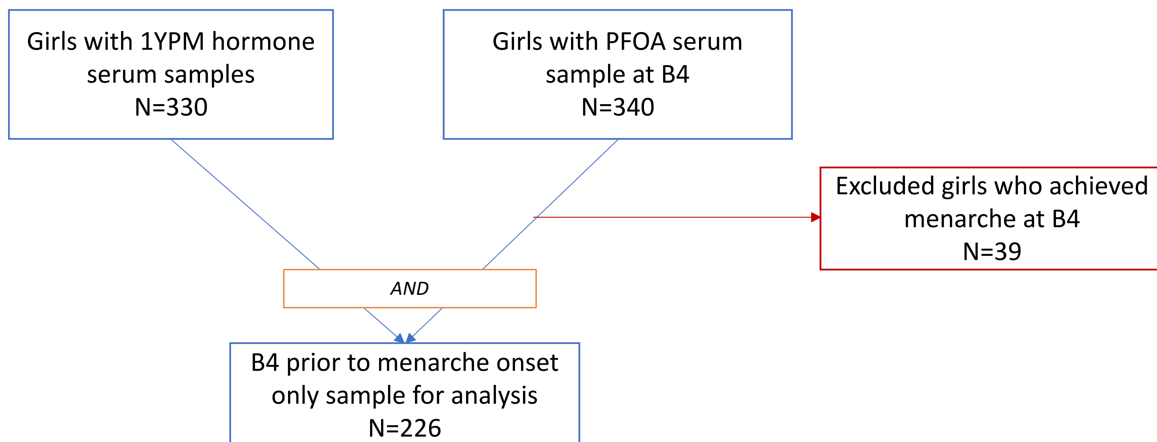


Figure 3A. 3 Flow diagram of Growth and Obesity Cohort Study (GOCS) study population in the assessment of perfluorooctanoic acid at B4 and 1YPM and hormones

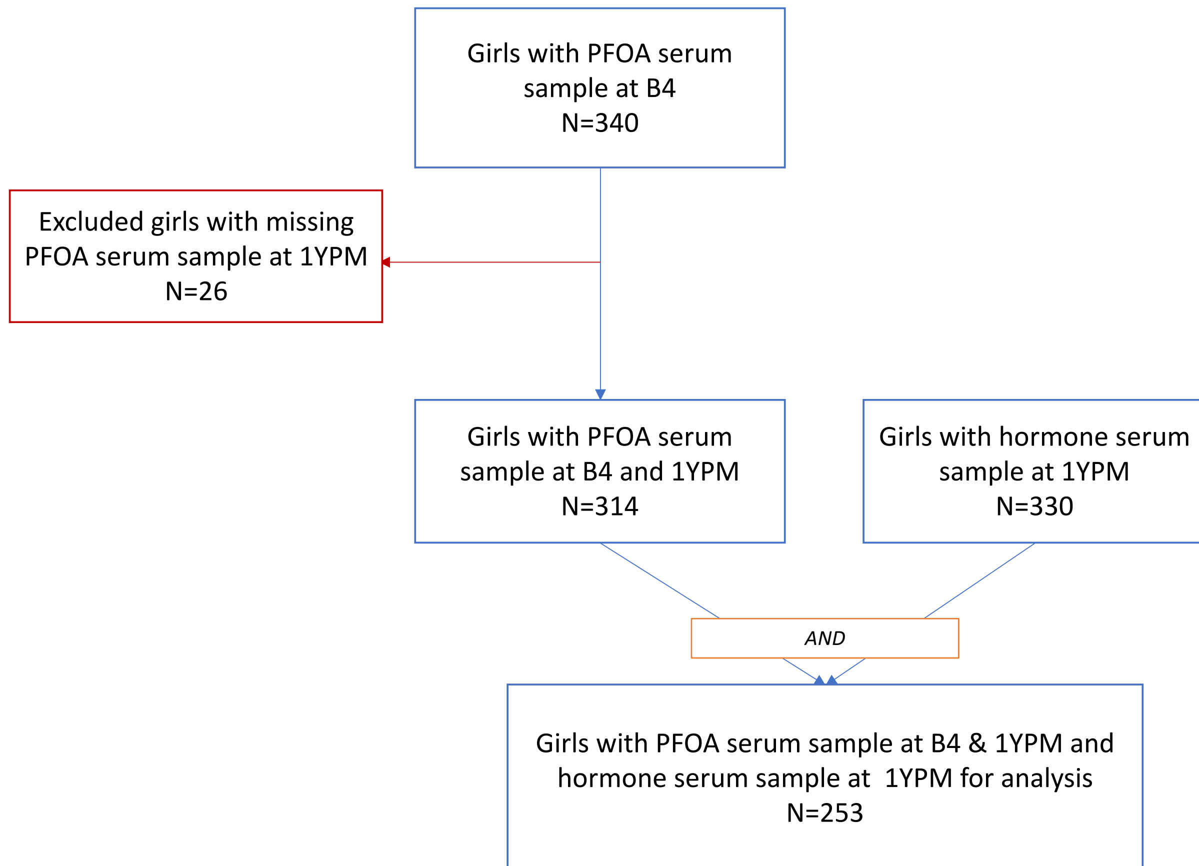


Figure 3B. 1 Directed acyclic graph of the study showing the relation between monobenzyl phthalate and hormones

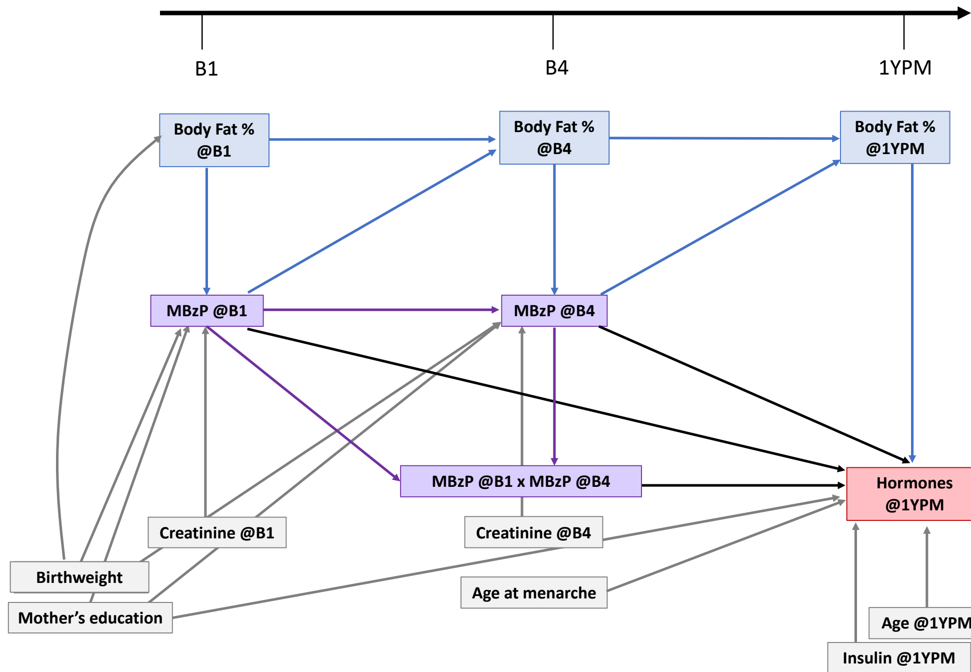


Figure 3B. 2 Directed acyclic graph of the study showing the relation between perfluorooctanoic acid and hormones

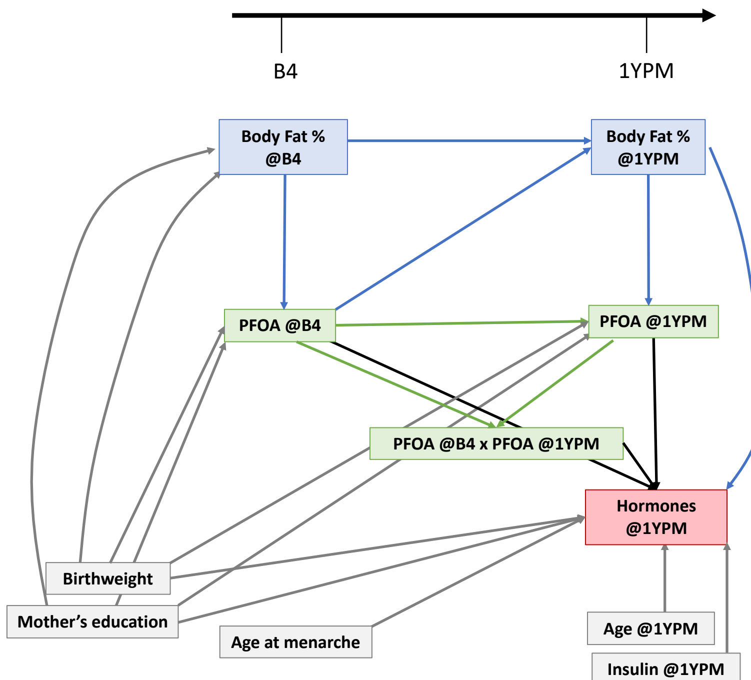


Table 3. 1 Characteristics of girls in the Growth and Obesity Cohort Study included in the present analysis assessed for monobenzyl phthalate at Tanner Stage B1 and B4

Characteristic	**Window of Susceptibility**		
	B1	B4[a]	
		Menarche Status	
		No	Yes
	(n=249)	(n=148)	(n=29)
Monobenzyl Phthalate, ng/ml (mean (SD))			
Tanner Stage B1	5.73 (13.46)	7.03 (16.73)	6.06 (8.63)
Tanner Stage B4	-	3.65 (7.93)	2.29 (2.02)
Age, years (mean (SD))			
Tanner Stage B1	7.39 (0.56)	7.39 (0.58)	7.45 (0.51)
Tanner Stage B4	-	11.14 (0.86)	11.86 (0.58)
Body fat percentage (mean (SD))			
Tanner Stage B1	25.52 (4.26)	25.04 (4.05)	26.28 (4.05)
Tanner Stage B4	-	26.66 (5.36)	28.61 (4.88)
Age at menarche, years (mean (SD))	12.07 (0.87)	12.44 (0.72)	12.21 (0.53)
Birthweight, kg (mean (SD))	3.33 (0.41)	3.33 (0.41)	3.24 (0.43)
Maternal education (n (%))			
Secondary education or less	190 (76.31)	115 (77.70)	23 (79.31)
Greater than secondary education	59 (23.69)	33 (22.30)	6 (20.69)
Insulin (mIU/ml)	10.97 (5.65)	9.36 (3.30)	8.99 (2.69)
Serum Hormone Levels at 1YPM			
Dehydroepiandrosterone Sulfate, ng/ml (mean (SD))	90.24 (48.71)	97.88 (52.41)	82.59 (39.66)
Insulin-like growth factor 1, ng/ml (mean (SD))	246.33 (58.83)	240.16 (59.66)	265.52 (49.31)
Testosterone, ng/ml (mean (SD))	0.19 (0.07)	0.20 (0.08)	0.19 (0.017)
Estradiol, pg/ml (mean (SD))	30.04 (28.43)	30.36 (23.10)	25.03 (11.27)
17 OH progesterone, ng/ml (mean (SD))	0.54 (0.34)	0.55 (0.36)	0.50 (0.35)

Androstenedione, ng/ml (mean (SD))	0.89 (0.35)	0.95 (0.35)	0.86 (0.38)
Sex hormone-binding globulin, nmol/L (mean (SD))	40.77 (17.71)	41.95 (17.76)	36.24 (17.02)

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

Table 3. 2 Simulated relative change[a] in hormone levels at 1 year post-menarche associated with log2 (ng/L) increase in urine monobenzyl phthalate among Growth and Obesity Cohort Study participants

Window of Susceptibility	Tanner Stage B1 N= 249			Tanner Stage B4 (prior to menarche) N= 148			Joint total effect of B1 and B4 (prior to menarche) N= 148		
	Estimate of relative change[b]	**SE[b]**	**95% Confidence Interval[b]**	**Estimate of relative change[c]**	**SE[c]**	**95% Confidence Interval[c]**	**Estimate of relative change[d]**	**SE[d]**	**95% Confidence Interval[d]**
DHEAS, ng/ml	-0.77	1.79	(-4.43, 2.78)	1.75	2.42	(-2.66, 6.30)	-1.61	3.68	(-8.33, 6.04)
IGF-1, ng/ml	-5.84	2.20	(-10.44, -1.58)	0.52	2.76	(-4.72, 5.93)	-8.42	4.08	(-16.23, -0.21)
Testosterone, ng/ml	0.002	0.003	(-0.004, 0.007)	-0.0005	0.0036	(-0.0069, 0.0063)	0.003	0.005	(-0.006, 0.014)
Estradiol, pg/ml	0.82	1.06	(-1.37, 2.86)	-0.24	1.10	(-2.28, 1.88)	1.68	1.56	(-1.26, 4.89)
17 OH progesterone, ng/ml	0.04	0.01	(0.01, 0.06)	-0.019	0.017	(-0.050, 0.013)	0.04	0.03	(-0.01, 0.09)
Androstenedione, ng/ml	0.008	0.013	(-0.020, 0.032)	-0.009	0.017	(-0.040, 0.023)	0.009	0.024	(-0.038, 0.060)
Sex hormone binding globulin, nmol/L	1.01	0.66	(-0.35, 2.32)	-0.54	0.85	(-2.13, 1.06)	0.12	1.27	(-2.29, 2.59)

[a] Estimates of relative change accounted for covariates mother's education, birthweight, body fat%, age at 1YPM, age at menarche, insulin at 1YPM, creatinine. Tanner Stage B4 only analysis additionally accounted for B1 MBzP concentrations.
[b] relative change in hormone levels when doubling MBzP exposure at Tanner Stage B1 only, regardless of subsequent exposure at Tanner Stage B4.
[c] relative change in hormone levels when doubling MBzP exposure at Tanner Stage B4 only, regardless of prior exposure at Tanner Stage B1.
[d] relative change in hormone levels when doubling MBzP exposure at both Tanner Stage B1 and B4.

