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The Associations of Endocrine-Disrupting Chemicals, the Gut Microbiome, and Breast Density in a Cohort of Adolescent Girls in Santiago, Chile

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The Associations of Endocrine-Disrupting Chemicals, the Gut Microbiome, and Breast Density in a Cohort of Adolescent Girls in Santiago, Chile

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

by

Lara Sumi Yoon

2021

ABSTRACT OF THE DISSERTATION

The Associations of Endocrine-Disrupting Chemicals, the Gut Microbiome, and Breast Density in a Cohort of Adolescent Girls in Santiago, Chile

by

Lara Sumi Yoon

Doctor of Philosophy in Epidemiology

University of California, Los Angeles, 2021

Professor Karin B. Michels, Chair

Introduction: Breast cancer is the most common invasive cancer among women. Percent breast density is a strong breast cancer risk factor in adult women. Few studies have assessed breast composition in adolescents. Yet, breast development begins during puberty, and adolescent breast composition may influence later-life breast cancer risk. Breast tissue density during puberty, a critical period of growth, may be particularly susceptible to the influence of endocrine-disrupting chemicals (EDCs), which interfere with hormonal pathways in the body. However, the mechanisms through which EDCs may influence breast density are not well understood. The microbiome has emerged as a potential regulator underlying the associations between environmental exposures and human health, including growth and development, metabolic disease, and cancer. The influence of EDCs on composition of the gut microbiome and the relation of the gut microbiome to breast composition remain unclear. The objective of this dissertation was to evaluate the associations of EDCs, the gut microbiome, and breast density in a cohort of adolescent Chilean girls. We hypothesize that: 1) EDCs are associated

with adolescent breast density; 2) EDCs influence the composition of the gut microbiome in adolescence; and 3) the gut microbiome composition and function is associated with breast composition during adolescence.

Methods: This dissertation includes biomarker, anthropometric, demographic, dietary, and breast composition data from 530 girls participating in the longitudinal Growth and Obesity Cohort Study in Santiago, Chile. EDC biomarker concentrations of 16 phenols, phthalates, and parabens were assessed by liquid chromatography mass spectrometry in urine samples. Microbiome composition was assessed by targeted sequencing of the V3-V4 hypervariable region of the 16S rRNA gene in self-collected stool samples. Breast composition was measured using dual-energy x-ray absorptiometry and evaluated as percent fibroglandular volume (%FGV), absolute fibroglandular volume (aFGV), and total breast volume (tBV). In Chapter 2, we evaluated the relation between urinary concentrations of suspected EDC biomarkers across three peripubertal time points (Tanner breast stage 1 [B1], Tanner breast stage 4 [B4], and 1-year post menarche [1YPM]) and breast composition (%FGV, aFGV, and tBV) measured at 2-years post-menarche in a longitudinal study design using generalized estimating equations. In Chapter 3, we assessed whether EDC biomarker concentrations were associated with composition of the gut microbiome in both single-chemical and chemical mixture analyses. In Chapter 4, we examined the relation between composition and predicted function of the gut microbiome and breast density measured at 2-years post-menarche using a cross-sectional study design. All three chapters identified potential confounding factors a priori using directed acyclic graphs and controlled for confounding in statistical analyses. Where appropriate, the Benjamini-Hochberg method was used to control for multiple hypothesis testing.

Results: There was high variability in EDC concentration across peripubertal time points. Select EDCs were associated with %FGV and aFGV; the association was modified by time point at which the urine sample was measured. When evaluated as single chemicals, select phenols

and phthalates were marginally associated with differences in gut microbial alpha diversity and beta diversity. Only a minor association was found for the relation of an EDC mixture to gut microbial alpha diversity. There were no differences in gut microbial composition nor were there differentially abundant microbial genera across categories of breast density (%FGV, aFGV).

Conclusions: Understanding the influence of environmental exposures during puberty, a critical period of growth and development, on adolescent breast composition is important for developing opportunities to reduce risks for breast cancer. Overall, these results suggest that there are pubertal windows of susceptibility to select EDCs for an association with gut microbial composition and breast density, though we cannot rule out significant findings by chance. We did not find evidence to suggest that breast density associated with composition and predicted function of the gut microbiome. Future research might consider the role of the gut microbiome in understanding how environmental chemicals may modify pubertal growth and development.

The dissertation of Lara Sumi Yoon is approved.

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2021

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"Variability in urinary phthalates, phenols, and parabens across childhood and relation to adolescent breast composition in Chilean girls" (Chapter 2) was submitted for publication. Co-authors on this manuscript include Alexandra M. Binder, Ana Pereira, Antonia M. Calafat, John Shepherd, Camila Corvalán, and Karin B. Michels. LSY, AMB, and KBM conceptualized and designed the study. AP, AMC, JS, CC, and KBM collected and analyzed data for the study. Statistical analyses were completed by LSY and supervised by AMB and KBM. LSY drafted the

manuscript, and all other authors were involved in critical interpretation and revision of the manuscript.

“Childhood concentrations of endocrine-disrupting chemicals in relation to adolescent gut microbial composition in the Chilean Growth and Obesity Cohort Study” (Chapter 3) is in preparation for journal submission. Co-authors on the study include Moira Bixby, Shoshannah Eggers, Shelley Liu, Chris Gennings, Alexandra M. Binder, Camila Corvalán, Antonia M. Calafat, Juan Cristóbal Gana, Jonathan P. Jacobs, and Karin B. Michels. LSY, AMB, and KBM conceptualized and designed the study. CC, AMC, JCG, and KBM collected and analyzed data for the study. Statistical analyses were completed by LSY, MB, SE, and SL with supervision by CG, AMB, JPJ, and KBM. LSY drafted the manuscript, and all other authors were involved in interpretation of results and critical revision of the manuscript.

“The association between breast density and gut microbiota composition at 2 years post menarche: A cross-sectional study of adolescents in Santiago, Chile” (Chapter 4) was submitted for publication. Co-authors on the manuscript include Jonathan P. Jacobs, Jessica Hoehner, Ana Pereira, Juan Cristóbal Gana, Camila Corvalán, and Karin B. Michels. LSY, JPJ, and KMB conceptualized and designed the study. AP, JH, JCG, CC, and KBM collected and analyzed data for the study. Statistical analyses were carried out by LSY and JH, with supervision by JPJ and KBM. LSY drafted the manuscript, and all other authors were involved in interpretation of results and critical revision of the manuscript.

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Yoon LS, Corvalán C, Pereira A, Shepherd J, Michels KB. Effect of sugar-sweetened beverage intake on adolescent breast density. Poster; Society for Epidemiologic Research Annual Meeting; 16-18 De 2020; Virtual.

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Chapter 1. Introduction

Epidemiology of breast cancer

Breast cancer is the most commonly diagnosed cancer and the leading cause of cancer mortality among women worldwide, accounting for an estimated expected 2,261,419 new cases and 684,996 deaths in 2020.[1] In Chile, the country in which the data for this dissertation was collected, the estimated number of new breast cancer cases in 2020 was 5,331, representing 21% of all new cancer cases among women and corresponding to an age-standardized incidence rate of 37.4 cases per 100,000.[1] Established risk factors for breast cancer account for an estimated 40% of the variability in breast cancer incidence. Of these known risk factors, only 5-10% of breast cancer cases is attributable to heritable or genetic risk factors such as the inherited *BRCA* mutations.[2] Biological risk factors for breast cancer include older age, family history of breast or ovarian cancer, endogenous hormone levels, early menarche, late menopause, and breast density. Behavioral risk factors for breast cancer include recent use of oral contraceptives, parity, age at first birth, breastfeeding, use of menopausal hormone therapy, physical inactivity, obesity, diet, and alcohol consumption.[3–5]

A lifecourse approach to breast cancer research

An increasing body of research suggests that early life events may be critical in later-life breast cancer risk.[6] Initiation of breast carcinogenesis typically involves mutations in cellular genes that control key regulator pathways of the cell and can be induced by internal factors, such as errors in the normal DNA replication process, or external factors, like effects from environmental exposures.[7] While most breast cancers are diagnosed among adult women, the breast develops and changes rapidly during several events throughout the lifecourse, including puberty, pregnancy, and menopause.[7] Certain environmental exposures occurring during these windows may have permanent effects on breast development and potential carcinogenic triggers and that

subsequently affect susceptibility to breast cancer later in life. In particular, breast tissue may be particularly sensitive to exogenous perturbation during puberty, a period of rapid growth and cellular differentiation driven by hormones and other growth factors.[8] Epidemiologic evidence indicates that menarche, height and weight gain during adolescence are associated with increased risk of breast cancer, while physical activity during adolescence is inversely associated with breast cancer risk. [6] Evidence for the effect of diet, alcohol use, and smoking during adolescence on breast cancer risk is mixed.[6] Additional research is needed to better understand how the effects of other factors, including environmental exposures, during the early life influence risk of breast cancer.

Breast density: a putative risk factor for breast cancer

Breast density is recognized as one of the strongest and most consistent predictors of breast cancer.[9] Breast density, thought to peak in young women following menarche, represents one of the few early life predictors of breast cancer risk that may be modified by exposure profile.[10] However, the majority of studies assessing breast density throughout the life course focus on adult women. Few studies have assessed breast composition in adolescents due to concerns over exposure to radiation during X-ray based mammography. As such, factors that contribute to breast composition during adolescence are not well-studied. The contribution of early life events to breast cancer, detailed previously, suggests an important role in for the assessment of adolescent breast density in contributing to an understanding of breast cancer. The prospective Growth and Obesity Cohort Study (GOCS) began in 2006 in Santiago, Chile and initially enrolled 515 girls.[11] Breast density assessments were completed at Tanner stage 4 (B4) using dual energy X-ray absorptiometry (DXA), a low-dose method.[12] Findings from this cohort indicate a positive association of adolescent breast density with select endocrine disrupting chemicals (EDCs) and certain dietary components (e.g., sugar), and an inverse association with other components of the diet including yogurt.[13,14] Other studies focused on breast density in

young women have emerged from the Dietary Intervention Study in Children (DISC), a randomized dietary intervention trial of 663 pre-pubertal children that lasted for 7 years.[15] Breast density assessments were done at age 25-29 during a follow-up study using non-contrast MRI.[16] Findings from the DISC suggest an inverse association of young adult breast density with adolescent body fatness, monounsaturated fat intake, and age at hormonal contraceptive use, and a positive association with saturated fat intake, dietary energy density, duration of hormone use, and select endogenous sex hormones.[17–20] Additional research to identify environmental influencers of breast development in adolescence is necessary.

Impacts of endocrine-disrupting chemicals on breast density

Endocrine disruptors are exogenous agents that interfere with processes in the endocrine system, including "synthesis, secretion, transport, metabolism, binding action, or elimination of natural blood-borne hormones that are present in the body and are responsible for homeostasis, reproduction, and developmental process."[21] The mechanisms through which endocrine-disrupting chemicals (EDCs) act are broad and varied. EDCs are a heterogeneous group in characterization (synthetic or natural chemicals), exposure source (food, water, occupation, or consumer products), and mechanism of action. EDCs may exert actions through nuclear hormone receptors, including estrogen receptors (ERs) and progesterone receptors (PRs), nonsteroidal receptors, or through any number of other metabolic or endocrine pathways.[21] Phthalates, the diesters of 1,2-benzenedicarboxylic acid (phthalic acid), and phenols are synthetic chemicals found in plastics, medical devices, personal care products (e.g., perfume and soap), and pharmaceuticals.[22] Common phthalates include di(2-ethylhexyl) phthalate (DEHP) and diethyl phthalate (DEP). Bisphenol a (BPA) is a widely recognized phenol; others include parabens and triclosan. Given the potential to interfere with endogenous hormones and impact the endocrine system, widespread exposure to EDCs during childhood and adolescence has caused concern over their health effects.

Experimental and epidemiologic evidence suggests an association between EDC exposure and breast health. *In vitro* studies have demonstrated that cells exposed to genistein show increased estrogen-sensitive mammary cancer cell growth and that diethyl phthalate (DEP) causes activation of estrogen-receptor alpha followed by proliferation of breast cancer cells.[23,24] Mice and rats exposed to Bisphenol A (BPA) have altered breast development, an increased number of epithelial cells, and develop preneoplastic lesions.[25–27] In a study of 76 adolescent Puerto Rican girls, higher levels of phthalates and mono-(2-ethylhexyl) phthalate were associated with premature thelarche.[28] Exposure to mono-benzyl phthalate has been associated with older age at breast development in a longitudinal study of US girls.[29] To date, one study has investigated the effect of childhood EDC exposure and adolescent breast density. Binder et al. evaluated the effect of urinary concentration of 26 select phenols and phthalates collected at Tanner stages 1 (B1) and 4 (B4) on breast density at B4. Monocarboxyisooctyl phthalate and monoethyl phthalate were positively associated with breast density.[14] Chapter 2 of this dissertation expands on the Binder et al. study of Chilean girls by 1) increasing the sample size, 2) describing the variability in EDCs across three peripubertal childhood time points, rather than two; and 3) elucidating the association of EDCs to a post-menarche measure of breast density, while taking into account the interaction with pubertal time point at which the EDC was measured.

Associations of EDCs with the gut microbiome

The gut microbiome, the collection of trillions of microorganisms living in in association with the human body in the gastrointestinal tract, has been increasingly linked to human health and disease.[30] Experimental and observational evidence suggests that gut microbiota are important for maintenance of health and development of disease, playing an important role in the immunology and metabolism through nutrient absorption, immune regulation, and metabolization of chemicals.[31–33] Hypotheses suggest a unique interplay between the immune

and metabolic systems in the body and gut microbiota. For instance, the gut microbiome is involved in estrogen metabolism, metabolic syndrome, and inflammation, indicating several pathways for contribution to breast development and breast cancer.[34–39] The role of the gut microbiota in transformation and metabolism of chemicals and compounds also suggests a potential association with EDCs. It is plausible that there is a link between EDC exposure and metabolization, gut microbial composition or function, and human health and disease. To date, the role of EDC exposure in gut composition and metabolism in the context of breast cancer has not been explored.

Growing evidence from animal models suggests that environmental EDCs such as phenols, phthalates, and parabens can influence composition and result in dysbiosis of gut microbiota.[40] Studies of mice, dogs, and rats have demonstrated that bisphenol-A (BPA), diethyl phthalate (DEP), diethylhexyl phthalate (DEHP), methyl paraben (MPB), and triclosan (TCS) exposure may alter gut microbial α-diversity (a measure of within-community microbial diversity).[41–45] Few studies have examined the relation of EDCs—specifically, phenols, phthalates, or parabens— to the gut microbiota among humans. A Taiwanese study of newborns found that medical exposure to DEHP through intravenous infusions was associated with altered microbial composition.[46] However, limitations of this study include a small sample size and limited generalizability. There is thus a significant lack of understanding of how EDCs may affect the gut microbiota in human population-based studies. It is currently unknown how childhood exposure to phenols, parabens, and phthalates may influence the composition of the gut microbiome in adolescence. Chapter 3 of this dissertation presents an exploration of the impact of single-chemical EDCs and an EDC mixture on the composition of the gut microbiome.

Relation of the gut microbiome to breast density

The gut microbiome represents a novel mechanistic pathway that may contribute to breast health. The gut microbiome is involved in estrogen metabolism, metabolic syndrome, and inflammation,

indicating several pathways for contribution to breast development and breast cancer.[34–39] Perturbations in microbiota composition resulting in a microbial imbalance, or dysbiosis, may increase vulnerability to pathogens through greater intestinal permeability and systemic inflammation.[47] Alterations to the abundance of microbes involved in estrogen metabolism may result in heightened circulating estrogen.[48] Estrogen levels are directly associated with breast development; therefore, the gut microbiome may play a mechanistic role in the development of breast density during puberty and adolescence.[49,50] It is unknown whether the gut microbiome is associated with breast density in adolescence. Chapter 4 of this dissertation investigates the relation of adolescent breast density to various measures of gut microbial composition.

Chapter 2. Variability in urinary phthalates, phenols, and parabens across childhood and relation to adolescent breast composition in Chilean girls

2.1. Abstract

Background: Epidemiologic evidence suggests that environmental factors that act as endocrine disrupting chemicals (EDCs) are associated with breast development and the risk of breast cancer. Exposure to EDCs during puberty, a period of rapid breast development, may affect susceptibility to breast carcinogenesis.

Methods: In a cohort of 366 Chilean adolescents participating in the Growth and Obesity Cohort Study, we evaluated the relation between urinary concentrations of 15 suspected EDCs biomarkers across three pubertal time points (Tanner breast stage 1 (B1), 4 (B4), and 1-year post-menarche) and breast fibroglandular volume (FGV; percent FGV [%FGV] and absolute FGV [aFGV]) and total breast volume (tBV) at 2-years post-menarche. We used linear mixed models to test differences in creatinine-corrected EDC biomarker concentrations at B4 and 1-year post-menarche compared to B1 and calculated intraclass correlation coefficients (ICC) of EDC concentrations across study time points to appraise the consistency of measurements over time. We fit multivariable generalized estimating equations (GEEs) to evaluate windows of susceptibility for the association between $\log_{10}$-transformed EDCs and $\log_{10}$-transformed breast outcomes. GEEs were adjusted for age, body fat percentage, total caloric intake, and maternal education.