Table 3. 3 Characteristics of girls in the Growth and Obesity Cohort Study included in the present analysis assessed for perfluorooctanoic acid at Tanner Stage B4 and 1-year post menarche

Characteristic	**Window of Susceptibility**		
	B4		B4 and 1YPM[a]
	Menarche Status		
	No	Yes	(n=264)
	(n=226)	(n=39)	
Perfluorooctanoic Acid, ng/mL (mean (SD))			
Tanner Stage B4	1.30 (0.80)	1.03 (0.59)	1.26 (0.78)
1 Year Post-Menarche	-	-	1.00 (0.58)
Age, years (mean (SD))			
Tanner Stage B4	10.82 (0.88)	11.49 (0.79)	10.92 (0.90)
1 Year Post-Menarche	12.50 (0.93)	12.23 (0.81)	12.46 (0.92)
Body fat percentage (mean (SD))			
Tanner Stage B4	26.82 (5.02)	28.87(4.98)	27.12 (5.06)
1 Year Post-Menarche	30.05 (5.11)	30.85 (4.76)	30.17 (5.06)
Age at menarche, years (mean (SD))	12.07 (0.86)	11.82 (0.77)	12.03 (0.85)
Birthweight, kg (mean (SD))	3.33 (0.42)	3.31 (0.49)	3.33 (0.43)
Maternal education (n (%))			
Secondary education or less	172 (76.44)	34 (87.18)	206 (78.03)
Greater than secondary education	53 (23.56)	5 (12.82)	58 (21.97)
Insulin at 1YPM (mIU/ml)	11.36 (5.96)	11.55 (6.10)	11.39 (5.97)
Serum Hormone Levels at 1YPM			
Dehydroepiandrosterone Sulfate, ng/ml (mean (SD))	95.07 (50.69)	74.22 (38.36)	91.99 (49.56)
Insulin-like growth factor 1, ng/ml (mean (SD))	246.69 (57.19)	251.90 (43.15)	247.46 (55.30)
Testosterone, ng/ml (mean (SD))	0.20 (0.08)	0.17 (0.06)	0.20 (0.08)
Estradiol, pg/ml (mean (SD))	33.96 (42.26)	27.62 (13.01)	33.02 (39.37)
17 OH progesterone, ng/ml (mean (SD))	0.54 (0.33)	0.51 (0.32)	0.53 (0.33)
Androstenedione, ng/ml (mean (SD))	0.92 (0.37)	0.75 (0.31)	0.90 (0.37)
Sex hormone-binding globulin, nmol/L (mean (SD))	39.33 (16.52)	39.82 (18.78)	39.41 (16.83)

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

Table 3. 4 Simulated relative change [a] in hormone levels at 1 year post-menarche associated with log2 (ng/mL) increase in serum perfluorooctanoic acid among Growth and Obesity Cohort Study participants

Window of Susceptibility	Tanner Stage B4 (prior to menarche) N= 226			1 Year Post Menarche N= 264			Joint total effect of B4 and 1YPM (prior to menarche) N= 264		
	Estimate of relative change[b]	**SE[b]**	**95% Confidence Interval[b]**	**Estimate of relative change[c]**	**SE[c]**	**95% Confidence Interval[c]**	**Estimate of relative change[d]**	**SE[d]**	**95% Confidence Interval[d]**
DHEAS, ng/ml	1.65	4.62	(-7.11, 10.86)	0.47	4.95	(-8.94, 10.10)	-5.21	9.35	(-23.23, 12.91)
IGF-1, ng/ml	6.12	5.35	(-4.37, 16.94)	25.22	5.57	(14.62, 35.91)	9.92	10.58	(-10.85, 30.56)
Testosterone, ng/ml	-0.008	0.007	(-0.023, 0.007)	-0.001	0.008	(-0.016, 0.014)	-0.006	0.015	(-0.036, 0.024)
Estradiol, pg/ml	1.59	3.93	(-6.15, 9.56)	5.03	4.15	(-3.08, 12.76)	-0.20	8.17	(-16.20, 15.18)
17 OH progesterone, ng/ml	-0.030	0.031	(-0.091, 0.032)	0.13	0.03	(0.06, 0.19)	0.02	0.06	(-0.10, 0.15)
Androstenedione, ng/ml	0.011	0.034	(-0.054, 0.079)	-0.03	0.04	(-0.10, 0.04)	0.01	0.07	(-0.13, 0.14)
Sex hormone binding globulin, nmol/L	-2.24	1.50	(-5.16, 0.68)	3.52	1.66	(0.38, 6.74)	-2.00	3.15	(-8.00, 4.27)

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

Table 3. 5 Simulated relative change[a] in hormone levels associated with log2 increase in both urine mono-benzyl phthalate (MBzP) and serum perfluorooctanoic acid (PFOA) at Tanner Stage B4

Window of Susceptibility	Tanner Stage B4 (prior to menarche) N= 225		
	Estimate of relative change[b]	**SE**	**95% Confidence Interval**
DHEAS, ng/ml	0.91	3.28	(-5.39, 7.28)
IGF-1, ng/ml	-1.00	3.90	(-8.74, 6.46)
Testosterone, ng/ml	0.001	0.005	(-0.010, 0.009)
Estradiol, pg/ml	-1.27	2.12	(-5.42, 3.06)
17 OH progesterone, ng/ml	-0.037	0.021	(-0.076, 0.004)
Androstenedione, ng/ml	-0.012	0.022	(-0.056, 0.031)
Sex hormone binding globulin, nmol/L	-1.26	1.06	(-3.30, 0.88)

[a] Estimates of relative change accounted for covariates mother's education, birthweight, body fat%, age at 1YPM, age at menarche, insulin at 1YPM, and creatinine (MBzP only).
[b] Joint total effect of MBzP and PFOA exposure at Tanner Stage B4 prior to menarche. Relative change in hormone levels when doubling both MBzP and PFOA at Tanner Stage B4 prior to menarche regardless of other window of susceptibility.

Chapter 4. Time-specific Impact of Trace Metals on Breast Density of Adolescent Latino Girls

4.1 Abstract

Introduction: Breast cancer is the leading cause of cancer deaths in women worldwide. Among the strongest predictors of breast cancer is high mammographic density, measured as percent and absolute fibroglandular volume. Epidemiologic studies have suggested that trace metals may be related to breast cancer. However, whether trace metals may modify breast density during critical developmental stages such as puberty remains unclear.

Objective: To prospectively evaluate the association between trace metals at specific pubertal time points on adolescent breast density.

Method: This study included Chilean girls from the Growth and Obesity Cohort Study with urine sample collection at Tanner breast stage B1 (n=291) and at stages both B1 and B4 (n=253) and breast density measurements at 2 years post menarche. Dual-energy X-ray absorptiometry was used to assess the volume of dense breast tissue (absolute fibroglandular volume (FGV)) and percent breast density (%FGV). Urine trace metals analyzed included arsenic, barium, cadmium, cobalt, cesium, copper, magnesium, manganese, molybdenum, nickel, lead, antimony, selenium, tin, thallium, vanadium, and zinc. Parametric G-formula was used to estimate the time-specific association between trace metals and breast density. The analyses accounted for the covariates age, body fat percentage, birthweight, age of menarche, maternal education, and creatinine.

Results: At B1, a doubling of thallium concentration resulted in 13.69 cm^3 increase in absolute FGV (β: 13.69, 95% confidence interval (CI): 2.81, 24.52), while a doubling of lead concentration was associated with a 7.76 cm^3 decrease in absolute FGV (β: -7.76, 95%CI: -14.71, -0.73). At B4, a doubling of barium concentration was associated with a 10.06 cm^3 increase (β: 10.06, 95% CI: 1.44, 18.60), copper concentration with a 12.29 cm^3 increase (β: 12.29, 95% CI: 2.78, 21.56) lead concentration with a 9.86 cm^3 increase (β: 9.86, 95% CI: 0.73, 18.98), antimony concentration with a 12.97 cm^3 increase (β: 12.97, 95% CI: 1.98, 23.79) and vanadium concentration resulted in a 13.14 cm^3 increase in absolute FGV (β: 13.14, 95% CI: 2.73, 23.58).

Conclusion: In this cohort of Latino girls, selected trace metals - barium, copper, lead, antimony, thallium, and vanadium – were associated with absolute breast density. We also observed an association between copper and percent FGV.

4.2 Introduction

Breast cancer is the leading cause of cancer deaths in women worldwide.[145] Among the strongest predictors of breast cancer is high mammographic density, which is determined by the percent dense fibroglandular volume relative to total breast size (%FGV).[146] Epidemiologic studies have underscored the important role of hormonal and reproductive factors as well as family history that may increase breast density and subsequently, breast cancer risk.[147–149] Anthropometric measures such as high body mass index and body fat distribution are also related to breast density, exerting direct and indirect influence on breast composition.[150] Recently, evidence from clinical and animal studies have suggested environmental exposures, including trace metals, to be associated with breast cancer risk.[58] However, whether exposure to trace metals is related to breast cancer incidence by affecting breast density remains unclear.

Trace metals are a subset of trace elements found naturally in the environment and are detected in minute amounts in the human body. Human exposure to trace metals occurs by ingestion of contaminated food or water, or inhalation of polluted air. In "trace" amounts, some trace metals (cobalt, copper, chromium, iron, magnesium, manganese, molybdenum, nickel, selenium, and zinc) are considered essential to humans, serving important functions in metabolic and other biologic processes.[151,152] For example, copper is required in hemopoiesis;[153] molybdenum in amino acid, uric acid, sulfuric acid metabolism;[154] manganese in bone, carbohydrate, lipid metabolism as well as reproductive and immune function processes;[155] and selenium in antioxidant and anti-cancer actions.[156] However, an abnormal concentration of these trace metals – both in excess and in deficit - has been associated with adverse health outcomes including cancer.[157] Epidemiologic and clinical studies have identified several metals ("metalloestrogens") mimicking estrogen and binding to estrogen receptors to be particularly harmful and linked with breast cancer risk.[58–60,158] Some of the known metalloestrogens include arsenic, barium, cadmium, cobalt, nickel, lead, selenium, and tin.[58]

Given trace metals' potential involvement in the pathogenesis of breast cancer, it is important to assess whether these metals may modify breast density during critical developmental stages. Puberty, a period of exponential growth of breast tissues, is an understudied window of susceptibility (WOS) during which stromal cells in the mammary gland are particularly sensitive to environmental exposures such as trace metals that may disrupt endocrine functions and interfere with proper pubertal development.[32,88] For example, cadmium and arsenite, well-studied metalloestrogens, induce cell division, and thus increase the expression of estrogen-regulated genes.[159–161] This increase in breast cell proliferation can lead to greater breast density.[161,162] A careful analysis of other highly prevalent trace metals in relation to breast density is needed to understand their potential role in breast cancer etiology.

The current study addresses key questions about the influence of trace metals on pubertal breast density. We evaluate the impact of trace metals on pubertal breast density at varying pubertal WOS in a cohort of adolescent Latino girls in Chile.

4.3 Methods

Study population

In 2006, the Growth and Obesity Chilean Cohort Study (GOCS) recruited children ages 3-4 years from low- and middle-income families in 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 grams (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). A total of 1,089 eligible children agreed to participate in the study, of which 601 were girls; a subset of these girls is included in the current study.[91] 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.[90] 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 2 year post-menarche (2YPM) timepoint.

Our study focused on girls who provided urine samples at Tanner stages B1 and B4 and breast density measurement at 2YPM (**Figure 1**). Our analysis of trace metals and breast density included two subsamples: 1) girls with urine samples collected at B1 and breast density measured at 2YPM (N=291) 2) girls with urine samples collected both at B1 and B4 and breast density at 2YPM (N=253). The study protocol was approved by the Ethics Committee of the Institute of Nutrition and Food Technology, University of Chile (INTA), and the Institutional Review Board of the University of California, Los Angeles. Informed written consent was obtained from all parents or guardians of the study participants.

Urine collection

At least 2mL of urine samples were collected from fasting study participants between 10 AM and 12 PM during visits to the clinic at INTA. They were collected in non-polycarbonate sterile cups and were immediately vortexed and aliquoted. The urine was temporarily stored at 4°C before processing for homogenization of the sample, followed by aliquoting and storage at -80°C before being shipped to a laboratory for biomarker quantification.

Urine analysis - trace metals

A panel of 17 trace metals was measured in fasting spot urine samples collected at B1 and B4. Samples were analyzed using tight quality control (QC) measures including analysis of the initial calibration, initial calibration verification, and continuing calibration verification standards: NIST traceable mixed-element standard solution at two concentration levels, procedural blanks and repeated analysis of 2% of samples. Matrix-appropriate SRMs were analyzed once in three months. Samples (200μL) were diluted 10 mL with diluent solution containing 0.05% Triton X-100, 0.5% Nitric acid and mixed internal standard. Samples were

mixed thoroughly analyzed using inductively coupled plasma – mass spectrometer – Triple quadrupole (ICP-MS) (Agilent 8900-QQQ) at the Mount Sinai Children's Health Exposure Analysis Resource (CHEAR) Network Laboratory Hub.[94] Trace metals analyzed included arsenic (As), barium (Ba), cadmium (Cd), cobalt (Co), cesium (Cs), copper (Cu), magnesium (Mg), manganese (Mn), molybdenum (Mo), nickel (Ni), lead (Pb), antimony (Sb), selenium (Se), tin (Sn), thallium (Tl), vanadium (V), and zinc (Zn). Recoveries were determined from analyses of matrix appropriate CHEAR-QC pools. Recoveries were 90-110% for As, Ba, Cd, Co, Cs, Cu, Mg, Ni, Pb, Sb, Se, Sn, Tl, V, and Zn; 80-120% for Mn and Mo. Data for all QC analysis including interday CV and intraday CVs were determined in each batch for internal urine pools fortified at mid- and high-level concentrations analyzed after initial calibration and every ten study samples. Interday CV range from 8 to 20% for most of the elements except for Mn at 37%. Intraday CV range from 1 to 12% for all elements. The limit of detection (LOD) for each trace metal is listed in **Supplementary Table 1**. For biomarker concentrations below LOD a value of the lab-specific LOD/sqrt(2) was imputed.

Urine creatinine was measured using a well-established colorimetric method with a LOD of 0.3125 mg/dL[93] and quantified in proficiency testing program conducted by G-EQUAS (The German External Quality Assessment Scheme for analyses in biological materials, http://www.g-equals.de/).[163] Quality control measures for the CHEAR lab assays have been previously described in detail.[164] Trace metal biomarkers missing creatinine values were excluded.

Trace metal and creatinine concentrations were $\log_2$ transformed for statistical analysis.

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).[98] Prior to DXA assessment, the girls were screened for pregnancy. In short, the left and right

breasts were scanned with Prodigy DXA system software (version 13.6, series 200674; GE Healthcare). Quality control and calibration were obtained using reference breast density materials. DXA assessed absolute FGV (cm^3) and total breast volume (cm^3). The %FGV was calculated as 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.[98,99]

<u>Covariates</u>

Covariates were selected *a priori* based on biological relevance of the trace metal-breast density relation (i.e., mother's education, birthweight, body fat%, age at 2YPM, age at menarche) based on biological relevance. A directed acyclic graph (DAG) was used to represent the relevant confounders and covariates utilized in the current analysis (**Figure 2**). Birthweight was obtained from health records. Anthropometric measures (e.g., weight, height) were measured every 6 to 12 months by trained dietitians. Percent body fatness was measured using a bioimpedance device. Age of menarche was surveyed by study dieticians every 6 months prior to B4 and every 3 months after reaching B4. Maternal education was collected through interviews with the girls' mothers. Missing covariate data were imputed using mean (continuous variables) or median (categorical variables) imputation.

<u>Statistical analysis</u>

Parametric G-formula (hereafter 'G-formula') was used for the statistical analysis of trace metals and breast density. G-formula, adjusting for both time-varying confounder and fixed covariates,[100] estimated time-specific associations of trace metals on breast density at 2YPM. Trace metals (continuous) and fat percentage (continuous) are measured longitudinally at multiple timepoints, which places fat percentage as both a confounder and a mediator in the association between trace metals and breast density. G-formula adjusts for body fat percentage,

an exposure-dependent confounder, without blocking the indirect path of trace metals to breast density through body fat percentage. Time-fixed covariates included age at 2YPM (continuous), birthweight (continuous), age at menarche (continuous), maternal education (categorical: secondary education or less, greater than secondary education). We used non-creatinine-adjusted trace metal biomarker concentrations and included creatinine as covariate in the models.[165] More details on the application of G-formula with time-varying confounders adjustment can be found in **Supplementary File 1**.

4.4 Results

The current analysis of trace metals and breast density include two subsamples, 291 girls with urinary samples in B1 and 253 girls with urinary samples with both B1 and B4 (**Figure 1**). Participants were on average 7.4 years old (SD 0.56) at B1, 11.0 years old (SD 0.94) at B4, and 13.7 years old (SD 1.1) at 2YPM (**Table 1**). In both subsamples, age of menarche was approximately 12.2 years and birthweight was 3.34 kg. Overall, girls' mean body fat percentage increased from 25% at B1 to 32% at 2YPM. The majority of participants' mothers (77%) reported secondary education or less.

The geometric means of the 17 trace metal biomarkers by study timepoint are listed in **Table 2**. For the majority of the biomarkers, concentrations at B1 were slightly higher than those of B4. The Spearman correlation coefficients between timepoints B1 and B4 for each trace metal ranged between 0.002 and 0.45. Almost all trace metals were positively correlated with each other, with no specific correlation patterns by element type (**Supplemental Figure 1**).

Under the hypothetical intervention of trace metal exposures at B1 only, a doubling of thallium concentration resulted in a 13.69 cm^3 increase in absolute FGV (β: 13.69, 95% Confidence Interval (CI): 2.81, 24.52) (**Table 3**). In contrast, a doubling of lead concentration was associated with a 7.76 cm^3 decrease in absolute FGV (β: -7.76, 95%CI: -14.71, -0.73).

Under the hypothetical interventions of trace metal exposure at B4 only, a doubling of barium concentration was associated with a 10.06 cm^3 increase (β: 10.06, 95% CI: 1.44, 18.60), copper concentration with a 12.29 cm^3 increase (β: 12.29, 95% CI: 2.78, 21.56) lead concentration with a 9.86 cm^3 increase (β: 9.86, 95% CI: 0.73, 18.98), antimony concentration with a 12.97 cm^3 increase (β: 12.97, 95% CI: 1.98, 23.79) and vanadium concentration resulted in a 13.14 cm^3 increase in absolute FGV (β: 13.14, 95% CI: 2.73, 23.58). No other significant associations were observed.

Under the hypothetical intervention of trace metal exposures at B4 only, a doubling of copper concentration resulted in a 1.92% unit increase in percent. We did not observe a relation between trace metal exposure and %FGV, under hypothetical interventions at B1 only (**Table 4**). We observed no joint total effect of trace metal exposures at B1 and B4 on breast density at 2YPM.

A sensitivity analysis of MBzP at B4 only restricting to girls with both B1 and B4 urine measurements (n=253) but without adjusting for MBzP measurement at B1 did not appreciably alter the results (data not shown).

4.5 Discussion

To date, this is the first study to consider the relation between trace metals and pubertal breast density at varying pubertal WOS. We observed that selected trace metals (barium, copper, lead, antimony, thallium, and vanadium) were associated with breast density among pubertal Chilean girls. Most of these associations were not consistent through pubertal timepoints, suggesting that breast development may have differential WOS for trace metals during puberty. With the except of copper, these results were not observed with the relative measure of breast density, %FGV, across all timepoints. In regard to temporal variability of trace

metal biomarkers, our results found low to moderate correlation between B1 and B4, which may depend on the biomarker's half-life as well as the commonness of the exposures.

In this study, known metalloestrogens, barium and lead were associated with absolute breast density. While our study is the first to suggest an association between these trace metals and breast density, previous mouse studies found barium and lead to disrupt estrogen function. One study found acute barium exposure to result in decreased ovary weight [166] and while another reported lead-water fed rats to have higher levels of docosahexaenoic acid concentration which are directly associated with estradiol concentrations.[167] Animal studies investigating metalloestrogens and breast density included cadmium and arsenic. Mouse studies by Parodi et al. examined the relation between *in utero* treatment to cadmium and arsenic dissolved in water and mammary gland development and found that cadmium and arsenic advanced puberty onset and mammary gland development prior to its effect on the onset of puberty. This important temporal relation was hypothesized to be induced by the metalloestrogens increasing the number of mammosphere-forming cells, epithelia cells, branch points and ultimately, breast density before its effect on the hypothalamic-pituitary-gonadal axis.[168,169] While we did not find an association between cadmium and breast density, it may be that different metals have different WOS (e.g., *in utero* and pubertal) for human breast development.

In contrast to our null finding between cadmium and breast density, a Polish study including women at 40-60 years of age and who were majority parous reported urine cadmium concentration to be inversely associated with %FGV (ß: -0.077, 95%CI: -0.142, -0.013), but not with absolute FGV.[170] In another study of premenopausal women ages 40-45 years, a doubling of urinary cadmium was associated with Breast Imaging- Reporting and Data System (BI-RADS) category of "extremely dense" breast (OR: 1.75, 95%CI: 1.14, 2.70).[171] The reasons for the mixed results in all of these studies are unclear. It is possible that our null finding is

attributable to our study population's lower mean cadmium concentrations as well the distinctly younger population of pubertal girls and the accompanying differences in covariates including parity and menopausal status.

Although we did not find any association between magnesium levels and breast density, a cross-sectional study of women aged 40-65 years with comparable mean urinary magnesium levels to our study found a doubling of urinary magnesium concentration to have greater breast density (OR: 1.36, 95%CI: 1.16, 1.59).[172] Magnesium deficiency has been reported to be associated with breast cancer, hypothesizing chronic and systematic inflammation as a underlying mechanism.[173] However, other studies have noted magnesium's favorable role in tumorigenesis.[174] The duality of magnesium's role in our bodies coupled with its potential heterogenous effect on pubertal girls and adult women may explain the discrepancy in study results.

Lastly, a study by White et al. evaluated the association between air toxics and breast density by using residential air assessment based on the Environmental Protection Agency National Air Toxic Assessment. The authors found that women living in areas with high concentrations of lead and cobalt were more likely to have dense breasts (OR: 1.60, 95%CI: 1.56, 1.64; OR:1.56, 95%CI: 1.52, 1.64, respectively).[175] It is important to note, however, the measured residential levels of air toxins may not necessarily reflect the actual individual exposure levels of the women in the study. In our study, both lead and cobalt exposures at B1 were associated with a decreased absolute FGV, but with an increased absolute FGV with exposures at B4. Different collection methods for trace metals (e.g. urine vs. toxic levels in air) as well as residual confounding or differences in underlying characteristics of the study populations may have accounted for the discrepancies in outcome.

Strengths and limitations

A limitation of our study is the collection of trace metals from a single urine sample. Trace metals are also commonly measured in blood, hair and toenails, all representing different exposure sources (e.g., diet, water, air) as well as varying affinity for specific metals. Studies have suggested analysis of trace metals in hair to be more representative of long-term exposure and stable levels.[176] Regardless, urinary biomarkers are a good, objective indicator of trace metal exposures that our study utilizes as a non-invasive, procedure to collect many analytes of interest at once.[177] Lastly, trace metals are present in mixtures, in which not all metals have homogenous effects on health outcomes. This makes it particularly challenging to interpret individual metal's impact on breast development, an outcome without clear disease manifestation.

A strength of our study is the longitudinal design which establishes a temporal relation between our trace metal exposures and our breast density outcome. Specifically, our study cohort was followed through critical pubertal periods, with data collected at clinically relevant breast developmental periods. Prior epidemiologic research on trace metals and breast density mostly used cross-sectional study designs with adult women participants which limits interpretation of the results. Additionally, our novel use of G-formula allowed estimation of the impact of trace metals on breast density at specific pubertal WOS while accounting for both fixed and time-varying covariates. This statistical approach reduces potential bias from over-adjusting for covariates that are both confounders and mediators.

4.6 Conclusion

Our evaluation of environmental exposures in relation to breast density provides important insights into the potential biological mechanisms of carcinogenesis in the breast during the critical period of puberty. In this cohort of Latino girls, trace metals -barium, copper,

lead, antimony, thallium, and vanadium – were associated with absolute breast density. We also observed an association between copper and percent FGV.