Results: Urinary EDC biomarker concentrations were highly varied across pubertal time points (ICC range 0.01-0.30). For 12 of the 15 evaluated EDCs, biomarker concentrations decreased over time. Triclosan measured at 1-year post-menarche was inversely associated with %FGV at 2-years post-menarche (β= -0.025, 95% confidence interval (CI) -0.041, -0.008). Monobenzyl phthalate measured at B1 was inversely associated with aFGV. Mono(2-ethyl-5-carboxypentyl) phthalate and the sum of di(2-ethylhexyl) phthalate metabolite concentrations at B4 were

positively associated with aFGV and tBV at 2-years post-menarche. No measured phenols were significantly associated with aFGV and tBV, while no measured parabens were associated with %FGV and aFGV.

Conclusions: Overall, our study suggests high variability in EDC biomarker concentrations across the peripubertal time period. We also found evidence to suggest that there are pubertal windows of susceptibility to select EDCs for the association with adolescent breast density.

2.2. Introduction

Endocrine disrupting chemicals (EDCs) are exogenous agents that interfere with processes in the endocrine system, including synthesis and metabolism of hormones.[21] Recent trends in breast cancer risk suggest that exposure to putative EDCs, including phthalates, parabens, and phenols, may play a role in increasing incidence of breast cancer.[51] The question of how EDCs are associated with breast cancer has been evaluated in a limited number of animal and epidemiologic studies.[52] In animal models, exposure to bisphenol A (BPA) has resulted in increased mammary gland growth, greater cell proliferation, and more tumor multiplicity.[53–55] Prenatal exposure to butyl benzyl phthalate in rats was associated with increased mammary gland susceptibility to carcinogens through modulation of gene expression.[56] Epidemiologic evidence for an association between EDCs and breast cancer risk is inconsistent. Medium-sized (N<500) studies of breast cancer cases and controls in adult populations of Mexican and Native Alaskan women have found positive associations between certain phthalate biomarkers, including monoethyl phthalate (MEP) and mono(2-ethylhexyl) phthalate (MEHP), and breast cancer risk.[57,58] In a larger nested-case control study within the Women's Health Initiative, urinary concentrations of 13 phthalate biomarkers were not associated with breast cancer risk.[59] Recently, a nested case-control study within the Multiethnic Cohort Study reported suggestive associations for increased breast cancer risk among women with a higher ratio of MEHP to oxidative di(2-ethylhexyl) phthalate (DEHP) metabolites compared to those with a lower ratio.[60]

Oxidative DEHP metabolites were presumed to be less toxic than MEHP, the hydrolytic DEHP metabolite, and thus would have lesser physiological effect on breast carcinogenesis.[60] In a US population-based study with 18 years of follow-up, higher urinary concentrations of methylparaben and propylparaben were associated with a 30 – 50% increase in breast cancer risk; the association was stronger among women with body mass index (BMI)<25.0 kg/m^2.[61] Other studies of Polish and American women have not found evidence to link urinary bisphenol-A concentrations to breast cancer risk.[62,63]

Endocrine disruptors, ubiquitous in personal care products, industrial materials, food packaging, and pharmaceuticals,[64–66] have the potential to interfere with breast carcinogenesis through their action on hormone receptors.[52] EDCs have been implicated in modulation of estrogenic and anti-estrogenic activity, modification of development of mammary tissue, and inhibition of sex steroids metabolism.[52] A strong risk factor for breast cancer is high mammographic breast density, a measure of the amount of fibroglandular tissue in the breast.[9] Breast tissue development begins early in the lifecourse and may be particularly sensitive to environmental perturbation during puberty, a period of rapid growth and cellular differentiation driven by hormones and other growth factors.[8] Exposure to EDCs during this window of sensitivity may have outsized impact on breast development and subsequent susceptibility to breast cancer, as pubertal breast development is thought to be an important determinant of adult mammographic density.[67] However, the effect of exposure to EDCs on breast density is understudied.

We have previously found associations between childhood exposure to select phenols and phthalates and breast composition at Tanner breast stage 4 (B4) in a cohort of Chilean adolescents. Specifically, B4 breast density was higher among girls with higher levels of monocarboxy-isooctyl phthalate measured at Tanner breast stage 1 (B1) and B4, and positively associated with MEP concentrations measured at B4.[14] In this study, we expanded on our earlier analysis by increasing the sample from 200 to 366 participants, increasing the number of

time points at which the EDC biomarkers were assessed from two to three, and utilizing a post-menarche measurement of breast density, when the breast has reached maturity. Breast density is thought to peak in young women following menarche; high density at this age may define breast density trajectories throughout the life course.[10] The goals of this study were to assess the variability of EDC biomarker concentrations across three peripubertal time points and to evaluate the relation between EDC biomarker concentrations breast composition at 2-years post-menarche. This study provides additional insight into potential windows of susceptibility to EDC exposures during childhood on adolescent breast density.

2.3. Methods

Study population

Participants in this study were part of the Growth and Obesity Cohort Study (GOCS), an ongoing longitudinal cohort study of children in Santiago, Chile. GOCS began in 2006 and included 1,196 children aged 2.5-4 years enrolled in preschool at the National Board of Preschool Council Program (Junta Nacional de Jardines Infantiles).[11] Participants in the study met the following criteria: (1) singleton birth born at term (37-42 weeks), (2) birthweight of ≥2500 and <4500 grams, and (3) without health conditions that might affect growth (e.g., metabolic or endocrine disorders). Of the children initially enrolled in the cohort, approximately half (n=601) were female. Once enrolled, participants visited the Instituto de Nutrición y Tecnología de los Alimentos (INTA) Health Clinic at the Universidad de Chile in Santiago, Chile at least one per year; in 2011, the visit frequency increased to twice per year to better capture pubertal maturation. At the clinic, trained dietitians evaluated anthropometry, bioimpedance, and pubertal development (thelarche, menarche, Tanner staging) in the children. Biological specimens (e.g., urine) were collected at defined follow-up time points: B1, B4, and 1-year post-menarche. Limited socioeconomic, demographic, and behavioral information was also collected via questionnaires completed by the mothers of the children. Diet was assessed via 24-hour recall

every six months beginning in 2013. Breast density was measured using dual X-ray absorptiometry when the girls reached B4 and again at 2-years post-menarche among 525 girls. The current study includes 366 girls with a breast assessment at 2-years post-menarche and at least one urine sample collected at B1, B4, or 1-year post-menarche. The study protocol was approved by the University of Chile Ethics Committee at INTA and the University of California, Los Angeles Institutional Review Board. Written informed consent was obtained from all parents or guardians of the children at enrollment and again before breast assessment. The analysis of blinded specimens by the CDC laboratory was determined not to constitute engagement in human subjects' research.

EDC biomarker assessment

Fasting spot urine samples were collected during visits to the clinic at INTA corresponding to three study time points: B1, B4, and 1-year post-menarche. During morning visits to the clinic, each participant collected at least 2 mL of urine in polypropylene sterile containers. The urine was temporarily stored at 4°C before processing for homogenization of the sample and measurement of urinary density, followed by aliquoting and storage at -80°C before being shipped to a laboratory for biomarker quantification. This protocol has shown to be effective with regards to temporal stability of metabolites in the urine.[68]

We selected 18 suspected EDC biomarkers a priori for measurement in urine, including 6 phenols (benzophenone-3, BPA, bisphenol F [BPF], bisphenol S [BPS], triclosan, triclocarban), 4 parabens (ethylparaben, butylparaben, methylparaben, and propylparaben), and 8 phthalate metabolites (mono(2-ethyl-5-carboxypentyl) phthalate [MECPP], mono(2-ethyl-5-hydroxyhexyl) phthalate [MEHHP], MEHP, mono(2-ethyl-5-oxohexyl) phthalate [MEOHP], MEP, mono-isobutyl phthalate [MiBP], mono-n-butyl phthalate [MBP], and the nonspecific metabolite of several phthalates mono-3-carboxypropyl phthalate [MCPP]). Urine samples collected at B1 and B4 from 200 randomly selected girls (400 samples total) were processed at the CDC National

Center for Environmental Health Laboratory using on-line solid phase extraction-high performance liquid chromatography-isotope dilution-tandem mass spectrometry as previously described.[69,70] Quality control pooled human urine materials were analyzed along with standards, blanks, and study samples. The limits of detection (LOD) ranged from 0.1 to 1.7 ng/mL depending on the analyte.[69,70] Additional funding supported the analysis of the remaining urine samples from 166 girls collected at B1 (93 samples), B4 (133 samples), and 1-year post-menarche (232 samples) at the Mount Sinai CHEAR Network Laboratory Hub using a previously described protocol.[71] EDC biomarkers needed to have been measured by both the CDC laboratory and the Mount Sinai laboratory in order to be included in this analysis. One EDC biomarker, mono-benzyl phthalate (MBzP), was excluded from this analysis and will be presented elsewhere. Creatinine quantification for all samples was performed at Mount Sinai. 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.[72,73] Three EDC biomarkers with ICC < 0.75 and with more than 50% replicates below the lab-specific LOD for both samples were excluded from further analysis (BPF, butylparaben, triclocarban). A total of 15 EDC biomarkers were used in the analyses; the mean ICC for biomarker pairs was 0.87. EDC concentrations below the lab-specific LOD were imputed a value of the LOD/sqrt(2).[74]

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. We also calculated the molar summation of several biomarkers: DEHP metabolites (MEHP, MEHHP, MECPP, MEOHP), ∑high- molecular weight phthalates (high-MWP) (MCPP, MECPP, MEHHP, MEHP, MEOHP), ∑low-MWP (MEP, MiBP, MBP), ∑phenols (benzophenone-3, BPA, BPS, triclosan), and ∑parabens (ethylparaben, methylparaben, propylparaben) by dividing each biomarker concentration by its molar mass and then summing the individual concentrations.

Breast composition assessment

Breast assessments were completed when the girls were 2-years post-menarche. Dual energy X-ray absorptiometry (DXA) was used to quantify dense breast tissue volume (fibroglandular volume; FGV) based on a breast scanning protocol developed by Shepherd and colleagues the University of California, San Francisco.[75] In this method, each breast was scanned with the Prodigy DXA system software (version 13.6, series 200674; GE Healthcare). The DXA system was continuously calibrated throughout the study using a quality control breast phantom. Values from the left and right breast were averaged to obtain single measures of absolute FGV (aFGV; cm^3) and total breast volume (tBV; cm^3). We derived percent FGV (%FGV; %) by calculating the proportion of absolute FGV among total breast volume. The DXA protocol has high validity and precision for breast density assessments among adolescent girls and is frequently used to evaluate bone density in children.[76,77] All breast composition assessments were log_{10}-transformed prior to analyses.

Covariates

Demographic, anthropometric, and nutritional information was collected during follow-up visits to the INTA health clinic. Body fat percentage was measured using bioelectrical impedance measurements (Tanita-BC-418 MA, Tanita-Corporation, Tokyo, Japan) and presented as a

continuous measure. Body fat percentage was also categorized (underfat/normal, overfat, obese) based on Tanita age- and sex-specific body fat reference curves.[78,79] Age at menarche was determined via phone interviews completed by study dietitians every 3 months during puberty and confirmed at the subsequent study visit. Information on birth mode (vaginal, caesarean), duration of predominant breast-feeding (<3 months, 3-6 months, >6 months), and maternal education (secondary education or less, more than secondary education) was collected via interviews with the mothers of the participants. Total caloric intake (g/day) was measured using 24-hour dietary recalls at each clinic visit and averaged across the recalls that occurred prior to each study time point (B1, B4, 1-year post-menarche) for each girl to reduce random measurement error.[80] Missing covariate data were imputed using last observation carried forward if available followed by mean or median imputation.

Statistical Analysis

To assess temporal variability in EDC biomarker concentration, we calculated geometric means and 95% confidence intervals at each study time point. The geometric mean is less influenced by extreme outliers than other measures of central tendency.[81] ICCs and corresponding 95% confidence intervals were calculated from a one-way random effects model for consistency to compare variability of each $\log_{10}$-transformed EDC biomarker concentration across the three study time points (B1, B4, 1-year post-menarche).[73] The ICC ranges from 0 to 1; a higher ICC indicates less intra-individual variability.[81] Individual linear mixed models (LMM) with random intercepts were used to evaluate differences in $\log_{10}$-transformed EDC biomarker concentrations at B4 and 1-year post-menarche compared to B1. These LMMs allow for intra-individual correlation across timepoints. Spearman correlation coefficients were calculated between log-transformed EDC biomarkers separately for each time point. EDC concentrations were creatinine-corrected to account for dilution and are presented in units of µg/g creatinine or µmol/g creatinine. For analyses of temporal variability, we corrected for creatinine using the

classical adjustment method by dividing urinary EDC biomarker concentration by creatinine concentration.[82]

To evaluate windows of susceptibility for the association between log_{10}(ng/ml)- EDC biomarker concentrations and breast outcomes, we fit generalized estimating equation (GEE) models with an identity link and independent correlation structure. This GEE approach is based on the multiple informants method and models subject-specific patterns of EDC biomarker concentrations (repeated measures) in relation to the breast composition outcome.[83,84] The multiple informants GEE approach can be used to examine whether the exposure of interest is associated with the outcome in the same manner for each study time point and provides a single coefficient estimate for each time point.[84] We examined the significance of the interaction between study time point and log_{10}-EDC biomarker concentration on breast outcomes using the F test. All models were adjusted for time-varying (age (years; continuous), body fat percent (continuous), total caloric intake (kilocalories (kCal) per day; continuous)) and fixed (maternal education (categorical)) factors selected a priori as potential confounders using directed acyclic graphs.[85] We used non-creatinine-adjusted EDC biomarker concentrations in the multivariable GEE analyses and additionally included creatinine as covariate in the models.[86] Log_{10} transformations of the EDC biomarker concentrations and breast outcomes were used to account for a non-linear relation and highly skewed variables. Therefore, beta coefficients represent the association between log_{10}(ng/ml)- EDC concentrations and log_{10}-transformed breast outcomes and can be interpreted as the percent chance in breast outcome when the EDC biomarker concentration increases by 1%. Exposure to EDC biomarkers often occurs in a cumulative, not isolated, manner; many EDC biomarkers are thus likely to be correlated. We therefore did not include more than one EDC biomarker in a model to avoid inducing large variance. We did not adjust for multiple comparisons for confidence intervals within the GEE models due to high shared variation between EDCs and to avoid reducing power.[87] However,

we did adjust p-values for analyses evaluating the significance of the interaction between study time point and $\log_{10}$-EDC biomarker concentration using the Benjamini-Hochberg procedure; false discovery rate (FDR) <0.25 was considered significant.[88] All analyses were performed in R version 4.0.4.[89]

2.4. Results

Participant characteristics at each study time point are presented in Table 2.1. Among the 366 girls with breast assessments at 2-years post-menarche included in the study, 293 provided a urine sample at B1; 333, B4; and 232, 1-year post-menarche. Girls included in the study did not noticeably differ from the 159 girls excluded from the study for missing breast composition assessments with respect to key study characteristics (Table A1. 1). Of the participants included at each of the study time points, the proportion providing a urine sample on at least one other time point was moderate to high. For instance, among girls who provided a urine sample at B4, 65.8 % also provided one at 1-year post-menarche; among the girls with a urine sample at 1-year post-menarche, 84.9% had provided one at B1. Participants were, on average, 7.9 years old (SD 0.45) at B1, 11.4 years old (SD 0.9) at B4, and 13.4 years old (SD 0.8) at 1-year post-menarche. Overall, participants' mean body fat percentage increased from B1 to 1-year post-menarche (25.6 % to 30.5 %), with the greatest proportion of obese girls at 1-year post-menarche. A majority of mothers of the participants reported secondary education or less, 3-6 months of predominant breast feeding, and vaginal births. Average caloric intake of the girls ranged from 1,745 to 1,873 kCal/day across the study time points.

Temporal variability of EDC biomarkers

Creatinine-adjusted EDC biomarker geometric means and standard deviations by study time point are presented in Table 2. 2. Apart from BPA, the distribution of the $\log_{10}$-transformed and creatinine-adjusted EDC biomarker concentrations at B1, B4, and 1-year post-menarche did not significantly differ between GOCS girls included in the analysis and girls excluded from the

analysis (Figure A1. 1). Overall, results from linear mixed models suggest that EDC concentrations were significantly lower at B4 and 1-year post-menarche compared to B1 for individual EDCs (methylparaben, ethylparaben, propylparaben, and all phthalate metabolites) and for summed EDC groupings (∑Phenols, ∑Parabens, ∑Low-MWP, and ∑High-MWP). However, benzophenone-3 concentrations were higher at B4 and 1-year post-menarche compared to B1. Spearman correlations of individual EDC biomarker concentrations within study time points ranged from low (close to 0) to high (close to 1) depending on the EDC biomarker grouping (Figure A1. 2, Figure A1. 3, Figure A1. 4). In general, EDC biomarker within the same chemical class were more likely to be highly correlated with each other than with biomarkers from other chemical classes. For instance, DEHP metabolites highly correlated with other DEHP metabolites; parabens highly correlated with other parabens. This pattern was consistent within each study time point. We also found evidence to suggest high variability of EDC biomarker concentrations across study time points (Table 2. 2). The ICC for phenols ranged from 0.01 to 0.11; for parabens, 0.07 to 0.15; for phthalates, 0.06 to 0.30.