Figure 4. 1 Flow diagram of Growth and Obesity Cohort Study (GOCS) study population in the assessment of trace metals and breast density

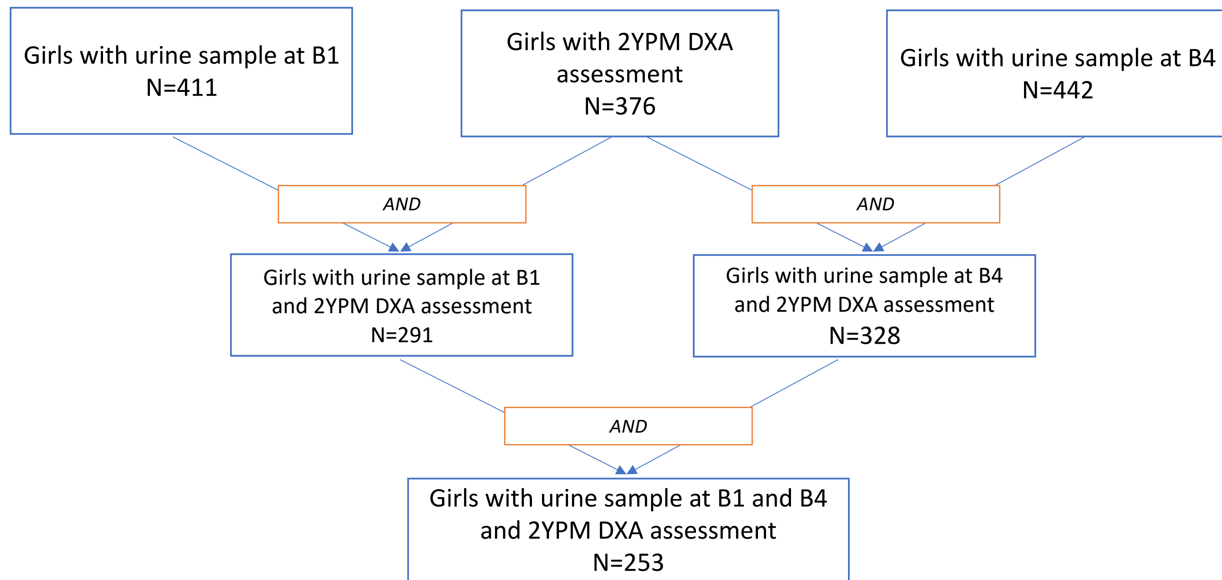


Figure 4. 2 Directed acyclic graph of the study showing the relation between trace metals and breast density

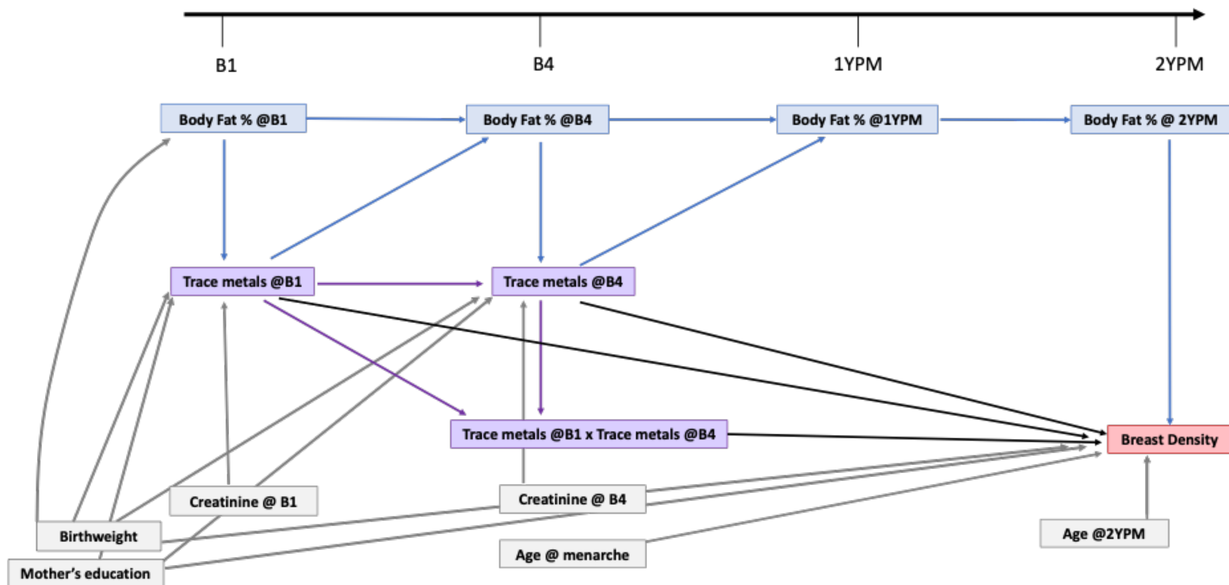


Table 4. 1 Characteristics of participants from the Growth and Obesity Cohort Study with breast density at 2-years post menarche and urine sample at Tanner breast stage B1 and B4

Characteristic	**Trace Metal Window of Susceptibility**	
	B1 only	B1 and B4[a]
	(n=291)	(n=253)
Age, years (mean (SD))		
Tanner Stage B1	7.40 (0.56)	7.41 (0.56)
Tanner Stage B4	-	11.02 (0.94)
Body fat percentage (mean (SD))		
Tanner Stage B1	25.57 (4.47)	25.24 (4.36)
Tanner Stage B4	-	26.72 (5.38)
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	224 (76.98)	195 (77.08)
Greater than secondary education	67 (23.02)	58 (22.92)
2 Year Post- Menarche (2YPM)		
Age at 2YPM	13.65 (1.10)	13.70 (1.06)
Body fat percentage at 2YPM (mean (SD))	32.49 (6.12)	32.08 (6.02)
Absolute Fibroglandular Volume, cm^3 (mean (SD))	213.90 (81.13)	214.46 (74.11)
Percent Fibroglandular Volume, % (mean (SD))	50.28 (15.44)	51.37 (15.25)

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

Table 4. 2 Urinary trace metal biomarkers (ng/ml) geometric means (95% confidence interval) by study time point among Growth and Obesity Cohort Study participants

	Tanner Stage B1	Tanner Stage B4	Spearman correlation coefficients
	n = 291	n = 253[a]	
Trace Metals			
Arsenic (As)	10.51 (9.66, 11.43)	9.29 (8.38, 10.29)	0.002
Barium (Ba)	2.47 (2.30, 2.66)	2.33 (2.11, 2.56)	0.22
Cadmium (Cd)	0.10 (0.09, 0.10)	0.08 (0.08, 0.09)	0.17
Cobalt (Co)	0.19 (0.17, 0.20)	0.21 (0.19, 0.23)	0.17
Cesium (Cs)	23.37 (21.72, 25.14)	16.52 (15.18, 17.97)	0.11
Copper (Cu)	10.36 (9.57, 11.21)	9.67 (8.85, 10.55)	0.10
Magnesium (Mg)	58030.80 (52919.10, 63636.20)	60076.90 (53386.80, 66362.40)	0.20
Manganese (Mn)	2.05 (1.94, 2.17)	1.61 (1.52, 1.69)	0.38
Molybdenum (Mo)	38.40 (35.24, 41.83)	33.95 (29.81, 36.71)	0.09
Nickel (Ni)	5.91 (5.55, 6.30)	5.30 (5.02, 5.58)	0.25
Lead (Pb)	2.18 (1.96, 2.42)	0.71 (0.65, 0.78)	0.16
Antimony (Sb)	0.12 (0.11, 0.13)	0.10 (0.09, 0.11)	0.11
Selenium (Se)	33.96 (31.60, 36.49)	33.91 (31.32, 36.71)	0.45
Tin (Sn)	1.24 (1.11, 1.40)	1.10 (0.96, 1.23)	0.22
Thallium (Tl)	0.38 (0.35, 0.41)	0.30 (0.28, 0.33)	0.14
Vanadium (V)	0.14 (0.13, 0.14)	0.12 (0.12, 0.13)	0.11
Zinc (Zn)	282.00 (256.70, 309.80)	346.3 (307.00, 390.60)	0.25

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

Table 4. 3 Simulated relative change[a] in absolute FGV (cm^3) associated with log2 (ng/ml) increase in urinary trace metal biomarkers among Growth and Obesity Cohort Study participants

Window of Susceptibility	Tanner Stage B1 only N= 291			Tanner Stage B4 only N= 253			Joint total effect of B1 and B4 N= 253		
	Estimate of relative change[b]	**SE[b]**	**95% Confidence Interval[b]**	**Estimate of relative change[c]**	**SE[c]**	**95% Confidence Interval[c]**	**Estimate of relative change[d]**	**SE[d]**	**95% Confidence Interval[d]**
Arsenic (As)	8.50	4.49	(-0.39, 17.50)	1.73	3.95	(-6.22, 9.62)	10.64	22.58	(-34.14, 54.51)
Barium (Ba)	-1.71	5.16	(-11.80, 8.61)	10.06	4.21	(1.44, 18.60)	8.17	9.62	(-10.74, 26.40)
Cadmium (Cd)	-3.22	6.51	(-15.99, 9.80)	0.01	5.69	(-11.44, 11.44)	74.87	63.91	(-39.86, 203.22)
Cobalt (Co)	-2.04	4.40	(-10.69, 6.84)	5.88	4.04	(-2.11, 13.87)	17.24	21.99	(-23.49, 63.44)
Cesium (Cs)	5.11	5.17	(-5.13, 15.52)	-4.71	4.85	(-14.36, 4.88)	-11.83	42.44	(-96.00, 68.28)
Copper (Cu)	-0.40	4.79	(-9.81, 9.22)	12.29	4.64	(2.78, 21.56)	-11.74	27.90	(-67.49, 41.00)
Magnesium (Mg)	-5.01	4.09	(-13.05, 3.15)	-7.10	4.14	(-15.50, 1.13)	-48.92	115.82	(-273.07, 174.90)
Manganese (Mn)	-1.71	6.58	(-14.69, 11.55)	14.08	7.62	(-0.61, 30.04)	8.61	12.37	(-16.26, 32.74)
Molybdenum (Mo)	-5.62	4.39	(-14.31, 3.11)	3.45	4.11	(-4.70, 11.82)	10.89	35.81	(-60.39, 77.93)
Nickel (Ni)	-5.78	5.95	(-17.50, 6.06)	-3.50	7.69	(-18.65, 11.82)	-21.58	42.85	(-107.22, 59.53)
Lead (Pb)	-7.76	3.55	(-14.71, -0.73)	9.86	4.53	(0.73, 18.98)	3.93	5.49	(-6.94, 14.79)
Antimony (Sb)	-2.41	5.24	(-12.69, 8.12)	12.97	5.41	(1.98, 23.79)	48.12	44.96	(-35.75, 139.50)
Selenium (Se)	-7.58	5.24	(-17.95, 2.82)	8.22	5.16	(-1.95, 18.54)	-122.80	52.05	(-224.51, -25.56)
Tin (Sn)	-1.47	3.17	(-7.67, 4.87)	-0.23	3.30	(-6.83, 6.27)	1.51	4.58	(-7.43, 10.80)
Thallium (Tl)	13.69	5.49	(2.81, 24.52)	0.61	5.06	(-9.47, 10.75)	11.95	25.51	(-36.18, 62.40)
Vanadium (V)	4.37	6.59	(-8.60, 17.48)	13.14	5.16	(2.73, 23.58)	29.66	50.62	(-63.50, 132.16)
Zinc (Zn)	-4.66	4.00	(-12.46, 3.36)	-3.28	3.39	(-10.03, 3.45)	-93.09	46.86	(-185.02, -3.71)

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

Table 4. 4 Simulated relative change[a] in percent FGV (%) associated with log2 (ng/ml) increase in urinary trace metal biomarkers among Growth and Obesity Cohort Study participants