Association of EDC concentrations with breast composition measures

A $\log_{10}$(ng/ml) increase in benzophenone-3 concentration at B1 was associated with a modest decrease (β:-0.024, 95% CI: 0.05, 0.000) in $\log_{10}$%FGV at 2-years post-menarche after adjusting for creatinine, maternal education, age, body fat percentage, and average daily caloric intake (Table 2. 3). We also found an inverse association between triclosan at 1-year post-menarche and %FGV at 2-years post-menarche (β:-0.025, 95% CI: -0.41, -0.008). The interaction between triclosan biomarker concentration and study time point was significant, suggesting differences in associations between triclosan concentrations and %FGV across B1, B4, and 1-year post-menarche (p=0.007). No other EDC biomarkers measured at B1 or 1-year post-menarche were associated with differences in %FGV at 2-years post-menarche, nor were

any EDC biomarkers measured at B4. We also did not report other significant interactions between EDC biomarker and study time point on %FGV at 2-years post-menarche.

A higher concentration of MBP measured at B1 was associated with lower aFGV at 2-years post-menarche (β: -0.09, 95% CI: -0.15, -0.024) (Table 2. 4). Higher aFGV at 2-years post menarche was also associated with higher concentrations of the single EDC biomarker MECPP (β: 0.055, 95% CI: 0.006, 0.104) and ∑DEHP metabolite biomarkers (β: 0.053, 95% CI: 0.003, 0.104) measured at B4. The association between EDC biomarker concentration and aFGV at 2-years post-menarche was significantly modified by study time point for several phthalates (MBP, MEHHP, and ∑DEHP) in models adjusted for confounding.

Biomarker concentrations of phenols measured at any time point were not associated with tBV (Table 2. 5). Propylparaben measured at 1-year post menarche was associated with higher tBV at 2-years post menarche (β: 0.021, 95% CI: 0.001, 0.041), and the relation of propylparaben to tBV was significantly modified by study time point (p=0.038). Several phthalate biomarkers MBP, MECPP, MEHP) were linked to differences in tBV; study time point significantly modified the associations. However, none of the summed phthalate groupings were significantly associated with tBV.

2.5. Discussion

In this longitudinal study of adolescent Chilean girls, urinary concentrations of select EDC biomarkers measured across different stages of puberty were weakly associated with breast outcomes measured at 2-years post-menarche. These associations were not consistent across time: for EDC biomarkers significantly associated with breast outcomes, those measured at B1 were generally associated with lower breast density and volume, while those measured at B4 and 1-year post-menarche were largely associated with higher breast density and volume. Our results also suggest differences in EDC biomarker concentration across time. For the majority of EDC biomarkers we evaluated, concentrations at B4 and 1-year post-menarche were

significantly lower than concentrations at B1. Taken together, these findings suggest that breast development may have windows of susceptibility to EDC exposure throughout puberty. However, given the number of hypotheses tested in our analyses, we cannot rule out the possibility of significant findings by chance.

Variability in EDC biomarker concentration across puberty

We reported geometric mean concentrations of creatinine-adjusted EDC biomarkers across time points corresponding to age 8 (B1), 11.5 (B4) and 13.5 (1-year post-menarche) years. Overall, urinary concentrations of EDC biomarkers were similar in our study population to those observed in other studies of young children or adolescents in the United States (U.S.), China, Sweden, and Mexico.[90–96] These observed differences in EDC biomarker concentration may reflect true geographic differences in population exposure to certain EDCs. For example, we observed lower concentration of BPA among the girls in our study (1.4 – 1.6 µg/g creatinine) compared to studies of U.S. children of similar age (1.1-4.2 µg/g creatinine), which might suggest decreased exposures through sources such as ultra-processed food (UPF) packaging.[92,96] EDCs such as BPA, DEHP, and other phthalates are ubiquitous in UPF packaging such as plastic containers and food lining.[97] UPFs account for roughly a quarter of total energy intake (kCal) in the general population in Chile.[98] In comparison, data from the U.S.-based National Health and Nutrition Examination Survey suggest that more than 50% of total energy intake among the general population and more than 65% of total energy intake among children and adolescents comes from UPF.[99] We also observed higher concentrations of paraben biomarkers (ethylparaben, methylparaben) in the Chilean girls compared to Swedish and Danish children of similar age, which might relate to differences in public and regulatory focus on parabens in the European Union compared to Chile.[95,100]

In the present study we observed decreasing EDC biomarker concentrations over time. With the exception of benzophenone-3 and BPA, creatinine-corrected concentrations of individual

phenols, parabens, and phthalate biomarkers were lower at B4 and 1-year post-menarche compared to B1. This trend is somewhat unexpected, as we might anticipate higher concentrations of certain parabens and phthalates biomarkers at older ages with increasing use of cosmetics and other personal care products. However, creatinine also increases with age; creatinine-adjusted concentrations of EDC biomarkers may decline with increasing age.[86] In contrast to our analysis, prior studies of Danish children reported higher concentrations of MEP among girls at older ages and more advanced pubertal stages (B4 and B5) compared to younger girls and those at less advanced stages (e.g., B1).[101,102] Notably, creatinine was either not measured or not mentioned in these studies. A U.S.-study observed higher metabolite concentrations of certain high-MWPs (MCOP, MCNP) among children aged 6-11 years compared to adolescents (12-19 years); these concentrations were also corrected for creatinine.[103] Though we did not quantify MCOP and MCNP in this analysis because they were only measured at one lab, we did observe a similar trend of higher concentrations at earlier pubertal stages (i.e., younger ages) among metabolites of other high-MWP (MCPP, MECPP, MEHHP, MEHP, MEOHP). While these studies confirm exposure to EDCs in childhood, it is difficult to disentangle changes in exposure to these chemicals from physiological differences such as changing body size.

Results from our study suggest relatively high variability in biomarker concentrations across the study period for all EDC biomarkers. Our ICCs generally agree with those reported in cohort studies in populations of children, with most studies reporting low ICCs, particularly when the period of time between urine collection is greater than several months. The HOME study of U.S.-based pre-school aged children reported relatively high variability of BPA and phthalate metabolite concentrations (ICC range 0.09 - 0.39) over an 8-year study period, with MEP exhibiting the lowest variability across time.[96,104,105] Overall, studies which have evaluated longitudinal EDC concentration in childhood or adolescence find relatively high variability across

time, particularly when the period between sample collections is greater than several months.[96,106,107] We also observed less temporal variability and lower concentrations of urinary MEHP, the hydrolytic metabolite of DEHP, across time compared to the other three DEHP metabolites (MECPP, MEHHP, MEOHP) measured in the study. Other observational studies have reported similar relative variability (ICC range 0.30-0.50) and lower concentration of MEHP relative to the oxidative DEHP metabolites.[108–113] The reasons for the lower variability of MEHP compared to other DEHP metabolites are unclear as MEHP has a shorter half-life compared to oxidative DEHP metabolites.[114] It is possible that DEHP exposure is relatively stable across childhood and the distribution of oxidative DEHP metabolites concentrations relates to changes in the body's metabolic activity with age and body size.[112,115]

Association of EDC biomarkers with breast outcomes

In our study, select EDC biomarkers measured across puberty demonstrated weak but significant associations with breast outcomes at 2-years post-menarche. Our results for the associations between these EDCs measured at B1, B4, and 1-year post-menarche and breast outcomes at 2-years post-menarche are not consistent with our prior study, which measured breast outcomes at B4. We had previously found an inverse association between triclosan concentration at B1 and B4 and aFGV at B4, a positive association between MEP concentration at B4 and aFGV at B4, and a non-linear association for the relation of BPA to aFGV and MCNP to tBV.[14] There are several major differences in this study and our prior study. In the current study, our sample size increased by more than 150 girls, increasing our study power. Girls included in this study differed from those in our prior study with respect to BMI Z-score: median BMI Z-score at B1 and B4 were 0.85 and 0.88, respectively. In contrast, girls included in the prior study had median BMI Z-score of -0.1 at B1 and 0.1 at B4. It is likely that the higher BMI Z-score in the current study sample better reflects the nutritional intake and obesity status of the cohort and of Chilean girls overall. While both studies adjusted for body size, we were able to

further adjust for total caloric intake to account for any residual confounding in the current study. We also evaluated three time points across the peripubertal window representing different periods of development, rather than two. Our breast composition outcome was assessed at 2-years post-menarche, after the exposure windows. In contrast, our prior study evaluated a simultaneous EDC measure and breast outcome assessment (B4). Looking at prospective exposure to EDCs such as diethyl phthalate, the parent compound of MEP, and later breast outcomes may have allowed us to better characterize the temporal nature of any potential effect of EDCs on breast development. Finally, we were able to capture a measure of breast density composition post-puberty when the breast has reached maturity.[10] Unpublished analyses from our cohort suggest a correlation between breast density measures at B4 and a 2-years post-menarche: girls in the highest category of breast density at B4 are likely to remain in the highest category at 2-years post-menarche. However, aFGV and tBV overall are significantly higher in girls at the 2-year post-menarche timepoint compared to the B4 timepoint.

While to our knowledge no other cohorts have evaluated the EDC-breast composition link in adolescents, several other studies have evaluated the relation between these chemical biomarkers and the timing of breast development. Evidence from longitudinal studies in the USA and UK suggests that timing of breast development is related to breast composition: earlier thelarche (i.e., first breast development) and greater time between thelarche and menarche have been associated with higher adult percent breast density.[116,117] Moreover, an increase in adult breast density and earlier thelarche are both associated with an increase in breast cancer risk.[118] It is unknown whether these risk factors are markers for each other or whether they might act through similar mechanisms to influence breast cancer risk. However, we might expect similar associations between EDCs and timing of breast development, and EDCs and breast composition. Support for this theory is inconsistent across other longitudinal cohorts. Two publications from the Breast Cancer and Environment Research Program (BCERP), a multi-

ethnic longitudinal cohort study of U.S. girls, report earlier breast development (i.e., age at Tanner breast stage 2 [B2]) for higher urinary concentrations of triclosan at age 6-8 years, but later breast development for higher benzophenone-3 and monobenzyl phthalate (MBzP) concentrations at age 6-8 years.[29,119] The BCERP studies and others have also observed null associations between select EDCs, including low-molecular weight phthalate biomarkers and phenols, and the timing of breast development. A study of 725 Danish girls did not find significant relations of age at B2 to concentrations of 12 phthalate biomarkers.[102] In the U.S.-based CHAMACOS longitudinal cohort of Latinos, peripubertal concentrations of benzophenone-3 and triclosan were not associated with age at B2.[120] Evaluating the literature on the association between EDCs and adolescent breast development should consider the difference in choice of breast or pubertal outcomes, study populations, sample sizes, and the timing of EDC biomarker measurements across studies.

It is notable that our current study was able to measure EDC biomarker concentrations at three different time points across puberty, compared to a single peripubertal window in other cohorts, allowing for examination of specific susceptible peripubertal periods. We observed significant interaction by study time point for the association between certain DEHP metabolites and breast outcomes, with significant positive associations for B4 concentrations of MECPP and both aFGV and tBV. It is plausible that the breast is more susceptible to EDC exposure during the B4 stage, in which the breast tissue is continuing to differentiate and proliferate, compared to B1, a pre-pubertal stage in which there is less rapid development, or 1-year post-menarche, when the breast is mature.[121] However, the significant associations observed with aFGV were among the phthalates with higher levels of exposure. A potential explanation for lack of significance at this stage for other phthalates is that we are limited in power to observe associations with lower concentrations. To our knowledge, no other studies have evaluated adolescent breast

composition with multiple pubertal exposure time points. Overall, these findings suggest Tanner breast stage B4 as a potential window of susceptibility to DEHP for aFGV.

Finally, a potential explanation for our findings is that EDCs are not associated with breast density in adolescence. Instead, it is possible that the use of multiple statistical tests related to the number of EDC biomarkers and breast outcomes measured increased the likelihood of falsely positive results by chance.[122] We attempted to control for type 1 errors by adjusting p-values from joint hypothesis testing of interaction using the Benjamini-Hochberg method and a standard false discover rate of 0.25.[88] However, EDC biomarker concentrations vary significantly across pubertal time periods; it is conceivable that our concentrations do not truly represent the average EDC concentrations among the cohort and that the timing between EDC biomarker measurement and breast outcome measurement significantly precludes any permanent effect. As previously discussed, other cohort studies have found null associations between EDC biomarkers and important pubertal breast outcomes.

Strengths and limitations

A limitation of our study is potential exposure misclassification from single spot urine collection at each study time point. While urine is the preferred biospecimen for characterizing concentrations of phthalate metabolites, parabens, and phenols as biomarkers, non-persistent chemicals such as EDCs are metabolized quickly and biomarker concentrations reflect recent exposure.[123,124] A single urine sample may not accurately represent an entire pubertal period. However, studies suggest that metabolite concentrations of certain phthalates and phenols have moderate to good correlation over time frames of weeks or months in children.[104,105] Because EDC exposures are often linked to consistent behavioral and dietary patterns such as use of personal care products and food choices, use of a single urine sample may reasonably reflect an exposure period. Additionally, though we did adjust for multiple comparisons for select joint hypotheses, it is possible that some our reported associations are due to type I error. However,

we may have increased type II error by adjusting for multiple comparisons if we believe that the relation between EDC biomarkers and breast outcomes are truly not null.[87] Urinary metabolite quantification represents a measure of internal dose that accounts for multiple parent compounds and routes of exposure.[107] We are unable to disentangle the specific source of the metabolite or parent compound exposure, particularly for nonspecific biomarkers that have more than one parent compound such as MCPP. However, biomarkers can be used to estimate the totality of exposure for a relevant time window and may provide a more accurate assessment of EDC exposure than assessment via lifestyle or dietary questionnaire.[125]

Our study has several strengths, including the large sample size and ability to prospectively study the relation between EDC exposure and adolescent breast density. We collected repeated samples of urine for EDC biomarkers assessment throughout puberty, which allowed us to examine the associations of interest at more than one time point and identify potential windows of susceptibility. There was moderate loss to follow-up in this study and only marginally varying sample sizes across time points. GOCS has longitudinal covariate information that allowed us to adjust for potential confounders such as maternal education and anthropometry. It is possible that there is residual confounding from diet due to the use of a single measure of total caloric intake. While we were unable to estimate dietary habits that may reflect source exposure to EDCs such as packaged food, we were able to control for factors such as maternal education as a surrogate for socioeconomic status, which may drive dietary choices.

2.6. Conclusions

We found strong temporal variability in urinary concentrations of phthalate metabolites, phenols, and parabens across pubertal time points. Urinary concentrations of a limited number of phenols and phthalates biomarkers across various pubertal stages were associated with differences in adolescent breast outcomes measured at 2-years post-menarche, which suggests

potentially varying windows of susceptibility during puberty. However, we cannot rule out findings of chance.

Table 2.1. Characteristics of 366 girls in the Growth and Obesity Cohort Study assessed for breast density at 2-years post menarche

Characteristic	**Study Time Point**		
	Tanner Stage B1	Tanner Stage B4	1-Year Post-Menarche
	(n=293)	(n=333)	(n=232)
Urine sample provided			
B1	293 (100.0)	261 (78.4)	197 (84.9)
B4	261 (89.1)	333 (100.0)	219 (94.4)
1Y PM	197 (67.2)	219 (65.8)	232 (100.0)
Age, years (mean (SD))	7.87 (0.45)	11.38 (0.88)	13.40 (0.82)
Age at menarche, years (mean (SD))	12.09 (0.91)	12.04 (0.89)	12.42 (0.76)
BMI Z-score (mean (SD))	0.85 (1.10)	0.88 (1.10)	0.86 (1.11)
Body fat percentage (mean (SD))	25.58 (4.41)	26.93 (5.11)	30.62 (5.61)
Body fat percentage category (count (%))			
Underfat/Normal	155 (54.4)	198 (62.3)	55 (40.1)
Overfat	82 (28.8)	68 (21.4)	39 (28.5)
Obese	48 (16.8)	52 (16.4)	43 (31.4)
Maternal education (%)			
Secondary education or less	236 (80.5)	271 (81.4)	192 (82.8)
Greater than secondary education	57 (19.5)	62 (18.6)	40 (17.2)
Duration of predominant breast feeding (count (%))			
< 3 months	90 (30.7)	107 (32.1)	74 (31.9)
3-6 months	171 (58.4)	188 (56.5)	134 (57.8)
> 6 months	32 (10.9)	38 (11.4)	24 (10.3)
Birth mode (%)			
Caesarean	85 (29.0)	91 (27.3)	73 (31.5)
Vaginal	208 (71.0)	242 (72.7)	159 (68.5)
Average caloric intake, kCal (mean (SD))	1878.81 (490.73)	1873.00 (457.64)	1745.07 (446.37)

Table 2. 2. Creatinine-adjusted urinary phenol, paraben, and phthalate biomarkers geometric means (95% confidence interval) by study time point.

	Tanner Stage B1	Tanner Stage B4	1-Year Post-Menarche	ICC (95% CI)
N	**293**	**333**	**232**	
Phenols				
Benzophenone-3	31.3 (27.7, 35.4)	50.5 (43.6, 58.5) [a]	47 (40, 55.4) [a]	0.07 (0, 0.16)
BPA	1.6 (1.4, 1.7)	1.4 (1.3, 1.6)	1.6 (1.4, 1.7)	0.11 (0.02, 0.2)
BPS	0.5 (0.5, 0.6)	0.5 (0.4, 0.6)	0.4 (0.3, 0.4) [a]	0.01 (0, 0.1)
Triclosan	12.3 (10.5, 14.5)	11.2 (9.6, 13.1)	10.1 (8.2, 12.5)	0.1 (0.02, 0.2)
∑Phenols [b]	0.2 (0.2, 0.3)	0.3 (0.3, 0.4) [a]	0.3 (0.3, 0.4) [a]	0.06 (0, 0.16)
Parabens				
Ethylparaben	1.8 (1.5, 2.2)	1.1 (1, 1.4) [a]	1 (0.8, 1.3) [a]	0.07 (0, 0.16)
Methylparaben	46.3 (38.4, 55.8)	31.7 (26.4, 38.1) [a]	20.8 (16.5, 26.1) [a]	0.14 (0.05, 0.24)
Propylparaben	3.9 (3, 5)	2.3 (1.8, 2.8) [a]	1.7 (1.3, 2.3) [a]	0.15 (0.06, 0.25)
∑Parabens [b]	0.4 (0.3, 0.5)	0.3 (0.2, 0.3) [a]	0.2 (0.1, 0.2) [a]	0.14 (0.05, 0.24)
Phthalates				
MBP	42.5 (38.8, 46.5)	29.6 (27, 32.4) [a]	32.6 (29.6, 35.9) [a]	0.10 (0.01, 0.19)
MCPP	3.3 (3, 3.7)	2.8 (2.5, 3.2) [a]	2.7 (2.4, 3.1) [a]	0.14 (0.05, 0.23)
MECPP	86.9 (79, 95.5)	46.3 (42.5, 50.4) [a]	36.8 (33.2, 40.7) [a]	0.06 (0, 0.15)
MEHHP	43.2 (39, 47.7)	22.8 (21, 24.8) [a]	19.2 (17.3, 21.3) [a]	0.07 (0, 0.17)
MEHP	4.4 (4, 4.9)	3.2 (2.9, 3.5) [a]	2.9 (2.6, 3.2) [a]	0.30 (0.21, 0.39)
MEOHP	26.1 (23.7, 28.9)	14.5 (13.3, 15.9) [a]	12.2 (11, 13.5) [a]	0.11 (0.02, 0.2)
MEP	168.3 (147, 192.7)	108.2 (95, 123.1) [a]	95.4 (81.9, 111.1) [a]	0.09 (0, 0.18)
MIBP	39.5 (35.9, 43.3)	34 (31.3, 37) [a]	28 (25.7, 30.5) [a]	0.25 (0.16, 0.35)
∑DEHP [b]	0.5 (0.5, 0.6)	0.3 (0.3, 0.3) [a]	0.2 (0.2, 0.3) [a]	0.07 (0, 0.16)
∑High-MWP [b]	0.6 (0.5, 0.7)	0.3 (0.3, 0.4) [a]	0.3 (0.2, 0.3) [a]	0.11 (0.02, 0.2)
∑Low-MWP [b]	1.5 (1.3, 1.6)	1 (0.9, 1.1) [a]	0.9 (0.8, 1) [a]	0.09 (0, 0.18)

a Significant difference ($p < 0.05$) in urinary EDC biomarker concentration compared to Tanner Stage B1 (reference) based on linear mixed models

b Concentrations are presented in µg/g creatinine except for ∑Parabens, ∑Phenols, ∑DEHP, ∑High-MWP, ∑Low-MWP (µmol/g creatinine).

Table 2. 3. Relative change in log10 percent FGV (95% CI) associated with log10 (ng/ml) increase in urinary phenol, paraben, and phthalate biomarkers[a]

	Tanner Stage B1	Tanner Stage B4	1-Year Post-Menarche	Interaction (p-value)[b]
Phenols				
Benzophenone-3	-0.024 (-0.048, 0.00)	0.004 (-0.012, 0.021)	0.006 (-0.015, 0.026)	0.15
BPA	0.005 (-0.029, 0.039)	0.001 (-0.026, 0.028)	-0.02 (-0.063, 0.023)	0.784
BPS	-0.016 (-0.055, 0.024)	0.019 (-0.007, 0.045)	0.011 (-0.019, 0.04)	0.395
Triclosan	0.009 (-0.009, 0.026)	0.009 (-0.007, 0.025)	**-0.025 (-0.041, -0.008)**	**0.007** [c]
∑Phenols	-0.016 (-0.031, 0.00)	-0.001 (-0.014, 0.013)	0.006 (-0.01, 0.022)	0.17
Parabens				
MEPB	-0.017 (-0.033, -0.002)	0 (-0.014, 0.014)	0.006 (-0.01, 0.022)	0.116
ETPB	0.002 (-0.012, 0.017)	-0.006 (-0.021, 0.01)	0.007 (-0.011, 0.024)	0.432
PRPB	-0.009 (-0.021, 0.003)	-0.001 (-0.012, 0.01)	-0.002 (-0.016, 0.011)	0.677
∑Parabens	-0.005 (-0.028, 0.017)	0.007 (-0.012, 0.025)	-0.008 (-0.029, 0.014)	0.76
Phthalates				
MBP	-0.029 (-0.061, 0.003)	0.017 (-0.01, 0.044)	-0.022 (-0.067, 0.024)	0.419
MCPP	-0.004 (-0.034, 0.027)	0.006 (-0.015, 0.027)	0.004 (-0.023, 0.03)	0.978
MECPP	0.016 (-0.015, 0.046)	0.013 (-0.016, 0.042)	0.007 (-0.032, 0.047)	0.910
MEHHP	0.01 (-0.018, 0.037)	0.017 (-0.013, 0.046)	0.006 (-0.033, 0.045)	0.996
MEHP	0.009 (-0.018, 0.036)	0.013 (-0.012, 0.039)	-0.01 (-0.045, 0.024)	0.705
MEOHP	0.01 (-0.017, 0.038)	0.016 (-0.013, 0.045)	0.01 (-0.029, 0.049)	0.985
MEP	0.013 (-0.007, 0.033)	-0.002 (-0.021, 0.017)	-0.001 (-0.024, 0.023)	0.422
MiBP	-0.02 (-0.052, 0.012)	0.01 (-0.022, 0.042)	0.002 (-0.045, 0.048)	0.742
∑DEHP	0.013 (-0.016, 0.043)	0.015 (-0.015, 0.044)	0.007 (-0.033, 0.047)	0.959
∑High-MWP	0.011 (-0.019, 0.04)	0.01 (-0.02, 0.041)	0.007 (-0.032, 0.045)	0.941
∑Low-MWP	0.014 (-0.012, 0.04)	0.001 (-0.023, 0.024)	-0.002 (-0.033, 0.029)	0.599

a Estimating average change (β) in %FGV associated with EDC biomarkers across study time points using a multivariable GEE model. Adjusted models include creatinine, maternal education, age at study time point, body fat percentage at study time point, and average daily caloric intake at study time point. Significant associations (95%CI does not include 0) are in bold.

b P-value for interaction between EDC biomarker concentration and study time point on %FGV (F test); $p<0.05$ are in bold.

c False Discovery Rate (FDR) <0.25

Table 2. 4. Relative change in log10 absolute FGV (95% CI) associated with log10 (ng/ml) increase in urinary phenol, paraben, and phthalate biomarkers[a]

	Tanner Stage B1	Tanner Stage B4	1-Year Post-Menarche	Interaction (p-value)[b]
Phenols				
Benzophenone-3	-0.024 (-0.074, 0.025)	-0.009 (-0.037, 0.019)	0.004 (-0.036, 0.043)	0.311
BPA	-0.018 (-0.078, 0.042)	0.006 (-0.04, 0.053)	-0.004 (-0.083, 0.076)	0.194
BPS	-0.007 (-0.073, 0.058)	0.004 (-0.04, 0.047)	0.025 (-0.035, 0.086)	0.654
Triclosan	0.001 (-0.032, 0.034)	-0.013 (-0.043, 0.016)	-0.02 (-0.052, 0.011)	0.969
∑Phenols	-0.013 (-0.041, 0.014)	-0.013 (-0.035, 0.009)	0.018 (-0.012, 0.048)	0.141
Parabens				
MEPB	-0.015 (-0.044, 0.013)	-0.012 (-0.034, 0.009)	0.016 (-0.014, 0.046)	0.167
ETPB	0.008 (-0.021, 0.036)	-0.012 (-0.037, 0.013)	0.021 (-0.01, 0.052)	0.231
PRPB	-0.013 (-0.033, 0.008)	-0.012 (-0.03, 0.006)	0.02 (-0.005, 0.046)	0.068 [c]
∑Parabens	-0.011 (-0.057, 0.035)	-0.016 (-0.046, 0.015)	-0.002 (-0.041, 0.037)	0.517
Phthalates				
MBP	**-0.087 (-0.15, -0.024)**	0.028 (-0.016, 0.071)	0.052 (-0.028, 0.133)	**0.0005**
MCPP	0.00 (-0.055, 0.056)	0.024 (-0.018, 0.067)	0.02 (-0.028, 0.068)	0.249
MECPP	0.014 (-0.043, 0.071)	**0.055 (0.006, 0.104)**	0.052 (-0.022, 0.126)	0.104
MEHHP	0.001 (-0.05, 0.051)	0.05 (-0.001, 0.1)	0.053 (-0.021, 0.126)	**0.041 [c]**
MEHP	0.007 (-0.043, 0.058)	0.039 (-0.005, 0.082)	0.04 (-0.023, 0.102)	0.056 [c]
MEOHP	0.004 (-0.047, 0.055)	0.047 (-0.003, 0.097)	0.053 (-0.019, 0.125)	0.051 [c]
MEP	0.014 (-0.022, 0.051)	0.012 (-0.02, 0.045)	0.011 (-0.031, 0.054)	0.701
MiBP	-0.054 (-0.113, 0.006)	0.001 (-0.049, 0.052)	0.04 (-0.045, 0.124)	**0.024 [c]**
∑DEHP	0.009 (-0.046, 0.064)	**0.053 (0.003, 0.104)**	0.054 (-0.021, 0.129)	0.068 [c]
∑High-MWP	0.006 (-0.05, 0.062)	0.042 (-0.014, 0.099)	0.05 (-0.022, 0.123)	0.094
∑Low-MWP	-0.006 (-0.052, 0.041)	0.019 (-0.021, 0.058)	0.029 (-0.026, 0.084)	0.136

a Estimating average change (β) in aFGV associated with EDC biomarkers across study time points using a multivariable GEE model. Adjusted models include creatinine, maternal education, age at study time point, body fat percentage at study time point, and average daily caloric intake at study time point. Significant associations (95%CI does not include 0) are in bold.

b P-value for interaction between EDC biomarker concentration and study time point on %FGV (F test); p<0.05 are in bold.

c False Discovery Rate (FDR) <0.25

Table 2. 5. Relative change in log10 total breast volume (95% CI) associated with log10 (ng/ml) increase in urinary phenol, paraben, and phthalate biomarkers[a]

	Tanner Stage B1	Tanner Stage B4	1-Year Post-Menarche	Interaction (p-value)[b]
Phenols				
Benzophenone-3	0 (-0.039, 0.04)	-0.013 (-0.04, 0.013)	-0.002 (-0.036, 0.031)	0.725
BPA	-0.029 (-0.082, 0.023)	0.005 (-0.037, 0.046)	0.014 (-0.036, 0.065)	**0.019** [c]
BPS	0.005 (-0.045, 0.055)	-0.014 (-0.06, 0.033)	0.015 (-0.036, 0.066)	0.724
Triclosan	-0.008 (-0.036, 0.02)	-0.022 (-0.046, 0.002)	0.004 (-0.021, 0.028)	0.27
∑Phenols	0.003 (-0.022, 0.028)	-0.012 (-0.03, 0.006)	0.011 (-0.015, 0.036)	0.362
Parabens				
MEPB	0.003 (-0.023, 0.028)	-0.012 (-0.03, 0.006)	0.008 (-0.017, 0.034)	0.444
ETPB	0.007 (-0.017, 0.031)	-0.007 (-0.029, 0.015)	0.014 (-0.012, 0.04)	0.45
PRPB	-0.003 (-0.022, 0.015)	-0.011 (-0.026, 0.004)	**0.021 (0.001, 0.041)**	**0.038** [c]
∑Parabens	-0.006 (-0.042, 0.03)	-0.022 (-0.049, 0.005)	0.005 (-0.026, 0.037)	0.255
Phthalates				
MBP	**-0.058 (-0.108, -0.007)**	0.01 (-0.03, 0.05)	0.065 (-0.017, 0.147)	**0.003** [c]
MCPP	0.007 (-0.043, 0.056)	0.02 (-0.012, 0.052)	0.014 (-0.027, 0.054)	0.207
MECPP	0.001 (-0.048, 0.051)	**0.045 (0.001, 0.089)**	0.043 (-0.009, 0.095)	**0.03** [c]
MEHHP	-0.007 (-0.048, 0.035)	0.036 (-0.009, 0.082)	0.045 (-0.005, 0.096)	**0.012** [c]
MEHP	-0.002 (-0.043, 0.039)	0.022 (-0.015, 0.059)	**0.049 (0.001, 0.098)**	**0.017** [c]
MEOHP	-0.004 (-0.046, 0.038)	0.034 (-0.01, 0.079)	0.042 (-0.009, 0.093)	**0.017** [c]
MEP	0.00 (-0.032, 0.033)	0.014 (-0.012, 0.041)	0.012 (-0.02, 0.045)	0.244
MiBP	-0.034 (-0.079, 0.011)	-0.008 (-0.05, 0.033)	0.025 (-0.041, 0.092)	0.055 [c]
∑DEHP	-0.002 (-0.049, 0.045)	0.042 (-0.004, 0.087)	0.046 (-0.007, 0.098)	**0.019** [c]
∑High-MWP	-0.002 (-0.05, 0.045)	0.035 (-0.011, 0.081)	0.042 (-0.009, 0.093)	**0.032** [c]
∑Low-MWP	-0.021 (-0.062, 0.02)	0.018 (-0.013, 0.05)	0.028 (-0.014, 0.07)	**0.021** [c]

a Estimating average change (β) in tBV associated with EDC biomarkers across study time points using a multivariable GEE model. Adjusted models include creatinine, maternal education, age at study time point, body fat percentage at study time point, and average daily caloric intake at study time point. Significant associations (95%CI does not include 0) are in bold.

b P-value for interaction between EDC biomarker concentration and study time point on %FGV (F test); p<0.05 are in bold.

c False Discovery Rate (FDR) <0.25 from Benjamini-Hochberg adjusted p-values

Chapter 3. Childhood concentrations of endocrine-disrupting chemicals in relation to adolescent gut microbial composition in the Chilean Growth and Obesity Cohort Study

3.1. Abstract

Background: Endocrine-disrupting chemicals (EDCs) have the potential to impact the composition of the gut microbiome. The objective of our study was to examine whether concentration of EDC biomarkers across three pubertal stages was associated with composition of the gut microbiome in adolescence in a longitudinal cohort of Chilean girls.

Methods: We quantified concentrations of 16 EDCs in urine collected at Tanner breast stage 1 (B1), Tanner breast stage 4 (B4), and 1-year post-menarche in the Growth and Obesity Cohort Study. Gut microbial composition of stool samples collected 3-years post-menarche was analyzed using 16s rRNA sequencing of the V3-V4 hypervariable region. Alpha diversity was quantified using the Shannon index. Beta diversity was quantified using Bray-Curtis dissimilarity. We assessed associations of single-chemical EDCs with the Shannon index using generalized linear regression. Permutational Analysis of Variance (PERMANOVA) was used to determine whether microbial communities differed by concentration of EDC using a Bray-Curtis based dissimilarity matrix. Multivariable generalized linear models (MaAsLin2) were used to identify differentially abundant amplicon sequence variants (ASVs) associated with concentrations of single-chemical EDCs. The relation of EDC mixtures to the Shannon index was conducted with weighted quantile sum (WQS) regression. All models were evaluated separately for each study time point and adjusted for potential confounders, including age, creatinine, body fat percentage, maternal education, average caloric intake, birth mode, breast feeding, and antibiotic use. A total of 257 girls were included in the analytic cohort.