Window of Susceptibility	Tanner Stage B1 only N= 291			Tanner Stage B4 only N= 253			Joint total effect of B1 and B4 N= 253		
	Estimate of relative change[b]	**SE[b]**	**95% Confidence Interval[b]**	**Estimate of relative change[c]**	**SE[c]**	**95% Confidence Interval[c]**	**Estimate of relative change[d]**	**SE[d]**	**95% Confidence Interval[d]**
Arsenic (As)	0.56	0.85	(-1.14, 2.21)	-1.15	0.79	(-2.69, 0.41)	-2.05	4.65	(-11.13, 7.12)
Barium (Ba)	0.53	0.98	(-1.45, 2.42)	-0.12	0.83	(-1.76, 1.47)	0.90	1.90	(-2.79, 4.55)
Cadmium (Cd)	-0.60	1.24	(-3.11, 1.74)	0.91	1.12	(-1.28, 3.14)	-1.04	12.95	(-26.06, 24.25)
Cobalt (Co)	0.38	0.84	(-1.29, 2.00)	1.25	0.79	(-0.27, 2.79)	5.61	4.44	(-3.15, 14.12)
Cesium (Cs)	0.67	0.98	(-1.29, 2.56)	-0.72	0.96	(-2.61, 1.17)	-0.20	8.65	(-17.14, 16.41)
Copper (Cu)	-0.14	0.91	(-1.96, 1.62)	1.92	0.92	(0.15, 3.72)	0.13	5.87	(-11.22, 11.52)
Magnesium (Mg)	0.71	0.78	(-0.84, 2.21)	-1.29	0.82	(-2.90, 0.32)	-12.50	23.05	(-57.95, 32.30)
Manganese (Mn)	-0.01	1.25	(-2.50, 2.43)	1.07	1.51	(-1.89, 4.07)	0.44	2.57	(-4.74, 5.33)
Molybdenum (Mo)	0.77	0.84	(-0.89, 2.39)	-1.24	0.81	(-2.86, 0.39)	-1.95	7.38	(-16.23, 11.87)
Nickel (Ni)	-0.47	1.13	(-2.76, 1.69)	2.16	1.51	(-0.81, 5.08)	7.54	8.60	(-9.14, 24.14)
Lead (Pb)	-0.09	0.69	(-1.47, 1.23)	1.04	0.90	(-0.72, 2.82)	0.86	1.21	(-1.41, 3.24)
Antimony (Sb)	0.12	1.00	(-1.87, 2.06)	1.10	1.07	(-0.99, 3.17)	15.45	9.15	(-2.56, 33.35)
Selenium (Se)	-1.01	1.00	(-2.99, 0.88)	-1.00	1.03	(-3.04, 1.04)	-17.35	11.28	(-39.13, 4.47)
Tin (Sn)	-0.42	0.60	(-1.65, 0.73)	0.002	0.65	(-1.28, 1.29)	-0.02	0.93	(-1.75, 1.78)
Thallium (Tl)	0.48	1.04	(-1.60, 2.52)	-0.19	1.00	(-2.15, 1.75)	0.44	5.16	(-9.85, 10.38)
Vanadium (V)	1.27	1.25	(-1.22, 3.69)	-0.45	1.04	(-2.48, 1.56)	3.60	10.56	(-18.02, 24.15)
Zinc (Zn)	-0.03	0.76	(-1.52, 1.48)	-0.66	0.67	(-2.00, 0.67)	-11.50	9.68	(-30.42, 6.68)

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

Chapter 5. Conclusions and Public Health Relevance

This dissertation examines the impact of endocrine disrupting chemical exposures (PFOA, MBzP, and trace metals) on breast density and hormones during puberty. The first study prospectively assessed the association between biomarker concentrations of BBP and PFOA at specific pubertal WOS and adolescent breast density. PFOA serum concentrations corresponded to a marginal increase in absolute FGV and decreased in % FGV, while no effect was observed between MBzP and breast density measures across pubertal WOS. The second study prospectively evaluated the association between MBzP and PFOA and hormones, individually and collectively, during puberty. In this study, MBzP was associated with insulin growth factor-1 (IGF-1) and 17-OH progesterone. PFOA was associated with IGF-1, 17-OH progesterone and sex hormone binding globulin. Associations between MBzP and PFOA and hormones varied by pubertal time point, suggesting differential effects of EDC exposure by specific window of susceptibility on pubertal hormone levels. The third study prospectively evaluated the association between trace metals at specific pubertal time points and adolescent breast density. Selected trace metals - barium, copper, lead, antimony, thallium, and vanadium – were associated with absolute breast density. Copper was also associated with percent FGV. Most of these associations were not consistent through pubertal timepoints, suggesting that breast development may have differential WOS for EDCs during puberty.

In all three studies, a novel computational method, G-formula was used to assess the association between EDCs and breast density and hormones. With additional identifiability assumptions, G-formula allows estimation of causal effect of these exposures on hormones and breast density in a longitudinal cohort of adolescent Latina girls. Additionally, this 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 confounders and a mediators between the EDCs and breast density. Lastly, under the numerous hypothetical interventions developed based on causal DAGs, we are able to estimate the effect of EDCs from a specific WOS (e.g. Tanner Stage B1 only, B4 only) 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).

In conclusion, by using the framework of life course epidemiology, this research contributes to an understanding of the variation in breast cancer risk associated with environmental exposures in childhood to help identify the most effective and appropriate time period for breast cancer prevention.

Appendix 1. Supplemental content for Chapter 2

Supplementary File 1. G-formula application

We considered four ‘hypothetical interventions’, two reflecting potential WOS (B1 and B4 separately for MBzP; B4 and 1YPM separately for PFOA) timepoints, one estimating the joint total effect of EDCs in both WOS timepoints (B1 and B4 for MBzP; B4 and 1YPM for PFOA), and one estimating the joint total effect of both MBzP and PFOA at B4 on breast density (**Supplementary Figure 1)**.

Here we describe a simplified application of the G-formula:

1) Obtain empirical parameters: a. Breast density at 2YPM is regressed on all potential time-fixed covariates, time-varying confounder and exposure of interest (e.g. E(Breast $Density_{2YPM}$|$MBzP_{B1}$, body fat $percentage_{B1}$, time-fixed covariates)) to obtain the regression coefficients and root mean square error (RMSE); b. Obtain observed marginal EDCs distributions at each timepoint and their standard deviations; c. Estimate the conditional distributions of each time-varying body fat percentage by regressing body fat percentage on observed EDC and relevant covariates to obtain the regression coefficients and RMSE.

2) Simulate the potential outcomes: a. We created 1000 copies of the original sample and simulated age at 2YPM, age at menarche, birthweight, maternal education that followed the same distributions as the observed variables; b. We simulated MBzP and PFOA variables at each timepoint that followed the observed MBzP and PFOA prevalence but was marginally independent of all simulated covariates; c. We simulated each potential body fat percentage as a function of the EDC intervention, birthweight,

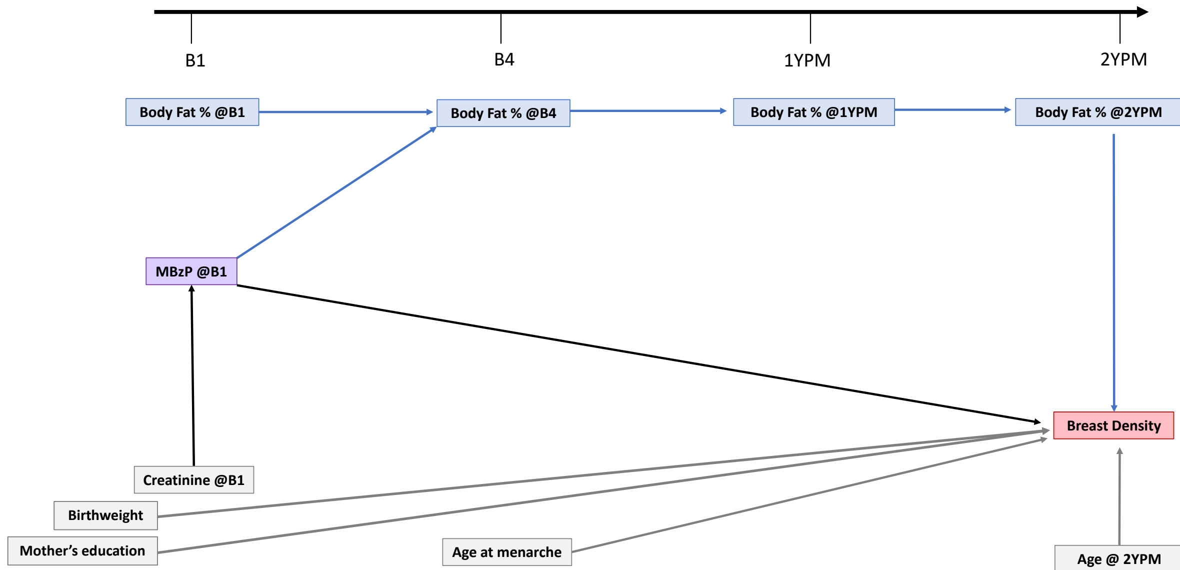
maternal education, age of menarche, and age of the same timepoint, using the regression coefficient and RMSE from step 1c; d. We simulate the potential breast density outcome at 2YPM as a function of the EDC intervention, potential body fat percentage from step 2c, product term between EDC intervention and potential body fat percentage, age at 2YPM, age at menarche, birthweight, maternal education using the regression coefficients from and RMSE from step 1a.

3) Fitting final marginal structural models (MSMs): We regressed each different potential breast density outcome on the EDC interventions to obtain point estimates of each marginal effect using the pooled sample. We repeat step 2-3 on 1000 bootstrapped samples of the same size taken at random with replacement from the original data to obtain Wald type 95% confidence interval (CI).

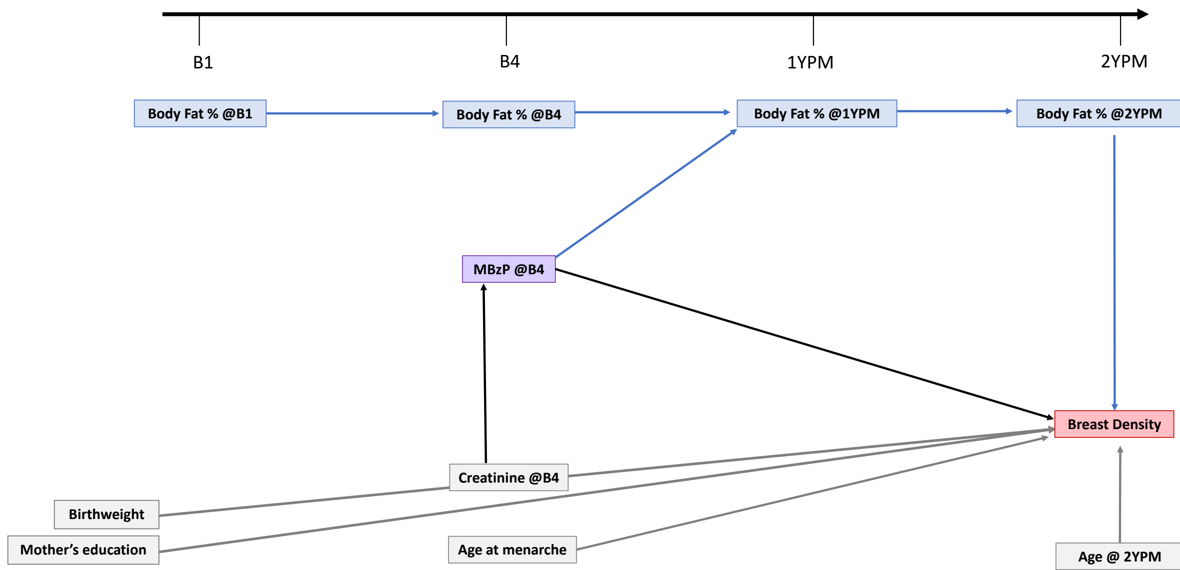
Log_2 transformations of both MBzP and PFOA biomarker concentrations were used to account for the skewness of the variables. Accordingly, beta coefficients can be interpreted as a relative increase in breast density outcome when the EDC concentrations are doubled.

It is important to note that causal interpretation of the results is only possible under the necessary set of identifiability assumptions: 1) no unmeasured or residual confounding between EDCs and breast density, 2) positivity assumption, 3) consistency, 4) no measurement error, and 5) no model misspecification. Further explanations on these assumptions can be found elsewhere.[47,48]

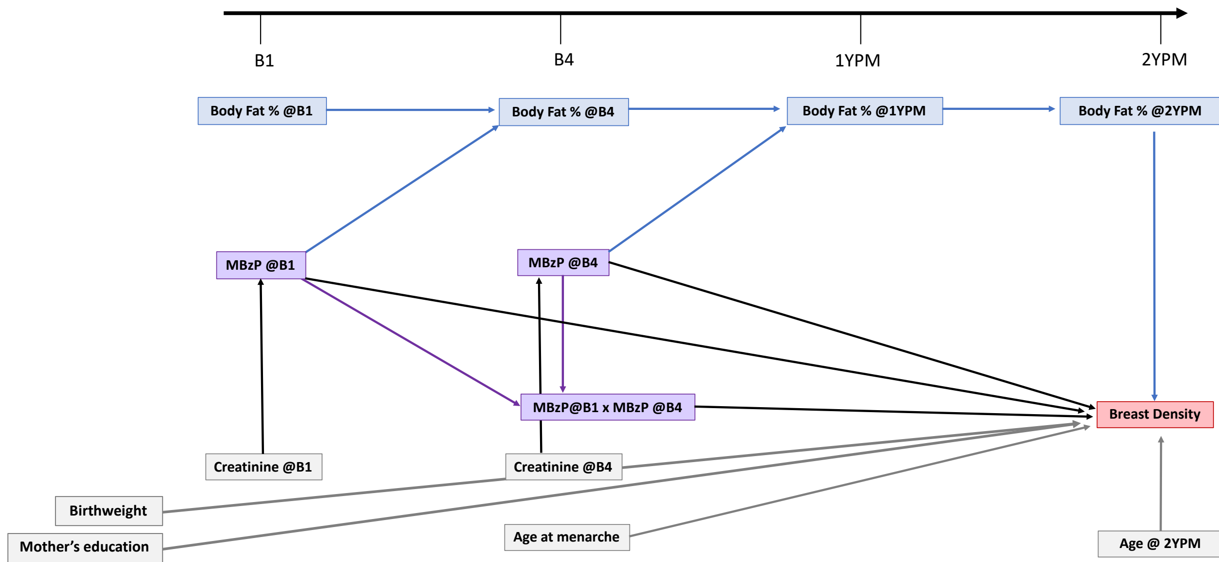
Supplementary Figure 1. 1 Directed acyclic graph of the study showing the hypothetical interventions of monobenzyl phthalate (MBzP) at B1 only and breast density



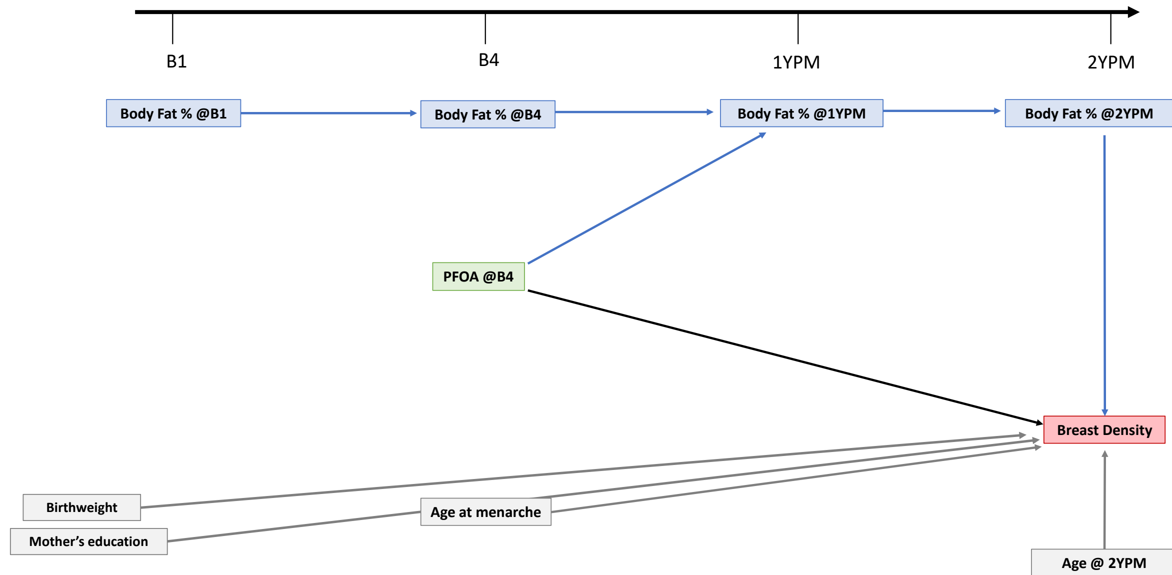
Supplementary Figure 1. 2 Directed acyclic graph of the study showing the hypothetical interventions of monobenzyl phthalate (MBzP) at B4 only and breast density



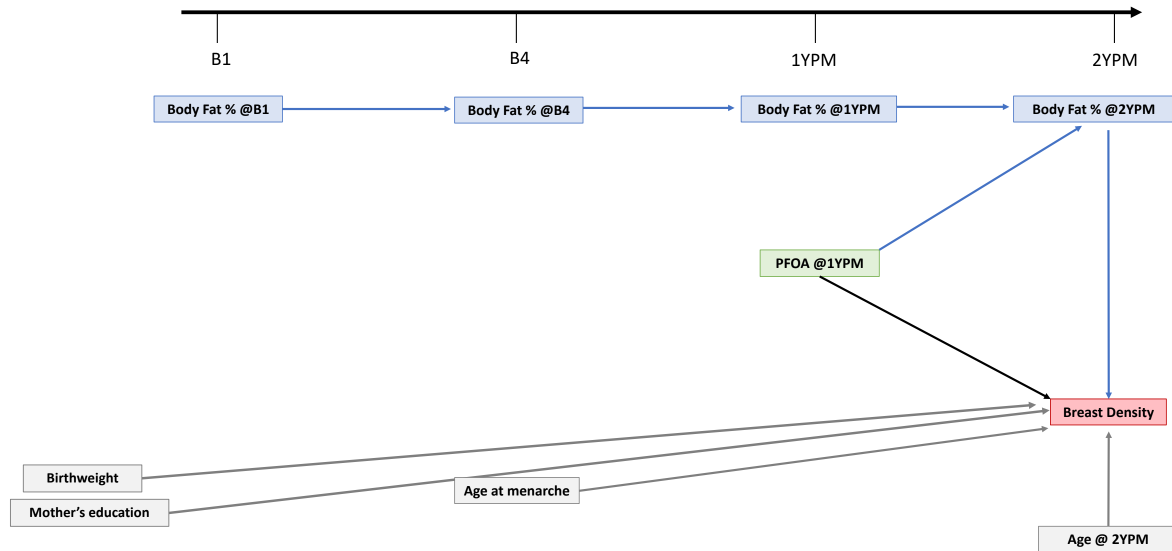
Supplementary Figure 1. 3 Directed acyclic graph of the study showing the hypothetical interventions of the joint total effect of monobenzyl phthalate (MBzP) at B1 and B4 and breast density



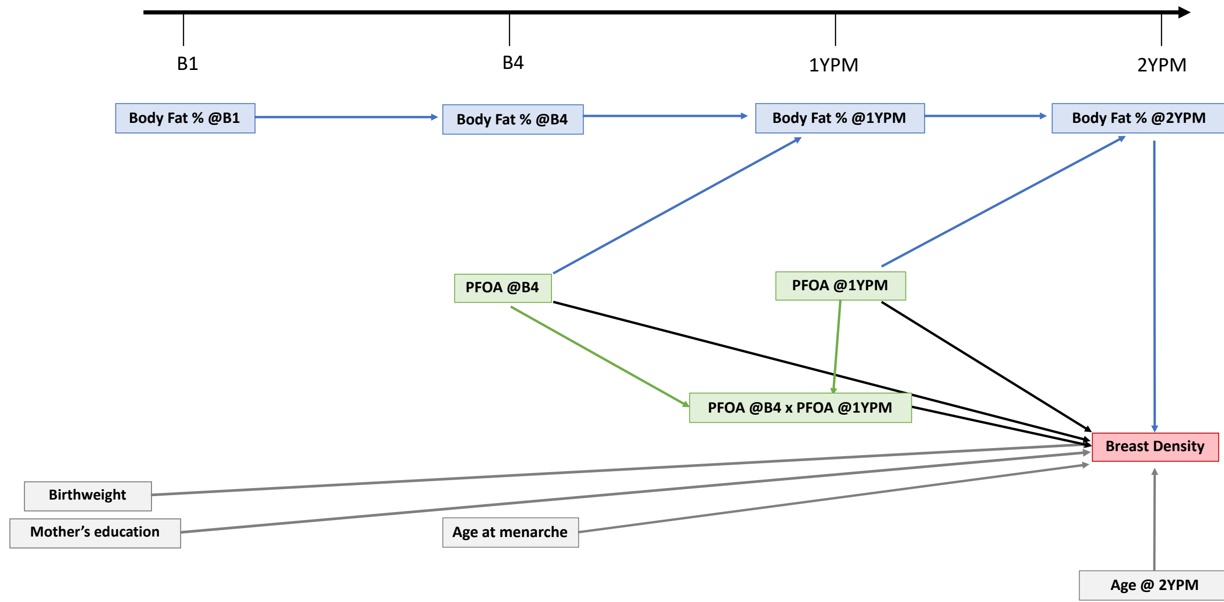
Supplementary Figure 1. 4 Directed acyclic graph of the study showing the hypothetical interventions of perflourooctanoic acid (PFOA) at B4 only and breast density



Supplementary Figure 1. 5 Directed acyclic graph of the study showing the hypothetical interventions of perflourooctanoic acid (PFOA) at 1YPM only and breast density



Supplementary Figure 1. 6 Directed acyclic graph of the study showing the hypothetical interventions of the joint total effect of perflourooctanoic acid (PFOA) at B4 and 1YPM and breast density



Appendix 2. Supplemental content for Chapter 3

Supplementary File 2. G-formula application

We considered two 'hypothetical interventions' for MBzP at B1 and B4 separately, two for PFOA at B4 and 1YPM, one estimating the joint total effect of EDCs in both WOS timepoints (B1 and B4 for MBzP; B4 and 1YPM for PFOA), and one estimating the joint total effect of both MBzP and PFOA at B4 on hormones (**Supplementary Figure 2)**.

Here we describe a simplified application of the G-formula:

1) Obtain empirical parameters: a. Hormones at 1YPM is regressed on all potential time-fixed covariates, time-varying confounder and exposure of interest (e.g. $E(Hormones_{1YPM}|MBzP_{B1}$, body fat $percentage_{B1}$, time-fixed covariates)) to obtain the regression coefficients and root mean square error (RMSE); b. Obtain observed marginal EDCs distributions at each timepoint and their standard deviations; c. Estimate the conditional distributions of each time-varying body fat percentage by regressing body fat percentage on observed EDC and relevant covariates to obtain the regression coefficients and RMSE.

2) Simulate the potential outcomes: a. We created 1000 copies of the original sample and simulated age at 1YPM, age at menarche, birthweight, maternal education, insulin at 1YPM that followed the same distributions as the observed variables; b. We simulated EDC variables at each timepoint that followed the observed EDC prevalence but was marginally independent of all simulated covariates; c. We simulated each

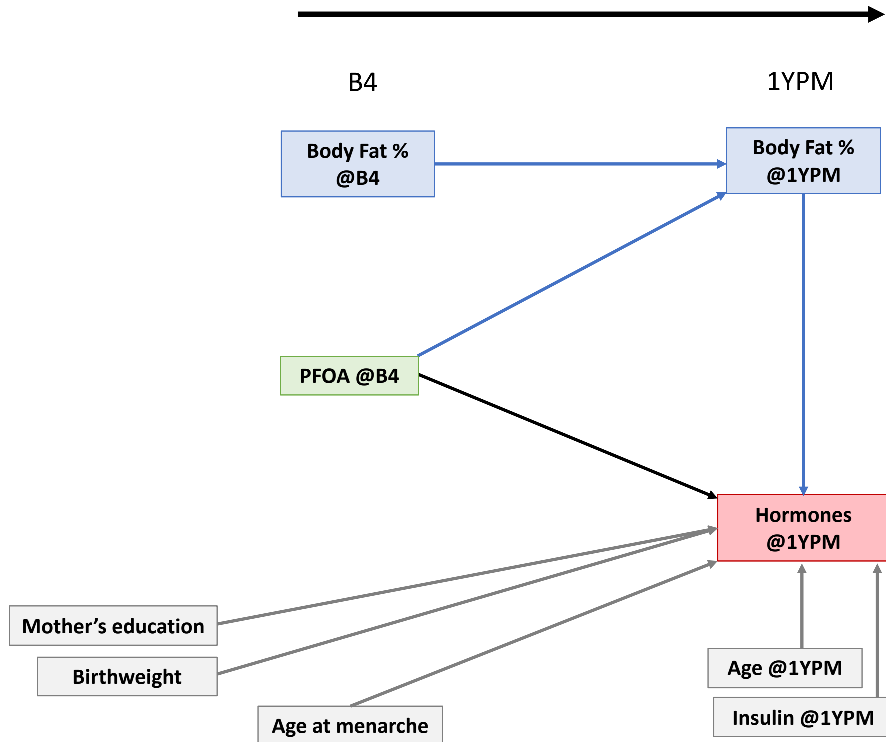
potential body fat percentage as a function of the EDC intervention, birthweight, maternal education, age of menarche, and age and insulin of the same timepoint, using the regression coefficient and RMSE from step 1c; d. We simulate the potential hormones outcome at 1YPM as a function of the EDC intervention, potential body fat percentage from step 2c, product term between EDC intervention and potential body fat percentage, age at 1YPM, age at menarche, birthweight, maternal education, insulin at 1YPM, using the regression coefficients from and RMSE from step 1a.

3) Fitting final marginal structural models (MSMs): We regressed each different potential breast density outcome on the EDC interventions to obtain point estimates of each marginal effect using the pooled sample. We repeat step 2-3 on 1000 bootstrapped samples of the same size taken at random with replacement from the original data to obtain Wald type 95% confidence interval (CI).

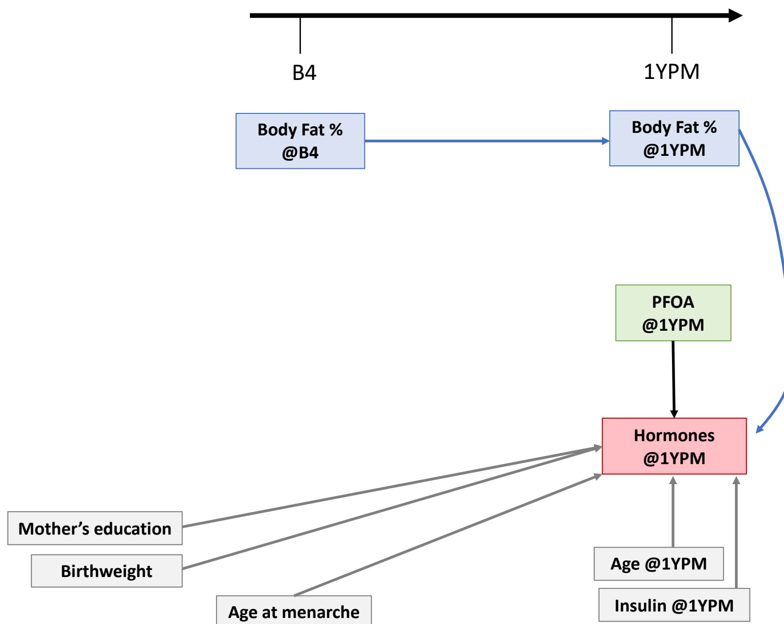
Log_2 transformations of EDC biomarker concentrations were used to account for the skewness of the variables. Accordingly, beta coefficients can be interpreted as a relative increase in hormone outcome when the EDC concentrations are doubled.