Results: Bisphenol A measured at Tanner stage 1 was positively associated with Shannon diversity adjusted for covariates (β=0.17, 95% confidence interval [CI]:0.02, 0.32), while mono-

butyl phthalate measured at 1-year post-menarche was inversely associated with Shannon index adjusted for covariates (β=-0.19, 95% CI:-0.34, -0.03). No differences in beta diversity were observed for any single EDC biomarker. Presence of three genera from the *Firmicutes* phylum were associated with concentrations of triclosan measured at B4 and MNBP measured at 1-year post-menarche. In WQS regression analyses with positive constraints, EDC mixtures measured at B1, B4, and 1-year post-menarche were not associated with the Shannon index

Conclusions: Our results suggest marginal associations for select individual EDCs with gut microbial composition. However, no relation was noted for an EDC biomarker mixture. The association of EDCs to gut microbial composition may be modified by the timing of the urine sample during puberty. Overall, our results provide support for biomarker-specific windows of susceptibility for gut microbial composition.

3.2. Introduction

The gut microbiota, the trillions of microbial organisms living in the human intestinal tract, has been increasingly linked to human health and disease.[30] A collection of experimental and observational evidence has demonstrated that the gut microbiota plays an essential role in nutrient absorption, immune regulation, and metabolization of chemicals.[31–33] An important aspect of the gut microbiota is the overall composition and function of the bacterial community.[30] Two metrics commonly used to quantify composition of the microbiota are alpha diversity, a measure of community variation within a sample that evaluates the number and evenness of species, and beta diversity, a measure of community dissimilarity between pairs of samples.[126] Perturbations to the gut microbiota and the resulting imbalance between microbes and host, or dysbiosis, have been linked to obesity, gastrointestinal disease, neurological disorders, and some estrogen-modulated diseases through observed differences in alpha diversity and beta diversity.[48,49,127–130] Host genetics, age, sex, and lifestyle factors like diet and antibiotic use have been associated with dysbiosis and gut microbial diversity.[131–134] Studies have also suggested

that the gut microbiota may be influenced by environmental chemicals and may modulate an association between environmental chemical exposure and health outcomes.[40,135]

Endocrine disrupting chemicals (EDCs), exogenous agents which interfere with the endocrine system, are ubiquitous in food packaging, consumer products, industrial processes, and medical devices around the world.[22,136,65] Increased exposure to EDCs has been linked to various human disorders, including male and female reproductive abnormalities, obesity, and cancer.[137–139] Given the overlap in human health outcomes associated with EDCs and the gut microbiota, and the oral route of exposure to EDCs and subsequent metabolization in the gut, it is plausible that the gut microbiota may mediate the association between exposure to EDCs and human diseases. A possible link between EDCs and the gut microbiota is estrogen synthesis. EDCs can interact with estrogen receptors to influence transcriptional activity and modulation of enzymes that are involved in estrogen metabolism.[140] The gut microbiota is influenced by circulating estrogen, while also impacting estrogen levels through β-glucuronidase secretion.[49] Despite this potential mechanistic link, few studies have evaluated the extent to which EDCs are associated with the gut microbiota. Evidence from animal models suggests that environmental EDCs such as phenols, phthalates, and parabens can influence composition and result in dysbiosis of gut microbiota.[40] In studies of mice and dogs, exposure to bisphenol A (BPA), a widespread EDC, has resulted in changes to alpha diversity and increased abundance of *Bacteroides* species and *Bifidobacterium* species (spp).[41–43] Other mouse and rat models have demonstrated that exposure to phthalates, including diethyl phthalate (DEP) and diethylhexyl phthalate (DEHP), methyl paraben (MPB), and triclosan (TCS) alters gut microbial community composition through changes in abundance of organisms like *Prevotella* spp., *Bacilli* spp., and *Lachnoclostridium* spp.[44,45] Evidence for the relation of EDCs to the gut microbiota among humans is lacking. A few studies have examined the relation of heavy metals or environmental pollutants to gut microbial outcomes [141,142]; however, only one study to date has examined gut

microbiota composition in relation to phthalate exposure. In a population of Taiwanese newborns, medical exposure to DEHP through intravenous infusions was associated with altered microbial composition, including decreased abundance of *Rothia* spp. and *Bifidobacterium longum.*[46] Limitations of this study include a small sample size (N=25) and limited generalizability to other age groups across the life course. There is thus a substantial gap in our understanding of how environmental EDCs may influence the gut microbiota in human population-based studies.

Given their potential to interfere with endogenous hormones and impact the endocrine system, exposure to EDCs are of particular importance during childhood and puberty– periods of rapid growth and development.[143] Research suggests that the gut microbiota continues to develop throughout childhood and adolescence before stabilizing during adulthood.[144–146] Therefore, the childhood and adolescent periods of development may represent opportunities to promote health through modulation of the gut microbiota. It is currently unknown how childhood exposure to phenols, parabens, and phthalates may influence the composition of the gut microbiota in adolescence. In this study, we examined the relation between 16 EDCs on adolescent gut microbial composition in a longitudinal study of Chilean girls. We first aimed to investigate the effects of individual phenols, parabens, and phthalates measured at three pubertal time points (Tanner breast stage 1 (B1), Tanner breast stage 4 (B4), and 1-year post-menarche) on the composition of the gut microbiota measured at adolescence (3-years post-menarche). Given the potential for multicollinearity, common exposure routes, and similar biologic mechanisms among the EDCs, we then aimed to investigate the combined effect of a phenol, paraben, and phthalate mixture on gut microbial composition.

3.3. Methods

Study population

The Growth and Obesity Cohort Study (GOCS) is an ongoing prospective cohort of 1,196 children in Santiago, Chile. The study design and enrollment have been described elsewhere.[11] Briefly, GOCS recruited children aged 3-4 years from low- and middle-income families enrolled in a public preschool program in the southern region of Santiago in 2006. Information was collected on demographic, anthropometric, and lifestyle factors through bi-annual clinic visits to the Instituto de Nutrición y Tecnología de los Alimentos (INTA) at the University of Chile. The current study was limited to female participants, comprising approximately 50% of the cohort. In 2009, trained dietitians began assessing pubertal development every 6 months using the Tanner rating scale.[147] Onset of menarche was evaluated at the clinic visit or by telephone. Urine biospecimens were collected three times throughout the study when the participant reached Tanner breast stage 1 (B1), Tanner breast stage 4 (B4), and 1-year post-menarche (1YPM). Stool specimens were collected once the participant was 13-15 years of age, corresponding to approximately 3 years post-menarche. Of the 601 girls enrolled at baseline, a total of 261 girls with at least one urine collection and a stool sample were eligible for inclusion in this analysis.

The study protocol was approved by the University of Chile INTA Ethics Committee and the Institutional Review Board at the University of California, Los Angeles. Parents or legal guardians of the participants provided written informed consent. The analysis of blinded specimens by the CDC laboratory was determined not to constitute engagement in human subjects' research.

Urinary EDC biomarker assessment

Study staff at INTA collected morning fasting spot urine samples from participants at B1, B4, and 1-year post-menarche. Urine samples were immediately processed, aliquoted, and stored at -80°C for later analysis. Urine samples were analyzed at either the National Center for Environmental Health Laboratory at the Centers for Disease Control and Prevention (CDC) in

Atlanta, GA, or the Children's Health Exposure Analysis Resource (CHEAR) Laboratory at the Icahn School of Medicine at Mount Sinai in New York, NY. A total of 19 EDC biomarkers (phthalates, parabens, phenols) selected a priori were quantified at each lab (Table A2. 1). From the 261 girls included in the analytic cohort, 198 samples from randomly selected girls collected at B1 (99 samples) and B4 (99 samples) were analyzed at CDC using previously described methods [69,14]; additional funding permitted the analysis of 415 urine samples from the remaining girls taken at B1 (106 samples), B4 (144 samples), and 1YPM (165 samples) at Mount Sinai using previously described methods.[71] A total of 40 samples analyzed at the CDC were also analyzed at Mount Sinai as duplicates to inform quality control and between-lab standardization. The intra-class correlation coefficient (ICC) for each EDC biomarker duplicate pair was estimated from a one-way random effects model measuring absolute agreement with multiple raters/measurements.[72,73] EDC biomarkers with ICC >0.75 and those with >50% of samples above the limit of detection (LOD) in both the CDC and Mount Sinai analyses were maintained in the analysis. Therefore, two EDC biomarkers (bisphenol F, triclocarban) were excluded from all analyses; butylparaben was excluded from analyses of the B4 study time point. EDC biomarker concentrations below the lab-specific LOD were imputed as LOD/sqrt(2).[74]

Before statistical analyses, we normalized CDC-analyzed biomarker data to the Mount Sinai-analyzed biomarker data using scaling parameters calculated with the 40 duplicate samples using a previously described method.[148] Specifically, we calculated the mean and relative standard deviation (SD) difference in biomarker concentration among the 40 duplicate samples, then shifted and scaled the mean and SD, respectively, in all the samples analyzed at the CDC to that of those analyzed at Mount Sinai. Samples were randomly chosen to be analyzed at the CDC; therefore, we assumed no significant differences in study participant characteristics in the normalization step. Creatinine was quantified for all samples at Mount Sinai to facilitate

correction for urine dilution. All EDC biomarker and creatinine concentrations were log_{10}-transformed prior to analyses. EDC biomarkers were evaluated both continuously and in quantiles (quartiles) in statistical analyses.

Stool gut microbiome assessment

Participants collected a fresh stool sample at home after receiving instructions and materials during their annual visit to the INTA health clinic. Immediately after collection, the stool sample was stored in the participants freezer before transfer to the INTA laboratory and storage in a -80°C freezer. A total of 279 samples (266 distinct samples, 13 duplicates) were shipped on dry ice to the National Exposure Assessment Laboratory at Emory University in Atlanta, GA, a CHEAR Laboratory, for sequencing of the gut microbiome.

At the National Exposure Assessment Laboratory, DNA was extracted from stool samples using the Qiagen DNeasy PowerSoil Kit (Qiagen; 12888) according to the manufacturer's protocol. The V3-V4 hypervariable region of the 16s ribosomal RNA (rRNA) gene was barcoded and amplified (PCR primer pair: 341F 5'-GTGCCAGCMGCCGCGGTAA-3'; 805R 5'-GACTACHVGGGTWTCTAAT-3'). Following amplification, 12.5 ng of DNA was used to generate and pool libraries based on fluorescence according to a standard workflow (Illumina, Inc.). Quantitation of final library pools was done using qPCR (Kapa Biosystems; KK4824). Pooled libraries were then sequenced on an Illumina MiSeq sequencer with MiSeq v3 600 cycle chemistry (Illumina MS-102-3003) with loading density of 6-8 pM and 20% PhiX following the manufacturer's instructions. Positive controls, negative controls, no template controls, and ZymoBIOMICS mock microbial community controls were also included.[149]

Raw demultiplexed amplicon sequence data were processed using QIIME2 (Quantitative Insights Into Microbial Ecology) version 2019.4.[150] Sequencing error rates were estimated using Divisive Amplicon Denoising Algorithm 2 (DADA2).[151] We trimmed the first 30 base pairs (bp)

from each read and truncated at position 290 based on Phred quality scores. Filtered sequences were dereplicated and chimeric sequences were removed to generate unique sequences, then processed to infer exact amplicon sequence variants (ASVs).[152] Sequences were assigned to ASVs using a naive Bayes classifier on the SILVA database (SILVA 132 release).[153]

Of the 266 study participants who provided fecal samples, 261 provided at least one urine sample at any of the three urine collection time points and were included in this analysis. ASV abundance and taxonomic classification for these samples were combined using the 'phyloseq' package in R, resulting in 261 samples, 6270 ASVs, and 7 levels of taxonomy for each ASV.[154,155] ASVs that were not present in at least 2 samples and with missing or uncharacterized taxonomy were filtered out, resulting in 5856 remaining ASVs. Samples that were below a read count of 10,000 were also dropped. The final analytic sample consisted of 257 participants.

Covariates

We selected covariates *a priori* as potential confounders of the EDC-microbiota relation using directed acyclic graphs. Variables included as potential confounding factors were age at clinic visit, maternal education (secondary education or less, more than secondary education), body fat percentage, mode of delivery at birth (vaginal, caesarean), duration of predominant breast-feeding (<3 months, 3-6 months, >6 months), antibiotic use in the 6 months prior to stool sample (yes, no, unknown), average caloric intake (g/day), and lab (CDC, Mount Sinai). Antibiotic use occurred after urine sample collection and therefore was unlikely to be a directly associated with EDC exposure; however, antibiotic use has a strong association with microbial composition and was therefore included as a covariate in analyses.[156] We calculated average caloric intake in the period prior to each clinic visit by taking the average values for all 24-hour dietary recalls prior to the sample. Diet data was not available prior to B1; therefore, we assumed that the average

caloric intake prior to B4 was representative of caloric intake prior to B1. Lab was not included as a covariate for the models evaluating the 1-year post-menarche exposure time point as all biomarkers collected at this time point were measured by Mount Sinai. Missing covariate data were imputed using last observation carried forward when possible; if prior observations were unavailable, we imputed values with the mean for normally distributed variables or median for skewed variables under the assumption that values were missing completely at random. . The percentage of missing values for covariates ranged from 0% to 8% for the cohort.

Statistical analysis

We calculated descriptive statistics for all covariates by study exposure time point (B1, B4, 1-year post-menarche). We visually evaluated the distribution of each EDC biomarker concentration using boxplots. Correlation between EDCs at each time point were calculated using Spearman correlation coefficients.

We quantified gut microbial alpha diversity as the Shannon index using the 'vegan' package in R [157–159]. The Shannon index is a measure of diversity of species in a given microbial community, accounting for both richness and evenness, and represents within-sample diversity.[160] A higher Shannon index generally implies higher diversity of the sample.[160] We evaluated Shannon index outliers as the 25th percentile (Shannon index=3.85) plus or minus the inter-quartile range (IQR=0.69). Six samples were outside this value— all less than 3.16— and were excluded from the analysis. We evaluated the relation of the Shannon index to single-chemical $\log_{10}$-transformed and quantiled (quartiles) EDC biomarker concentrations using generalized linear models with an identity link structure. Models were stratified by study time point and adjusted for potential confounders, including age, creatinine, maternal education, body fat percentage, breast feeding, birth mode, average caloric intake, antibiotic use, and lab. As a sensitivity analysis, because antibiotic use is strongly associated with gut microbial

outcomes, we ran the same regression models while excluding girls who reported antibiotic usage in the 6 months prior to the stool sample (N=13-24 by time point).

To evaluate beta diversity, permutational multivariate analysis of variance (PERMANOVA) models were used to examine the contribution of single-chemical log_{10}-transformed EDC biomarker concentrations to gut microbiota composition using the adonis2 function in the 'vegan' package in R.[154,157] All models assessed the marginal effect of the EDC biomarker, accounting for age, creatinine, maternal education, body fat percentage, breast feeding, birth mode, average caloric intake, antibiotic use, and lab. In this approach, PERMANOVA partitioned a Bray-Curtis distance matrix and specified 999 permutations and marginal effects in the function.

To identify microbial taxa that might be driving differences in alpha diversity or beta diversity, we performed multivariable linear regression analyses to evaluate associations of the log_{10}-transformed single-EDC biomarkers with genus-level microbial taxa using the 'MaAsLin2' package in R.[161] We first agglomerated the microbial ASV data to the taxonomic rank of genus using the 'phyloseq' package in R, which resulted in 358 genera represented in the data.[154,155] We ran log-transformed generalized linear models with the MaAsLin2 default specifications (minimum abundance of 0.01% in 10% of samples, total sum scaling normalization, arsine square-root transformation). These parameters have recently been observed to perform equally as well as compositionality-corrected and specialized normalization or transformation methods.[161] Models were adjusted for the same set of potential confounders as in prior analyses.

Because we evaluated hypotheses for many different biomarker-diversity and abundance relations across three time points, the probability of type 1 error was considerable. Therefore, we also evaluated our single-chemical EDC biomarker relations to alpha and beta diversity and abundance outcomes while controlling for multiple comparisons using the Benjamini-Hochberg

false discovery rate (FDR) in supplementary analyses.[88] FDR-corrected statistical significance was defined as q-values less than 0.25.