It is important to note that causal interpretation of the results is only possible under the necessary set of identifiability assumptions: 1) no unmeasured or residual confounding between trace metals and breast density, 2) positivity assumption, 3) consistency, 4) no measurement error, and 5) no model misspecification.

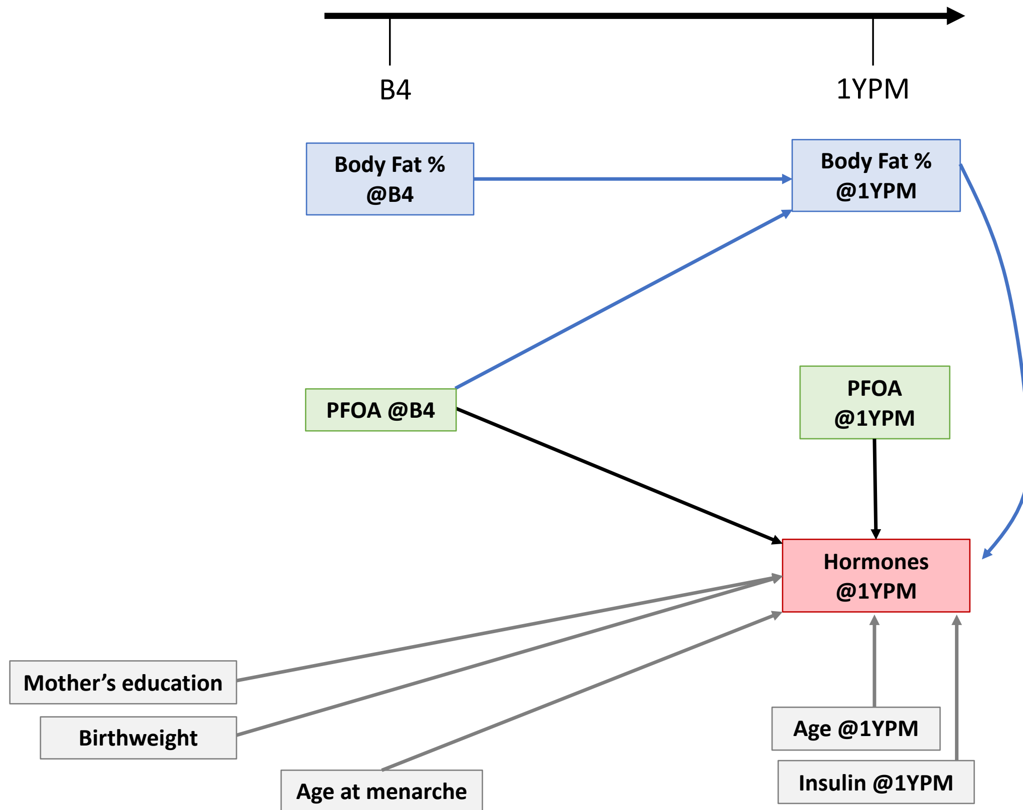
Supplementary Figure 2. 1 Directed acyclic graph of the study showing the hypothetical interventions of perflourooctanoic acid (PFOA) at B4 only and hormones



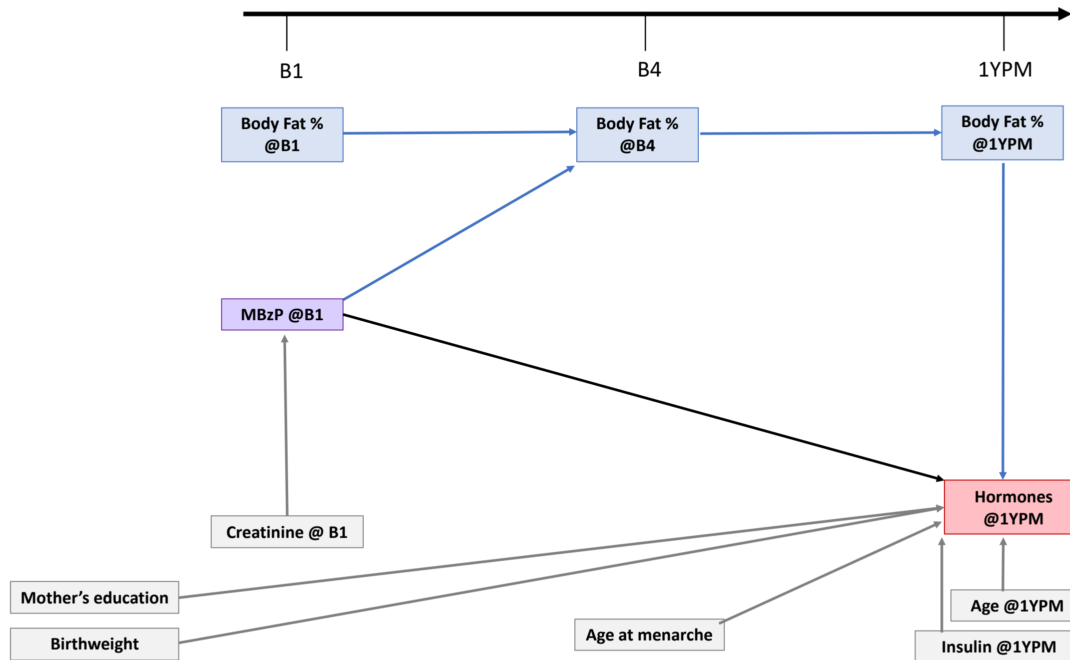
Supplementary Figure 2. 2 Directed acyclic graph of the study showing the hypothetical interventions of perflourooctanoic acid (PFOA) at 1YPM only and hormones



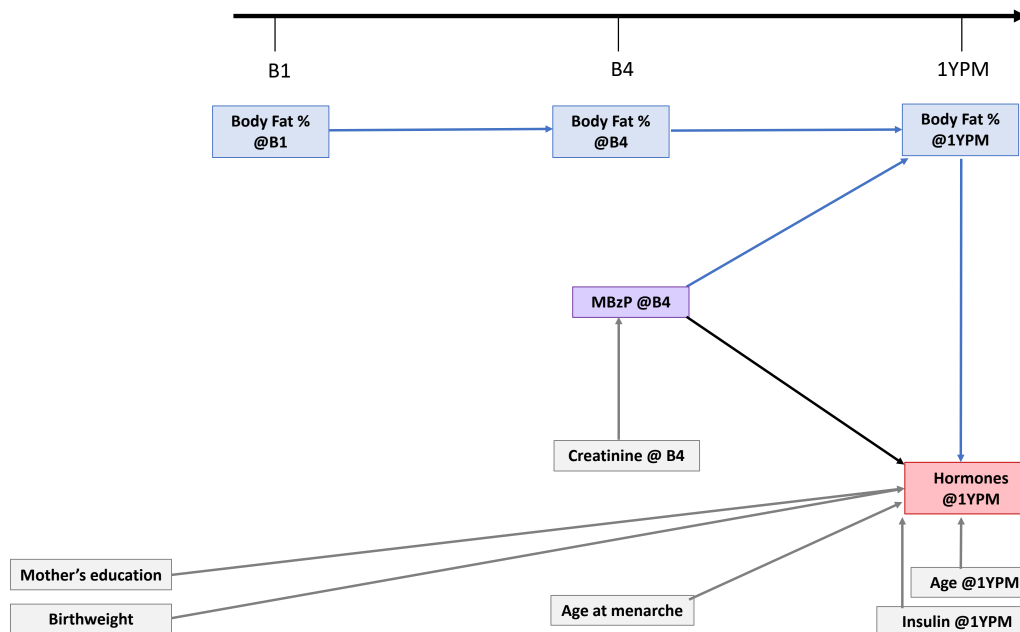
Supplementary Figure 2. 3 Directed acyclic graph of the study showing the hypothetical interventions of the joint total effect of perflourooctanoic acid (PFOA) at B4 and 1YPM and hormones



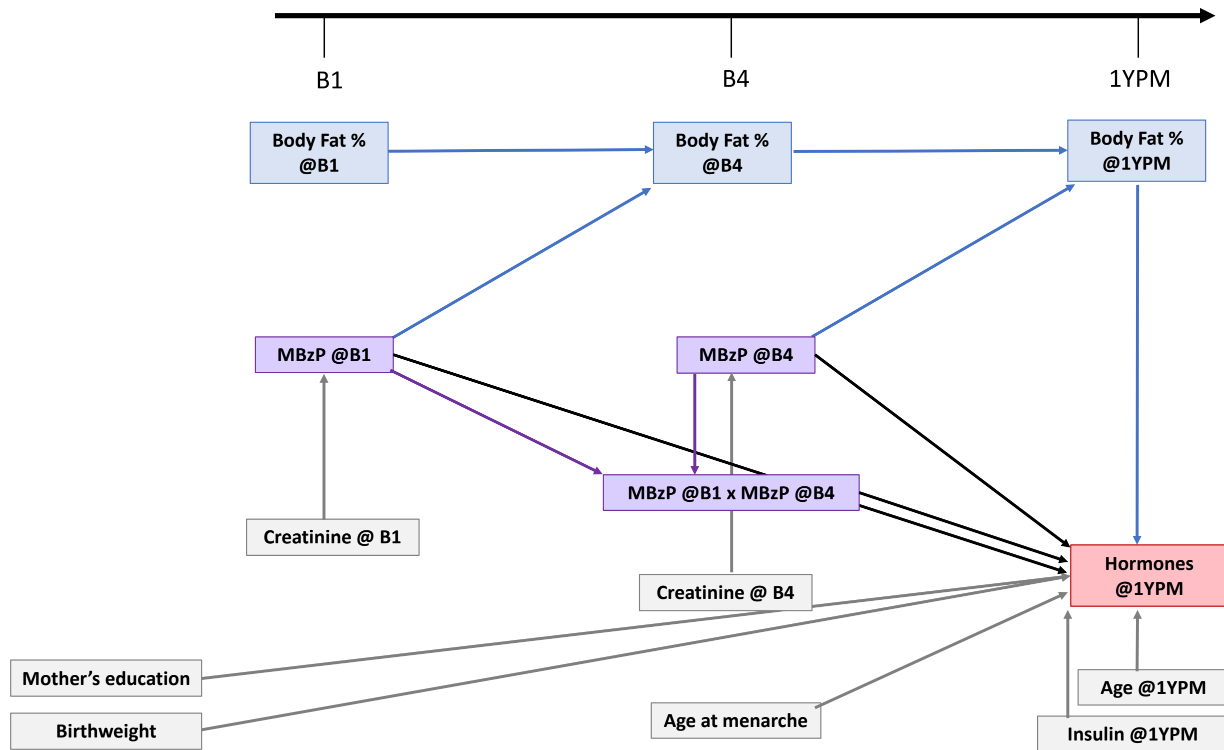
Supplementary Figure 2. 4 Directed acyclic graph of the study showing the hypothetical interventions of monobenzyl phthalate (MBzP) at B1 only and hormones



Supplementary Figure 2. 5 Directed acyclic graph of the study showing the hypothetical interventions of monobenzyl phthalate (MBzP) at B4 only and hormones



Supplementary Figure 2. 6 Directed acyclic graph of the study showing the hypothetical interventions of the joint total effect of monobenzyl phthalate (MBzP) at B1 and B4 and hormones



Appendix 3. Supplemental content for Chapter 4

Supplementary Table 1. 1 Trace metal limit of detection values at the Mount Sinai CHEAR Network Laboratory Hub

Analyte	Average Limit of Detection (LOD), ng/ml
Arsenic (As)	0.166
Barium (Ba)	0.333
Cadmium (Cd)	0.0421
Cobalt (Co)	0.138
Cesium (Cs)	0.240
Copper (Cu)	1.15
Magnesium (Mg)	6.73
Manganese (Mn)	1.15
Molybdenum (Mo)	0.156
Nickel (Ni)	2.24
Lead (Pb)	0.0745
Antimony (Sb)	0.0427
Selenium (Se)	0.834
Tin (Sn)	0.0451
Thallium (Tl)	0.0461
Vanadium (V)	0.0272
Zinc (Zn)	39.8

Supplementary File 1. G-formula application

We considered three 'hypothetical interventions', reflecting potential WOS (B1 and B4 timepoints) and one estimating the joint total effect of trace metals in both WOS timepoints (B1 and B4) (**Supplementary Figure 1 and 2)**.