To build upon our single-chemical analyses, we additionally examined the EDC biomarker concentration profile of our cohort using a mixture approach. We first used weighted quantile sum (WQS) regression to assess the mixture effect (i.e., joint action) of EDC biomarkers on the outcome of Shannon diversity. The WQS method allows for a complex correlation structure among highly-correlated EDCs and has been previously described.[162] In all WQS models, data were divided into 40% training and 60% validation sets; 1000 bootstrap samples were used for parameter estimation such that the final reported beta coefficient is the mean across simulated distributions. In the multistep WQS approach, the model first built a weighted index with empirical weights based on each EDC biomarker's association with the Shannon index, representing an alpha diversity associated EDC mixture effect. The association of the WQS index to the Shannon index was then evaluated in a generalized linear model with an identity link function. The coefficient associated with the WQS index is interpreted as the change in Shannon index associated with a quartile-increase in the WQS index. While few studies have evaluated the association of these chemicals to alpha diversity, they have demonstrated similar mechanisms of effect.[135] We hypothesized *a priori* that these chemicals would act in the same direction on the outcome; therefore, the WQS approach, which limits constraints to one direction of effect, is appropriate for this analysis. To explore the potential direction of effect, we included separate WQS regression models with constraints in the positive and negative directions when estimating the weights. Like the single-chemical analyses, the WQS analyses were stratified by time point (B1, B4, and 1-year post-menarche) and adjusted for age, creatinine, maternal education, body fat percentage, breast feeding, birth mode, average caloric intake, antibiotic use, and lab. For models with statistically significant WQS estimates ($p<0.05$), we replicated the

analysis with an alternate seed for reproducibility. The R package 'gWQS' was used for WQS analyses.

Statistical analyses were conducted in SAS version 9.4 and R version 4.0/RStudio version 1.4.1106.

3.4. Results

Study characteristics

Among the 257 participants with at least one urine sample and a stool sample included in this analysis, 197 provided a urine sample at Tanner stage B1; 233, Tanner stage B4; and 167, 1-year post-menarche (Table 3. 1). The mean (SD) age of participants who provided urine samples at B1, B4, and 1-year post-menarche was 7.9 years (SD=0.46), 11.3 years (SD=0.82), and 13.3 years (SD=0.76), respectively; the mean age at stool sample was 15.36 years (data not shown). Across the cohort, roughly 77% of participants had mothers with a secondary school education or less. With respect to birth-related variables, more than half of participants were breast fed for 3-6 months and more than 75% of participants were born vaginally. Average caloric intake ranged from 1,872 kCal at B1 and B4 to 1,779 kCal at 1-year post-menarche. Approximately 52-58% of the urine samples were measured at CDC at B1 and B4; at 1-year post-menarche, all urine samples were measured at Mount Sinai. Approximately half of participants reported having no antibiotic usage in the six months prior to stool sample collection, 10% reported antibiotic use, and the remaining 40% reported not knowing whether they had taken antibiotics. Log_{10}-transformed EDC concentrations are presented in Figure A2. 1.

The overall mean Shannon index of the stool samples was 4.0 (SD=0.31) (data not shown). The mean (SD) Shannon index was consistent across study time point strata (Table 3. 1). The relative composition of gut microbiota by taxonomic classification is presented in Table A2. 2.

Overall, *Firmicutes* was the most highly represented microbial phyla, comprising on average 65.9% of sample relative abundance, followed by *Bacteroidetes* (18.1%) and *Actinobacteria* (10.3%). At the class level, *Clostridia* and *Bacteroidia* represented 57.9% and 18.3% of microbial taxa, respectively. *Bacteroides* (12.2%) was the most abundant genus.

Single-chemical analyses

Single-chemical analyses using log_{10}-transformed EDC biomarker concentrations suggested that bisphenol A (BPA) measured at B1 was positively associated with the Shannon index (β=0.17, 95% confidence interval [CI]:0.02, 0.32), mono-isobutyl phthalate (MIPB) at B1 was inversely associated with the Shannon index (β=-0.17, 95%CI:-0.32, -0.02), and mono-n-butyl phthalate (MNBP) measured at 1-year post-menarche was inversely associated with the Shannon index (β=-0.19, 95%CI:-0.34, -0.03) (Table 3. 2). The associations of BPA, MIPB, and MNBP measured at other study time points to the Shannon index were not significant. We also found evidence to suggest non-significant but trending inverse associations for bisphenol S (BPS) (β=-0.14, 95%CI:-0.29, 0.01), mono-(2-ethyl-5-carboxypentyl) phthalate (MECPP) (β=-0.15, 95%CI:-0.32, 0.01), mono (2-ethyl-5- hydroxyhexyl) phthalate (MEHHP) (β=-0.14, 95%CI:-0.29, 0.01), mono (2-ethyl-5-oxohexyl) phthalate (MEOHP) (β=-0.14, 95%CI:-0.3, 0.02), and monoethyl phthalate (MEP) (β=-0.09, 95%CI:-0.19, 0.01) measured at 1-year post-menarche and the Shannon index. When evaluating single-chemical quartiled EDC biomarker concentrations, BPA measured at B1 remained positively associated with Shannon index, while MNBP, MECPP, and MEOHP were similarly inversely associated with the Shannon index (Table 3. 2). In supplementary analyses which excluded the 10% of the cohort who reported using antibiotics in the six months prior to stool sample, the relations of MIBP measured at B1 (β=-0.18, 95% CI -0.33, -0.03) and MNBP measured at 1-year post-menarche (β=-0.17, 95% CI -0.34, -0.01) to the Shannon index remained significant (Table A2. 3). Additionally, controlling for multiple comparisons using the Benjamini-Hochberg FDR method resulted in fewer

statistically significant associations among the total cohort (Table A2. 4). However, the significance of the relation of MNBP, MECPP, MEHHP, and MEOHP measured at 1-year post-menarche to the Shannon index persisted for both log_{10}-transformed values and quartiled values ($q < 0.25$).

PERMANOVA analyses using the single-chemical log_{10}-transformed EDC biomarkers indicated marginally significant sample dissimilarity associated with MNBP measured at 1-year post-menarche (Bray-Curtis distance PERMANOVA, R^2=0.008, p=0.05) (Table 3. 3). However, this result was no longer significant after correction for multiple comparisons. No other biomarkers were observed to contribute significantly to differences in gut microbial composition in PERMANOVA models.

In multivariable linear models, four single-chemical log_{10}-transformed EDC biomarkers were associated with microbial genera (Table 3. 4). Among the EDC biomarkers quantified at B4, triclosan was associated with higher abundance of microbes from the *Anaerofustis* genus (β= 0.13, *p*= 0.0001, *q*= 0.15), while mono-ethyl phthlate (MEP) was associated with lower abundance of microbes from the *Bifidobacterium* genus (β= -0.20, *p*= 0.0002, *q*= 0.23). MNBP measured at 1-year post-menarche was inversely associated with the *Ruminiclostridium.9* genus (β= -0.33, *p*= 0.0001, *q*= 0.07) and positively associated with the *Tyzzerella.4* genus (β= 0.12, *p*= 0.0007, *q*= 0.18). No other ASVs were associated with concentrations of single-chemical EDCs after FDR correction.

WQS mixture analyses

Mixture analyses using WQS regression with quartiled EDC concentrations suggested no association between the EDC mixture when measured at B1 and B4 and the Shannon index, regardless of the direction or constraints applied to the β coefficient (Table 3. 5). Though we observed a statistically significant inverse association between the EDC mixture measured at 1-

year post-menarche and the Shannon index in a WQS analysis with positively constrained β values (β=-0.14; 95% CI: -0.25, -0.03; *p*=0.02), we did not observe this relation was not observed in secondary analyses for reproducibility. When evaluating the same positively constrained WQS model with a different seed for reproducibility, the association was no longer statistically significant (β=-0.09; 95% CI: -0.21, 0.04; *p*=0.18) (Table 3. 5).

3.5. Discussion

To our knowledge, this is the first investigation of childhood phenol, phthalate, and paraben biomarker concentrations in relation to adolescent gut microbial composition. In this study, we found that childhood concentrations of select EDC biomarkers at B1 and 1-year post-menarche were differentially associated with adolescent gut microbial alpha diversity and weakly associated with beta diversity. In single-chemical analyses, higher bisphenol A (BPA) concentration measured at B1 was associated with higher Shannon diversity in adolescence, while higher mono-n-butyl phthalate (MNBP) concentration measured at 1-year post-menarche was associated with lower Shannon diversity in adolescence. Higher concentration of select phthalates measured at 1-year post-menarche were also borderline associated with lower Shannon diversity in adolescence. An EDC biomarker mixture at 1-year post-menarche was not significantly associated with lower Shannon diversity in adolescence. Taken together, these results suggest higher EDC concentration in childhood is associated with small variation in global gut microbial diversity in adolescence in a chemical-specific manner and provide support for the idea of windows of susceptibility to EDC exposure in relation to gut microbial composition.

Little epidemiologic evidence exists for the relation between our selected EDCs and the gut microbiome. Previous studies using single-EDC analytic strategies have found mixed results for the relation of a few select EDC to gut microbial composition. For example, triclosan (TCS) exposure was associated with lower gut microbial alpha diversity (Shannon index) in infants

exposed to TCS-containing breast milk [163], but was not associated with the Shannon index among mothers and infants in a randomized crossover study of TCS-containing household products.[164] While we did not find an association with TCS measured at B1, B4, nor 1-year post-menarche and the Shannon index, it is difficult to compare our results to prior studies due to differences in study population (infants versus children), sample sizes (<50 versus >200), and source of TCS (breast milk versus urine). Similar important differences exist for when comparing our study to a study of phthalates and the gut microbiome. The study of Taiwanese infants that found an association between DEHP and gut microbial dysbiosis did not calculate alpha diversity, had a small sample size, and limited generalizability beyond infants.[46] In a review of the literature, we found no studies in humans that have evaluated the relation of parabens or other phenols to the gut microbiome. To our knowledge, few studies have characterized childhood or peripubertal exposure to EDCs using a mixture approach, and none have evaluated the association with gut microbiota. Recently, studies evaluating EDC mixture effects have focused on the prenatal exposure window in relation to infant or early childhood health outcomes, including neurodevelopment and body size.[165–170] Two other studies have evaluated a phthalate mixture in early childhood (after infancy) on child behavior.[171,172] While the study from Li et al. also collected urine samples and quantified EDC biomarkers at multiple time points in a large cohort, the EDC mixture index represented the weighted exposure intensity across all collections, rather than each childhood time window separately.[172] In contrast, the study from Daniel et al. of more than 400 inner-city mothers and their children evaluated an EDC mixture at 3 years of age and 5 years of age on child behavior outcomes.[171] The pubertal window, much like the prenatal or early childhood window, is an important critical period of development in the lifecourse.[173] EDC exposure during these windows of childhood may have heightened impact on later life health and disease. Therefore, evaluation of EDC mixture exposure across different windows of time provides valuable contributions to our understanding of windows of susceptibility throughout the lifecourse.

This study is not without limitations. Phenol, phthalate, and paraben biomarkers have short half-lives and metabolize quickly in the body; a single fasting spot urine thus reflects the period shortly before sample collection and may not be representative of long-term exposure.[123,124] However, any misclassification of EDC exposure is unlikely to be associated with the outcome of interest and will thus be non-differential. Moreover, although these EDCs have short half-lives, pervasive availability and chronic exposure suggests that a single urine sample may reasonably reflect a longer period of time.[123] Additionally, while we were able to prospectively assess EDC biomarker concentration over three study time points, it is difficult to gauge the appropriate amount of follow-up time necessary to see a potential effect on the gut microbiota. Although we have detailed longitudinal covariate data, it is possible that we did not completely control for confounding factors. While we were able to include important variables which may affect gut microbial composition, like diet and antibiotic usage, we cannot rule out that other perturbations of the gut microbiota occurred. In this temporal context, it is possible that the most-recent EDC assessment at 1-year post-menarche more accurately reflects the association between EDC exposure and gut microbial composition. Given this limitation, we evaluated models with constraints in both the positive and negative direction to allow for future hypothesis generation.

Our study has many strengths. Notably, it is one of the earliest population-based human studies with a large sample size and prospective study design to investigate the relation between childhood exposure to EDCs and the gut microbiome. We were able to control for a number of potential confounding factors known to be associated with the gut microbiome and related to EDC exposure, including birth mode, breast feeding, and diet. Finally, we were able to consider three separate exposure windows throughout childhood, allowing for potential identification of important windows of susceptibility for development of the gut microbiome.

3.6. Conclusions

In this longitudinal cohort of Chilean girls, we found evidence to indicate a biomarker-specific association between childhood and pubertal EDC exposure and adolescent gut microbial composition. This association differed across exposure time points in puberty, suggesting potential windows of susceptibility to EDCs. We additionally report the results of an EDC mixture approach, which is highly appropriate when considering the totality of EDC exposure.

Table 3. 1. Demographic and anthropometric characteristics of girls in the Growth and Obesity Cohort Study by study time point (N=257)

Characteristic	**Study Time Point***		
	Tanner Stage B1	Tanner Stage B4	1 Year Post-Menarche
	(n=197)	(n=233)	(n=167)
Lab of urine sample analysis			
CDC	95 (48.2)	98 (42.1)	0
Mount Sinai	102 (51.8)	135 (57.9)	160 (100.0)
Age, years (mean (SD))	7.91 (0.46)	11.33 (0.82)	13.26 (0.76)
Body fat percentage (mean (SD))	25.48 (4.21)	26.80 (4.89)	30.23 (5.35)
Maternal education (%)			
Secondary education or less	151 (76.6)	182 (78.1)	126 (78.8)
Greater than secondary education	46 (23.4)	51 (21.9)	34 (21.2)
Duration of predominant breast feeding (count (%))			
< 3 months	65 (33.0)	71 (30.5)	53 (33.1)
3-6 months	113 (57.4)	134 (57.5)	91 (56.9)
> 6 months	19 (9.6)	28 (12.0)	16 (10.0)
Birth mode (%)			
Caesarean	49 (24.9)	57 (24.5)	38 (23.8)
Vaginal	148 (75.1)	176 (75.5)	122 (76.2)
Average caloric intake, kCal (mean (SD))	1871.61 (464.50)	1872.98 (503.44)	1779.36 (479.95)
Antibiotic use in 6 months prior to stool sample			
Yes	18 (9.1)	24 (10.3)	13 (8.1)
No	103 (52.3)	131 (56.2)	89 (55.6)
Unknown	76 (38.6)	78 (33.5)	58 (36.2)
Shannon index (mean (SD))	4.08 0.33)	4.07 (0.33)	4.08 (0.33)

* Not mutually exclusive

Table 3. 2. Results from linear regression of single-chemical EDC concentration (log10-transformed, quartiled) on Shannon diversity by study time point (N = 251).

	Tanner Stage B1 (n=197)	**Tanner Stage B4 (n=233)**	**1 Year Post-Menarche (n=167)**
	β[a] (95%CI)	**β[a] (95%CI)**	**β[b] (95%CI)**
Log10-transformed EDC concentration			
BP3	-0.05 (-0.17, 0.06)	-0.04 (-0.11, 0.03)	0.02 (-0.08, 0.11)
BPA	0.17 (0.02, 0.32)*	0.07 (-0.05, 0.17)	-0.03 (-0.2, 0.14)
BPS	0.04 (-0.1, 0.18)	-0.03 (-0.14, 0.08)	-0.14 (-0.29, 0.01)**
BUPB	0.00 (-0.06, 0.07)	-0.02 (-0.08, 0.05)	0.02 (-0.07, 0.10)
ETPB	0.00 (-0.07, 0.07)	-0.02 (-0.08, 0.04)	0.02 (-0.05, 0.09)
MBP	-0.11 (-0.25, 0.05)	-0.03 (-0.16, 0.07)	-0.19 (-0.34, -0.03)*
MBZP	-0.01 (-0.12, 0.11)	-0.04 (-0.13, 0.07)	-0.1 (-0.23, 0.02)
MCPP	-0.01 (-0.14, 0.13)	-0.01 (-0.09, 0.07)	-0.08 (-0.2, 0.04)
MECPP	-0.01 (-0.16, 0.16)	0.05 (-0.11, 0.17)	-0.15 (-0.32, 0.01)**
MEHHP	-0.04 (-0.18, 0.12)	0.04 (-0.11, 0.17)	-0.14 (-0.29, 0.02)**
MEHP	0.00 (-0.13, 0.13)	0.07 (-0.06, 0.18)	-0.09 (-0.24, 0.05)
MEOHP	-0.07 (-0.21, 0.09)	0.04 (-0.1, 0.16)	-0.14 (-0.3, 0.02)**
MEP	-0.03 (-0.14, 0.07)	-0.04 (-0.13, 0.04)	-0.09 (-0.19, 0.01)**
MEPB	0.01 (-0.07, 0.08)	0.00 (-0.05, 0.06)	0.00 (-0.07, 0.07)
MIBP	-0.17 (-0.32, -0.02)*	0.00 (-0.13, 0.14)	-0.13 (-0.31, 0.04)
PRPB	0.00 (-0.05, 0.05)	-0.01 (-0.06, 0.04)	0.02 (-0.03, 0.08)
TCS	0.01 (-0.07, 0.1)	0.03 (-0.04, 0.11)	-0.03 (-0.1, 0.05)
Quartiled EDC concentration			
BP3	-0.03 (-0.07, 0.02)	-0.02 (-0.06, 0.03)	-0.01 (-0.06, 0.05)
BPA	0.06 (0.00, 0.11)*	0.03 (-0.02, 0.08)	0.01 (-0.05, 0.07)
BPS	0.01 (-0.03, 0.05)	-0.01 (-0.05, 0.03)	-0.04 (-0.1, 0.03)
BUPB	-0.02 (-0.06, 0.03)	-0.03 (-0.07, 0.02)	0.02 (-0.05, 0.08)
ETPB	0.00 (-0.05, 0.04)	-0.03 (-0.07, 0.02)	0.00 (-0.06, 0.05)
MBP	-0.05 (-0.11, 0.0)**	-0.02 (-0.08, 0.03)	-0.07 (-0.13, 0.00)*
MBZP	-0.02 (-0.07, 0.04)	-0.04 (-0.09, 0.01)	-0.02 (-0.08, 0.03)
MCPP	0.00 (-0.06, 0.05)	0.00 (-0.05, 0.05)	-0.02 (-0.08, 0.03)
MECPP	-0.02 (-0.07, 0.04)	0.02 (-0.03, 0.08)	-0.07 (-0.13, -0.01)*
MEHHP	-0.01 (-0.06, 0.05)	0.02 (-0.03, 0.07)	-0.06 (-0.12, 0.00)**
MEHP	-0.01 (-0.06, 0.05)	0.03 (-0.02, 0.08)	-0.04 (-0.1, 0.01)
MEOHP	-0.03 (-0.08, 0.03)	0.02 (-0.03, 0.07)	-0.07 (-0.13, 0.00)*
MEP	-0.02 (-0.06, 0.03)	-0.03 (-0.07, 0.02)	-0.03 (-0.09, 0.02)
MEPB	0.00 (-0.05, 0.04)	-0.01 (-0.05, 0.04)	0.02 (-0.02, 0.07)
MIBP	-0.05 (-0.11, 0.00)**	-0.02 (-0.07, 0.03)	-0.05 (-0.11, 0.02)
PRPB	0.00 (-0.04, 0.04)	0.00 (-0.05, 0.04)	0.00 (-0.05, 0.05)
TCS	-0.01 (-0.05, 0.04)	0.01 (-0.03, 0.06)	0.00 (-0.05, 0.05)

a: Adjusted for age at clinic visit, maternal education (secondary education or less, more than secondary education), body fat percentage, (vaginal, caesarean), duration of predominant breast-feeding (<3 months, 3-6 months, >6 months), antibiotic use in the 6 months prior to stool sample (yes, no, unknown), average caloric intake (g/day), and lab (CDC, Mount Sinai)

b: Adjusted for the same set of covariates but excluding lab

*: P-value <=0.05

**: P-value <=0.10

Table 3. 3. Results from PERMANOVA of single-chemical EDC concentration (log10-transformed) by study time point (N = 251).

	Tanner Stage B1 (n=197)			**Tanner Stage B4 (n=233)**			**1 Year Post-Menarche (n=167)**		
	R2	**P-value**	**FDR**	**R2**	**P-value**	**FDR**	**R2**	**P-value**	**FDR**
Log10-transformed EDC concentration									
BP3	0.005	0.45	0.9	0.004	0.38	0.9	0.008	0.06	0.9
BPA	0.005	0.33	0.9	0.004	0.62	0.9	0.005	0.72	0.9
BPS	0.005	0.59	0.9	0.004	0.28	0.9	0.006	0.55	0.9
TCS	0.004	0.92	0.93	0.004	0.45	0.9	0.007	0.27	0.9
MEPB	0.004	0.86	0.9	0.004	0.77	0.9	0.006	0.63	0.9
ETPB	0.004	0.93	0.93	0.003	0.84	0.9	0.007	0.13	0.9
PRPB	0.005	0.43	0.9	0.005	0.17	0.9	0.006	0.52	0.9
MBP	0.004	0.69	0.9	0.004	0.58	0.9	0.008	0.05	0.9
MBZP	0.005	0.29	0.9	0.003	0.86	0.9	0.006	0.35	0.9
MCPP	0.004	0.85	0.9	0.004	0.76	0.9	0.006	0.47	0.9
MECPP	0.004	0.64	0.9	0.004	0.68	0.9	0.006	0.45	0.9
MEHHP	0.005	0.6	0.9	0.004	0.71	0.9	0.005	0.79	0.9
MEHP	0.005	0.33	0.9	0.004	0.62	0.9	0.006	0.69	0.9
MEOHP	0.005	0.5	0.9	0.004	0.61	0.9	0.006	0.67	0.9
MEP	0.005	0.45	0.9	0.005	0.08	0.9	0.006	0.45	0.9
MIBP	0.005	0.36	0.9	0.003	0.77	0.9	0.008	0.06	0.9

a: Adjusted for age at clinic visit, maternal education (secondary education or less, more than secondary education), body fat percentage, (vaginal, caesarean), duration of predominant breast-feeding (<3 months, 3-6 months, >6 months), antibiotic use in the 6 months prior to stool sample (yes, no, unknown), average caloric intake (g/day), and lab (CDC, Mount Sinai)

b: Adjusted for the same set of covariates but excluding lab

*: P-value <=0.05

**: P-value <=0.10

Table 3. 4. Multivariable MaAsLin2 modeling of the association between microbial taxa (ASVs) and log10-transformed EDC by study time point.

EDC	**Taxa** (Phylum; Class; Order; Family; Genus)	**β**	**SE**	**p-value**	**q-value**
	B4				
TCS	Firmicutes;Clostridia;Clostridiales;Eubacteriaceae;Anaerofustis	0.13	0.03	0.0001	0.15
MEP	Actinobacteria;Actinobacteria;Bifidobacteriales;Bifidobacteriaceae;Bifidobacterium	-0.20	0.05	0.0002	0.23
	1-year post-menarche				
MBP	Firmicutes;Clostridia;Clostridiales;Ruminococcaceae;Ruminiclostridium.9	-0.33	0.08	0.0001	0.07
MBP	Firmicutes;Clostridia;Clostridiales;Lachnospiraceae;Tyzzerella.4	0.12	0.04	0.0007	0.18

Table 3. 5. Results from WQS regression of EDC concentration on Shannon diversity by study time point with EDC concentrations quartiled in generalized linear models (N = 251).

	Tanner Stage B1 (n=197)	**Tanner Stage B4 (n=233)**	**1 Year Post-Menarche (n=167)**
Positive, no constraints[a]			
β (95%CI)	-0.03 (-0.16, 0.1)	-0.01 (-0.11, 0.09)	-0.07 (-0.18, 0.04)
p-value	0.65	0.82	0.22
Positive, with constraints[b]			
β (95%CI)	0.04 (-0.06, 0.13)	-0.01 (-0.11, 0.09)	-0.14 (-0.25, -0.03)
p-value	0.45	0.81	0.02[c]
Negative, no constraints[a]			
β (95%CI)	-0.12 (-0.27, 0.02)	0 (-0.12, 0.12)	-0.02 (-0.15, 0.12)
p-value	0.10	0.97	0.81
Negative, with constraints[a]			
β (95%CI)	0.02 (-0.11, 0.15)	0 (-0.12, 0.12)	-0.06 (-0.22, 0.1)
p-value	0.79	0.96	0.48
Positive, with constraints and different seed[b]			
β (95%CI)			-0.09 (-0.21, 0.04)
p-value			0.18

a: Models for time points B1 and B4 are adjusted for age at clinic visit, maternal education (secondary education or less, more than secondary education), body fat percentage, (vaginal, caesarean), duration of predominant breast-feeding (<3 months, 3-6 months, >6 months), antibiotic use in the 6 months prior to stool sample (yes, no, unknown), average caloric intake (g/day), and lab (CDC,Mount Sinai). Models for time point 1-year post-menarche did not include lab as a covariate.

b: Secondary analysis with different seed for evaluation of reproducibility

c: P-value <=0.05

Chapter 4. The association between breast density and gut microbiota composition at 2-years post menarche: A cross-sectional study of adolescents in Santiago, Chile

4.1. Abstract

Background*:* The gut microbiome has been linked to breast cancer via immune, inflammatory, and hormonal mechanisms. We examined the relation between adolescent breast density and gut microbial composition and function in a cohort of Chilean girls.

Methods: This cross-sectional study included 218 female participants in the Growth and Obesity Cohort Study who were 2 years post-menarche. We measured absolute breast fibroglandular volume (aFGV) and derived percent FGV (%FGV) using dual energy X-ray absorptiometry. All participants provided a fecal sample. The gut microbiome was characterized using 16S ribosomal RNA sequencing of the V3-V4 hypervariable region. We examined alpha diversity and beta diversity across terciles of %FGV and aFGV. We used MaAsLin2 for multivariable general linear modeling to assess differential taxa abundance and differential MetaCyc pathway abundance between %FGV and aFGV terciles. All models were adjusted for potential confounding variables and corrected for multiple comparisons.

Results*:* The mean %FGV and aFGV was 49.5% and 217.0 cm^3, respectively, among study participants. Similar median alpha diversity levels were found across %FGV and aFGV terciles when measured by the Shannon diversity index (%FGV T1: 4.0, T2: 3.9, T3: 4.1; aFGV T1: 4.0, T2: 4.0, T3: 4.1). No genera were differentially abundant when comparing %FGV nor aFGV terciles after adjusting for potential confounders ($q > 0.56$ for all genera). We found no associations between predicted MetaCyc pathway abundance and %FGV and aFGV.

Conclusions*:* Breast density measured at 2-years post menarche was not associated with composition and predicted function of the gut microbiome among adolescent Chilean girls.

4.2. Introduction

Breast cancer is the most common cancer among women worldwide, however, one third of affected women have no known or suspected risk factors.[1] Greater breast density is strongly associated with increased risk of breast cancer among adult women.[174] Breast density, measured as the relative proportion of fibroglandular tissue to fatty tissue in the breast, is inversely related to age, parity, and later menopause and also associated with childhood and adolescent body fatness.[175,176] Initial peak breast density is hypothesized to be established during adolescence [177], and breast tissue may be particularly vulnerable to exposures during puberty, a period of rapid breast development.[178,179] Adolescent breast density has been associated with pubertal maturation and body fatness.[180] However, few studies have characterized other important associates of breast development and composition during this vulnerable period.

A novel mechanistic pathway that may contribute to development of the breast is the gut microbiome. Collectively the trillions of microbes living in the human intestinal tract, the gut microbiome plays important roles in numerous biological processes including immune regulation, dietary metabolism, epithelial barrier function, and hormone regulation.[129,181–183] Experimental and epidemiologic evidence suggests that the gut microbiome may be associated with different diseases such as metabolic disorders and cancers, including breast cancer, through direct and indirect mechanisms.[184–189] For instance, perturbations in microbiota composition, or dysbiosis, may lead to systemic inflammation resulting in an increased vulnerability to pathogens.[47] Carcinogenesis may follow from dysbiosis-induced permeability of the intestinal epithelium and resulting extracellular vesicle circulation throughout the body in biofluids.[190] Changes in the composition of gut microbiome, and specifically in microbes involved in estrogen metabolism, may influence breast cancer development through an increase in circulating estrogen levels.[48] Evidence for an association between circulating estrogens and adult mammographic breast density is mixed.[191–194] However, we have shown that levels of

prepubertal estrogen, measured with an ultra-sensitive method, are associated with earlier thelarche, which is in turn related to breast density at the end of puberty.[50] Estrogen levels are directly associated with breast development; therefore, the gut microbiome may play a mechanistic role in the development of breast density during puberty and adolescence.[49,50]

It is unknown whether the gut microbiome is associated with breast density in adolescence. The current study examined the association between breast density at two years post-menarche and the gut microbiome in a cohort of adolescent Chilean girls. We hypothesized that the microbial composition and function of the gut would differ across densities of the breast.

4.3. Methods

Study design and population

We conducted a cross-sectional analysis of a subset of female participants in the Growth and Obesity Cohort Study (GOCS). The original GOCS, which began in 2006, has been described previously.[11] In brief, 1,196 children aged 2.5 to 4 years from low- and middle- income families and enrolled in preschool at the National Board of Preschool Council Program (Junta Nacional de Jardines Infantiles) were enrolled in the study; approximately half (601) were girls. Participants in the study visit the Institute of Nutrition and Food Technology (INTA) Health Clinic at the Universidad de Chile in Santiago, Chile at least once per year for anthropometric assessments, pubertal (Tanner) evaluation, collection of biospecimens, and to complete 24-hour dietary recall interviews. A limited set of behavioral and demographic information was also collected via questionnaire. Breast composition was measured when the participants were two years post-menarche (2PM). The current study included a subset of 218 girls randomly selected to provide a fecal sample and who had a breast composition measurement at 2PM. The 2PM breast assessment and fecal sample collection occurred between 2018 and 2019. The study protocol and written consent forms were approved by the University of Chile Ethics Committee at INTA.

Breast composition measurement

Breast composition was measured at the clinic visit corresponding to a timepoint of 2PM for each girl using the dual energy X-ray absorptiometry (DXA) breast scanning protocol developed by Shepherd et al. at the University of California, San Francisco.[75] The Prodigy DXA system software (version 13.6, series 200674; GE Healthcare) was used to scan each breast for quantification of adipose fat and fibroglandular (FG) tissue. Stable calibration of the system was continuously performed using a quality control breast phantom. Absolute fibroglandular volume (FGV; cm^3) and total breast volume for each breast were derived from a two-compartment model of adipose fat and fibroglandular tissue. The percentage of FGV tissue in the breast was derived by dividing the absolute FGV by total breast volume and multiplying by 100. Percent FGV and absolute FGV for the left and right breast were averaged to obtain two single measures of breast density: percent FGV (%FGV) and absolute FGV (aFGV). DXA is frequently used in studies of bone density in children; exposure to ionizing radiation from the DXA protocol is low.[77] The DXA approach for breast composition assessment has high validity and precision among adolescent girls.[76] Both breast density outcomes were categorized into terciles ('T1', 'T2', 'T3') based on the distribution of the sample for statistical analyses.

Fecal collection

Fecal samples were collected after annual visits to the INTA health clinic occurring when the girls were between 13 and 15 years old. During the visit, girls were provided with materials (sealed plastic bag, stool catcher, a plastic, sterile container with a spoon to manipulate the sample and procedure gloves) and instructions for at-home fecal collection. Briefly, after the stool deposit, girls were asked to collect a part of the stool approximately the size of a walnut and place it in the plastic sterile container and label with the date and time of sample collection. Within 15 minutes of collection, the samples were sealed and then stored temporarily in the participant's freezer, at which time study personnel were contacted to schedule the sample pick

up. Samples were retrieved no later than three days after initial collection, labeled with a de-identified key, and stored at INTA in a -80°C freezer prior to shipment.

Fecal processing and 16s rRNA sequencing

Fecal processing of 279 samples (266 unique samples and 13 duplicates) was performed at the National Exposure Assessment Laboratory at Emory University, a Children's Health Exposure Analysis Resource Laboratory. DNA was extracted from fecal samples using the Qiagen DNeasy PowerSoil Kit (Qiagen; 12888). Composition of the gut microbiome was determined through sequencing and amplification of the V3-V4 hypervariable region of the 16S rRNA gene. Libraries were made from 12.5 ng of DNA following a standard 16S Metagenomic Library Preparation Workflow from Illumina, Inc. Libraries were pooled in equal amounts based on fluorescence quantification, resulting in a 630 bp amplicon. Final library pools were quantitated via qPCR (Kapa Biosystems; KK4824). The pooled library was sequenced on an Illumina MiSeq using MiSeq v3 600 cycle chemistry (Illumina MS-102-3003) at a loading density of 6-8 pM with 20% PhiX, generating roughly 20M, 300 bp paired-end reads. In addition to the 279 experimental samples, traditional negative, no template control (NTC) negative, positive, and ZymoBIOMICS mock microbial community controls were included in the assays.[149] The Emory Integrated Genomics Core performed the assays.

After sequencing, demultiplexed raw amplicon sequences were processed using QIIME2 (Quantitative Insights Into Microbial Ecology) version 2019.4.[150] Denoising and dereplication, including chimera removal and trimming of reads based on quality scores, were performed using the Divisive Amplicon Denoising Algorithm 2 (DADA2) module.[151] Amplicon sequence variants (ASVs) were inferred using DADA2 to increase resolution and allow for intrinsic biologic meaning.[152] Taxonomy was assigned using a naive Bayes classifier on the SILVA database (SILVA 132 release).[153] We excluded samples from girls that did not have breast assessments at 2PM (n=48). The end product yielded a total of 6,270 unique ASVs from 218 samples.

Standard preprocessing (filtering, subsetting, agglomeration) with the R package 'phyloseq' was used to exclude undefined or ambiguous taxa and taxa below a prevalence threshold of 2% of total samples.[154,155] The final ASV feature table comprised 18,628,903 total reads (mean per sample = 87,872, range 28,118 to 278,065) and a total of 1,600 unique ASVs across 218 samples.