Here we describe a simplified application of the G-formula:

1) Obtain empirical parameters: a. Breast density at 2YPM is regressed on all potential time-fixed covariates, time-varying confounder and exposure of interest (e.g. E(Breast Density$_{2YPM}$|Trace Metal$_{B1}$, body fat percentage$_{B1}$, time-fixed covariates)) to obtain the regression coefficients and root mean square error (RMSE); b. Obtain observed marginal trace metal distributions at each timepoint and their standard deviations; c. Estimate the conditional distributions of each time-varying body fat percentage by regressing body fat percentage on observed trace metal and relevant covariates to obtain the regression coefficients and RMSE.

2) Simulate the potential outcomes: a. We created 1000 copies of the original sample and simulated age at 2YPM, age at menarche, birthweight, maternal education that followed the same distributions as the observed variables; b. We simulated trace metal variables at each timepoint that followed the observed trace metal prevalence but was marginally independent of all simulated covariates; c. We simulated each potential body fat percentage as a function of the trace metal intervention, birthweight, maternal education, age of menarche, and age of the same timepoint, using the regression coefficient and RMSE from step 1c; d. We simulate the potential breast density outcome

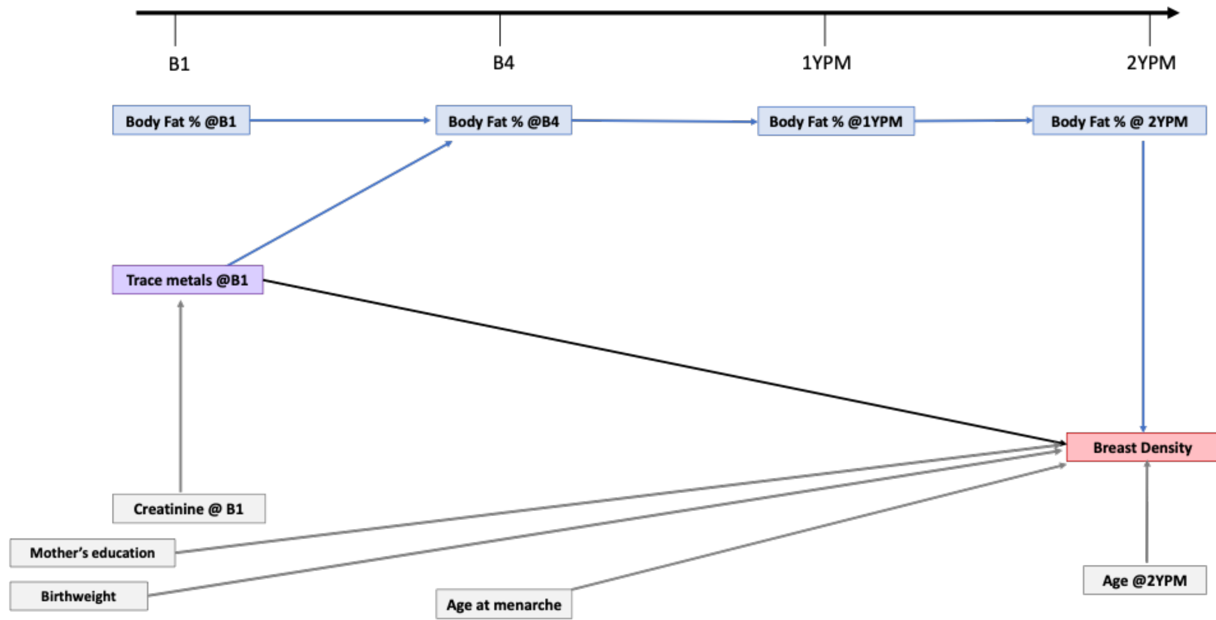
at 2YPM as a function of the trace metal intervention, potential body fat percentage from step 2c, product term between trace metal intervention and potential body fat percentage, age at 2YPM, age at menarche, birthweight, and maternal education, using the regression coefficients from and RMSE from step 1a.

3) Fitting final marginal structural models (MSMs): We regressed each different potential breast density outcome on the trace metal interventions to obtain point estimates of each marginal effect using the pooled sample. We repeat step 2-3 on 1000 bootstrapped samples of the same size taken at random with replacement from the original data to obtain Wald type 95% confidence interval (CI).

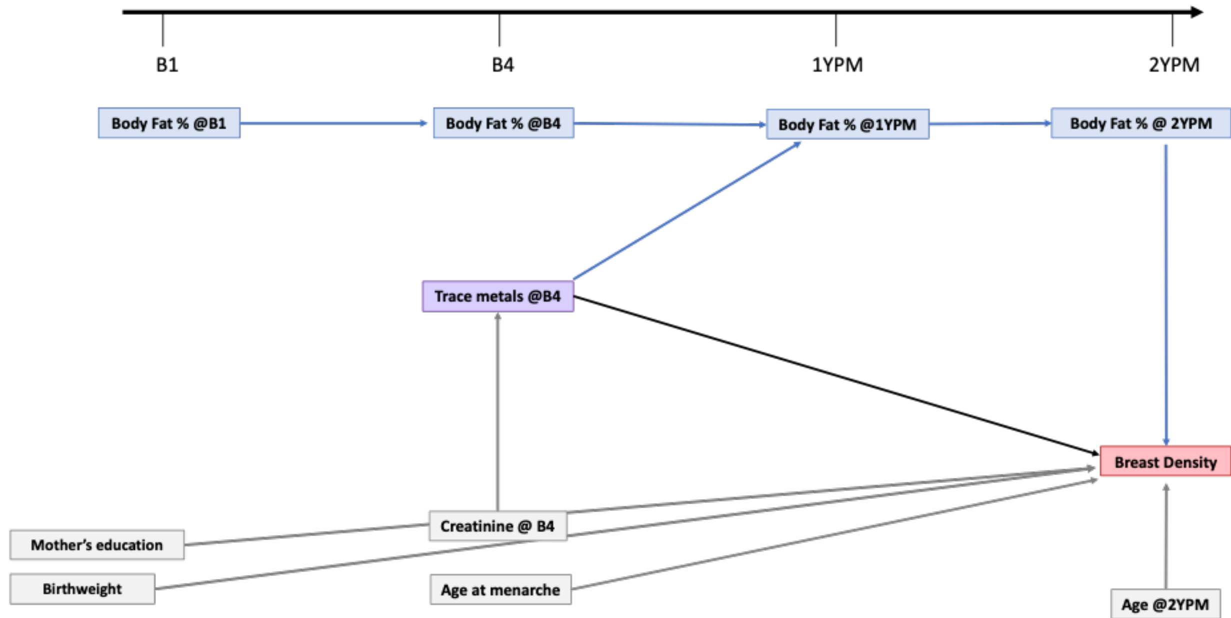
Log_2 transformations of trace metal biomarker concentrations were used to account for the skewness of the variables. Accordingly, beta coefficients can be interpreted as a relative increase in breast density outcome when the trace metal concentrations are doubled. We used SAS 9.4 software (SAS Institute Inc.) for all analyses.

It is important to note that causal interpretation of the results is only possible under the necessary set of identifiability assumptions: 1) no unmeasured or residual confounding between trace metals and breast density, 2) positivity assumption, 3) consistency, 4) no measurement error, and 5) no model misspecification.

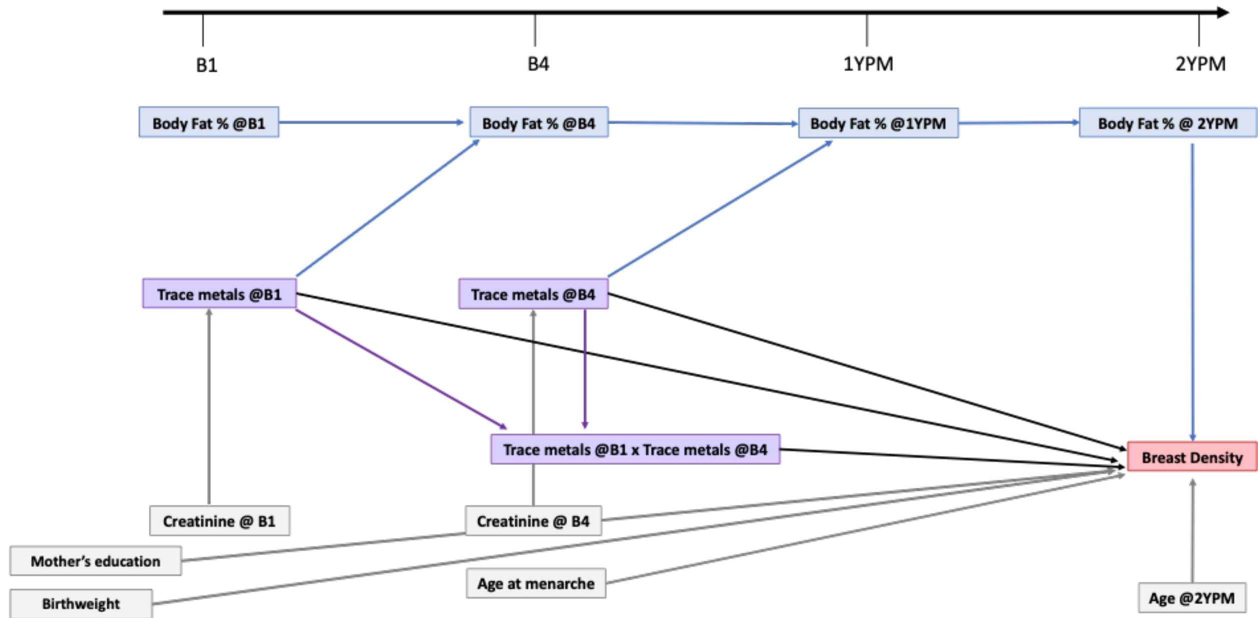
Supplementary Figure 3. 1 Directed acyclic graph of the study showing the hypothetical interventions of trace metals at B1 only and breast density



Supplementary Figure 3. 2 Directed acyclic graph of the study showing the hypothetical interventions of trace metals at B4 only and breast density

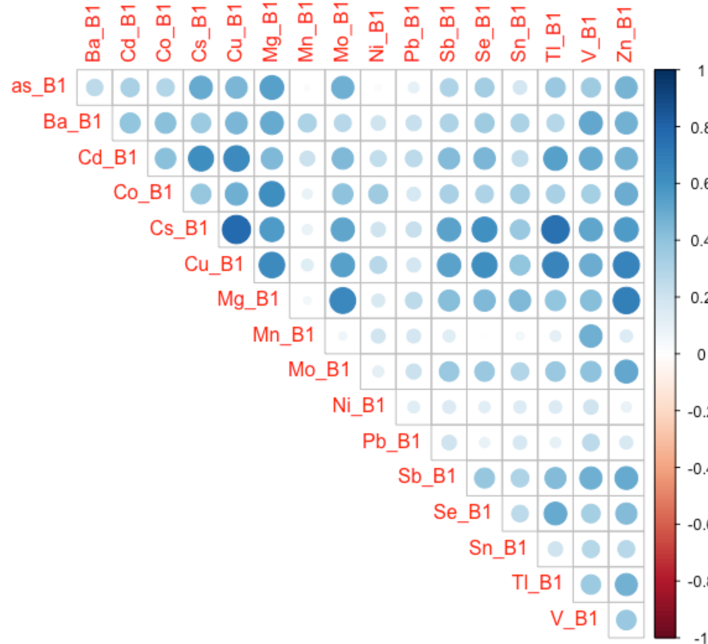


Supplementary Figure 3. 3 Directed acyclic graph of the study showing the hypothetical interventions of the joint total effect of trace metals at B1 and B4 and breast density

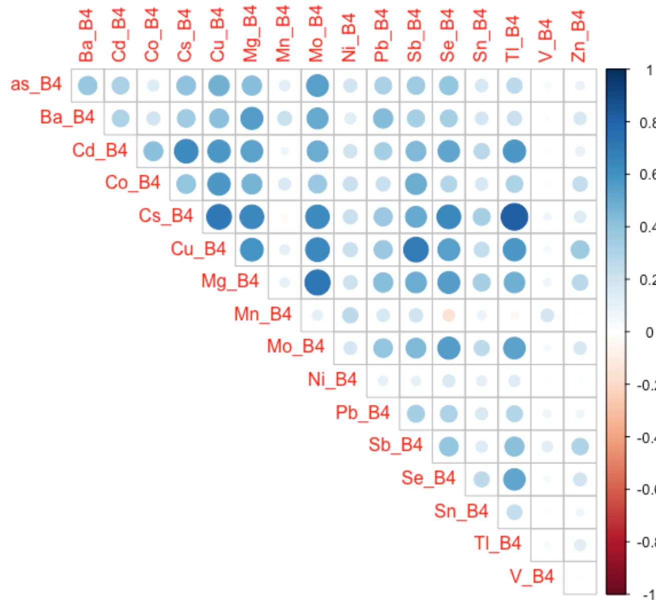


Supplementary Figure 3. 4 Spearman correlation coefficients for trace metals between timepoints B1 and B4

a) Trace metal correlations at B1 timepoint



b) Trace metal correlations at B4 timepoint



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