Covariates

Demographic, anthropometric, and nutritional data were collected by trained dietitians during the annual study visit at the health clinic. Age- and sex- adjusted body mass index (BMI; kg/m^2) Z-scores were calculated using the World Health Organization growth reference data and categorized into Norma/Underweight (Z-score ≤ 1), Overweight (1 < Z-score ≤ 2), and Obese (2 < Z-score). Body fat percentage was estimated using Tanita-BC-418 MA bioelectrical impedance measurements (Tanita-Corporation, Tokyo, Japan) and categorized into Healthy, Overweight, and Obese based on Tanita children's age- and sex-specific body fat reference curves.[78,79] Age at menarche was determined via phone interviews completed by study dietitians every three months during puberty and dichotomized (≤ 12 years, > 12 years). Mothers of the participants were present at the clinic visits and were asked to complete short questionnaires with information on their highest level of education (ssecondary education or less, post-secondary education), birth mode of the participant (cesarean, vaginal), and months of exclusive breast feeding (<3 months, 3-6 months, >6 months). Dietary 24-hour recalls were collected longitudinally beginning in April 2014 by trained dietitians using the USDA multiple-pass method. Food and nutrient information were obtained using a harmonization process that mapped Chilean foods to the USDA Food and Nutrient Database for Dietary Studies. Daily intake of five major food groups (vegetables [g], fruit [g], red or processed meat [g], yogurt [g], and whole grains [g]) and total energy intake (kCal) were averaged over the data collection period and up to the date of the 2PM breast composition assessment to reduce random-

measurement error and to obtain a more accurate assessment of long-term diet.[80] We also collected data on ethnicity (Mapuche [native Chilean indigenous] and non-Mapuche, according to last name), average daily hours of television during the week (≤1 hour, 1-3 hours, >3 hours) as a proxy of physical activity, and antibiotic use in the 6 months prior to fecal sample (yes, no). Missing covariate data were imputed using last observation carried forward where applicable and regression imputation in the R package 'mice' with available covariates otherwise.[195]

Statistical analysis

Relative abundance at the phyla level was plotted for all samples and across %FGV and aFGV terciles. We estimated alpha diversity as the observed richness (the number of species per sample) and the Shannon index (a measure of richness and evenness) on unfiltered data.[158,196] Rarefaction without replacement was used to standardize library sizes and account for uneven sampling depth prior to estimating alpha diversity metrics.[197] Overall differences in observed richness and the Shannon index by %FGV and aFGV terciles were tested using the Kruskal-Wallis (KW) followed by post-hoc pairwise testing with the Wilcoxon Rank Sum in cases where data were compatible with evidence to reject the KW null hypothesis. Beta diversity was visualized using principal coordinate analysis (PCoA) plots with Bray-Curtis dissimilarity. Single and multivariate permutational analysis of variance (PERMANOVA) were used to test for overall differences in microbial composition between %FGV and aFGV terciles.[198] Homogeneity of variance was used to test whether differences in community structure were due to dissimilar dispersions. Microbial diversity metrics were estimated using the R packages 'phyloseq' and 'vegan'.[154,157]

ASVs were agglomerated to the genus level prior to associating microbial community features with breast density outcomes. We used the R package 'MaAsLin2' (Microbiome Multivariable Associations with Linear Models), which relies on a modified generalized linear model for compositional data, to identify differentially abundant microbe genera across %FGV and aFGV

terciles.[161] All models specified minimum abundance of 0.01% in 10% of samples, total sum scaling normalization, and arcsine square root-transformation.[161] The models were adjusted for a set of potential confounders including age, body fat percentage, antibiotic use, maternal education, total calories, hours of TV watching, ethnicity, birth mode, and breast feeding. Where appropriate, the Benjamini and Hochberg (BH) correction method was used control the false discovery rate (FDR) and produced q-values.[88]

Functional metabolic pathways of microbial communities were predicted from 16S rRNA marker sequencing data using PICRUSt2 implemented as a QIIME2 plugin.[199] Default parameters were used in the pipeline, including SATe-enabled phylogenetic placement, hidden-state prediction, and a distance cut-off of 2. PCoA ordination with Bray-Curtis dissimilarity was used to visualize MetaCyc pathway abundance predictions. Differences in MetaCyc pathway abundance between %FGV and aFGV terciles were testing using multivariable PERMANOVA. Multivariable MaAsLin2 was used to evaluate differential abundance of predicted MetaCyc pathways with default parameters and BH correction for multiple comparisons.

4.4. Results

Study sample characteristics

The association between breast density and gut microbial composition and function was assessed among 218 GOCS participants. The mean age of the participants was 14 years at the time of breast assessment and 12 years at reported menarche (Table 4. 1). The participants were primarily non-indigenous (Non-Mapuche, 82.5%) and 77.5% of the mothers had a secondary school educational attainment or less. The mean %FGV among the sample was 49.5% (SD=14.5); for tercile 1 (T1), mean %FGV was 33.7%; tercile 2 (T2), 48.9%; and tercile 3 (T3), 66.2%. The mean aFGV among the sample was 217.0 cm^3; for T1, mean aFGV was 136.2 cm^3; T2, 209.9 cm^3; and T3, 306.2 cm^3. More than half the cohort was considered overweight or

obese. Overall, participants had a mean BMI Z-score of 1.0 and a mean body fat percentage of 32.6%. Additional study population characteristics are reported in Table 4. 1.

Phylum-level microbial composition of the sample

The relative composition of the gut microbiome for each %FGV and aFGV tercile is displayed in Figure 4. 1. Overall, *Firmicutes* was the most highly represented bacterial phylum and comprised 66% of the sample abundance. Microbes from the *Bacteroidetes* and *Actinobacteria* phyla represented 18.1% and 10.4% of the abundance, respectively. We found minor differences in relative abundance across %FGV terciles for *Bacteroidetes* (T1: 20.4%, T2: 15.4%, T3: 18.5%), *Actinobacteria* (T1: 8.9%, T2: 10.9%, T3: 11.5%) and *Euryarchaeota* (T1: 1.4%, T2: 0.9%, T3: 0.9%) (Figure A3. 1). There were no differences in relative abundance at the phylum level across aFGV terciles (Figure A3. 2).

Microbial diversity analyses

We calculated alpha diversity for each sample using observed richness and the Shannon diversity index. Overall, the mean number of observed species present in the fecal samples was 214 and the mean Shannon index was 4.0 (Figure 4. 2). We observed differences in median observed species richness across %FGV terciles (T1: 226; T2: 200, T3: 223). There were no differences in the observed number of species across aFGV terciles nor in the Shannon index for both %FGV and aFGV terciles.

Beta diversity analysis was performed using Bray-Curtis dissimilarity and visualized by principal coordinate analysis (PCoA) (Figure 4. 3). PCoA axes 1 and 2 represented 9.2% and 5.8% of the total variance, respectively. Multivariable PERMANOVA analyses included body fat percentage, age, antibiotic use, birth mode, breast feeding, daily caloric intake, maternal education, and ethnicity along with either with %FGV and aFGV to evaluate associations of microbial

composition with participant characteristics (Table 4. 2). Only 1.3% of the variability in microbial composition was associated with %FGV, while 0.8% was associated with aFGV.

Differential abundance of microbial taxa and predicted functional pathways

In multivariable linear modeling with MaAsLin2, no genera were associated with %FGV nor with aFGV after FDR correction ($q < 0.25$) (data not shown). We also noted no shifts in global predicted microbial pathway abundances (Figure 4. 3). Altogether, cohort characteristics reflected a small proportion of sample variation; %FGV and aFGV explained 0.9% and 0.8% respectively (Table 4. 4). There were no major differences in abundance of predicted MetaCyc pathways by %FGV nor aFGV when adjusting for potential confounders (data not shown).

4.5. Discussion

In this cross-sectional study of Chilean adolescents, we found no associations of microbial composition nor predicted function with breast density. Our results suggest minimal differences in alpha diversity: girls in the lowest and highest breast %FGV terciles had slightly higher observed species richness compared to those in tercile 2. However, this pattern was not observed for aFGV and when examining different alpha diversity metrics (Shannon index).

Several recent clinical studies have examined the association between the gut microbiome and breast cancer. In a case-control study of 96 post-menopausal women, pre-treatment breast cancer patients had altered microbial composition (beta diversity) and increased abundance of *Clostridiaceae*, *Faecalibacterium*, and *Ruminococcaceae* compared to controls.[48] A cross-sectional study of incident pre- and post-menopausal breast cancer patients reported a less diverse microbiome and differential abundance of *Firmicutes* in women with human epidermal growth factor receptor 2 (HER2) positive breast cancer compared to HER2 negative breast cancer.[200] Other studies also support associations of specific microbial taxa (e.g., *Bacteroidetes*, *Blautia spp*.) with breast cancer staging and clinical characteristics, including body size.[201,202]

Much less information is available from epidemiologic studies on the possible association between the gut microbiome and breast density. A study of healthy menopausal women in the United States found no association between mammographic breast density and gut microbial beta diversity and *Firmicutes* to *Bacteroidetes* (F/B) ratio, and suggestive differences in alpha diversity.[203] These results are comparable to those from a study of cancer-free postmenopausal women, which found that alpha diversity and relative abundance did not differ in women with high versus low mammographic density.[194] To our knowledge, our study is the first that examines the association between the gut microbiome and breast density in adolescents. Though our study differs in several ways from the prior studies of the breast density and gut microbiome relation, notably in study population and breast density assessment method, we found similar null associations for alpha diversity and relative abundance with respect to breast density alone. Null findings specifically in studies of the gut microbiome-breast density association might reflect the complex interaction between body composition (e.g., body fatness), the gut microbiome, and breast density. Obesity is strongly inversely associated with breast density and with composition of the gut microbiome.[129,181,204] It is plausible that an association is mediated by body composition, such that any affect is cancelled out when controlling for body fat percentage.[205] However, we did not note any breast density and microbial composition associations when stratified by category of body fatness. It is also possible that studies of the microbiome-breast cancer association are reflective of alterations to the composition of the gut following the disease state, rather than the hypothesized effects of the gut microbiome in contributing to breast cancer pathogenesis through early alteration of estrogen metabolism.

Limitations and Strengths

Our study has several limitations. The cross-sectional design does not allow for a temporal specification of the gut microbiome – breast density association, so we cannot rule out reverse causation. However, the human gut microbiome is thought to be relatively stable after infancy

and early childhood.[30] We cannot fully preclude that other perturbations of the microbiome, such as major dietary changes, occurred in the time just prior to sample collection. However, we collected information on antibiotic usage and clinical diagnoses of disease in the six months prior to stool collection, and less than 2% of the sample reported any probiotic consumption. We did not have comprehensive information on nutritional supplements; however, use of vitamins or nutritional supplements is uncommon in Chile, particularly among girls. We had limited information on physical activity, which is associated with gut microbial composition and many anthropometric characteristics (e.g., body fatness) associated with breast density. We also lack direct information on functional microbiome data which may show relations with breast density that were not apparent when examining composition alone. However, we were able to approximate functional potential of the community using PICRUSt2.

Our study also has several strengths, including a large sample of geographically, socioeconomically, and behaviorally similar girls. We were also able to comprehensively assess the gut microbial community using fecal 16s rRNA gene sequencing. The GOCS study has collected longitudinal data on lifestyle, socioeconomic, and anthropometric factors, allowing for specific control of potential confounders. MaAsLin2, a novel general linear modeling approach, allows for preserved statistical power with multiple covariates and control for false-discovery rate.

4.6. Conclusions

In conclusion, we found no important association between the gut microbiome and breast density at 2 years post-menarche in female adolescents.

Table 4. 1. Population characteristics of 218 girls participating in the Growth and Obesity Cohort Study

Characteristic	**Distribution**
Percent FGV (%), mean (SD)	49.5 (14.5)
Tercile 1, median (range)	34.0 (19.1, 41.4)
Tercile 2, median (range)	48.8 (41.5, 56.3)
Tercile 3, median (range)	65.3 (56.6, 98.0)
Absolute FGV (cm3), mean (SD)	217.0 (78.1)
Tercile 1, median (range)	140.2 (74.2, 178.0)
Tercile 2, median (range)	210.8 (178.1, 249.2)
Tercile 3, median (range)	292.7 (250.1, 546.4)
Total breast volume (cm^3), mean (SD)	472.0 (236.4)
Age (years), mean (SD)	14.0 (0.9)
Age at menarche (years), mean (SD)	12.0 (0.8)
BMI Z-score, mean (SD)	1.0 (1.0)
Body fat percentage (%), mean (SD)	32.6 (5.8)
Energy intake per day (kCal), mean (SD)	1709.4 (376.5
Ethnicity, n (%)	
Non-Mapuche	181 (83.0)
Mapuche	37 (17.0)
Birth mode, n (%)	
Cesarean	54 (24.8)
Vaginal	164 (75.2)
Antibiotic use in prior 6 months, n (%)	
No	198 (90.8)
Yes	20 (9.2)
Maternal education, n (%)	
Secondary education or less	171 (78.4)
Post-secondary education	47 (21.6)
Hours of TV per day, n (%)	
≤1 hour	77 (35.3)
1-3 hours	109 (50.0)
>3 hours	32 (14.7)

Figure 4. 1. Microbial relative abundance at the phylum level stratified by (**A)** %FGV tercile and **(B)** aFGV tercile in fecal microbiota samples from 218 GOCS participants

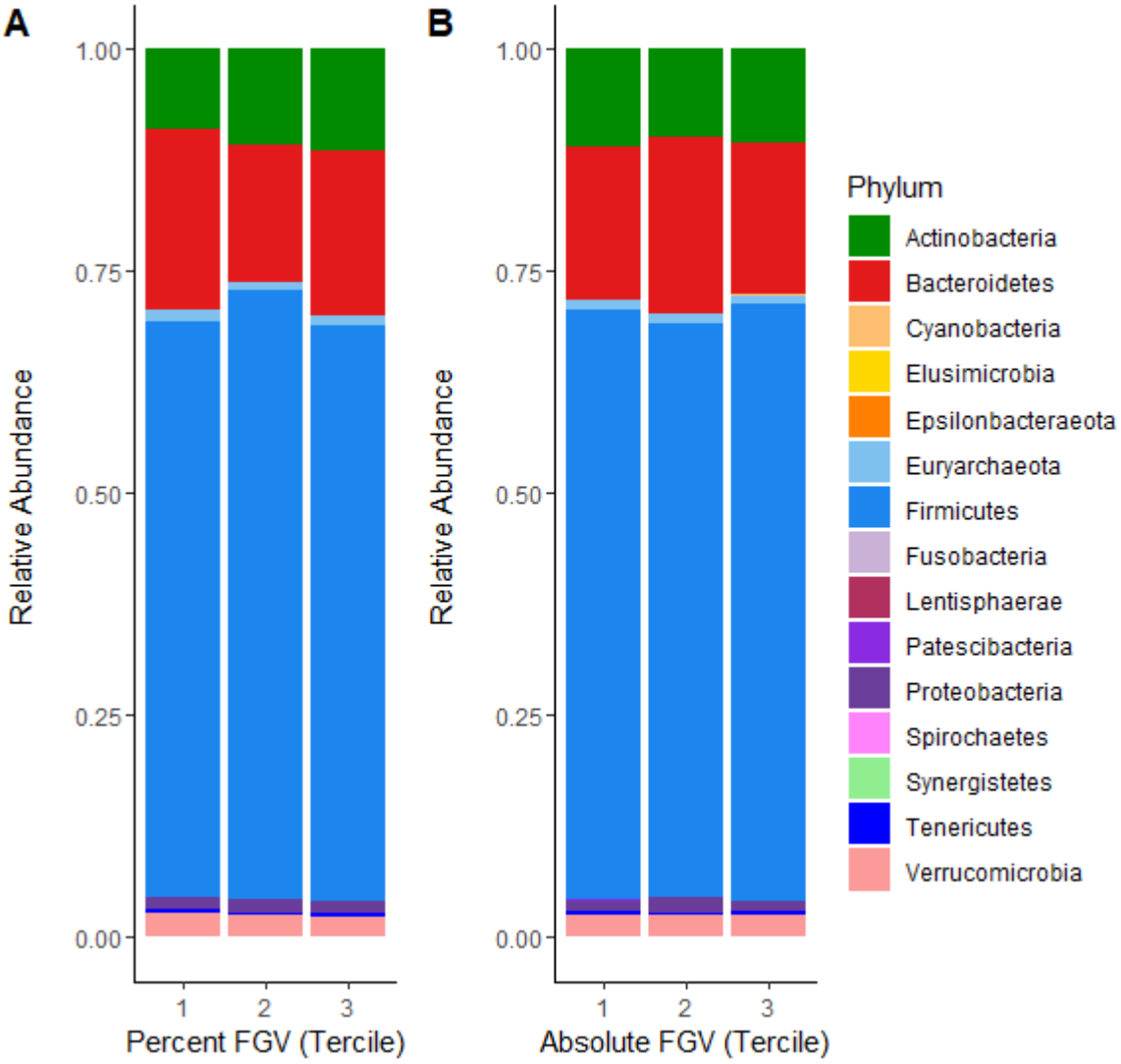


Figure 4. 2. Box plots of alpha diversity metrics for observed richness and Shannon index across terciles of %FGV (**A, B)** and aFGV **(C, D)**.

P-values are presented for overall differences in alpha diversity metrics (Kruskal-Wallis) and post-hoc pairwise differences. Boxes represent the lower, median, and upper quartile of the data and whiskers are 1.5*interquartile range.

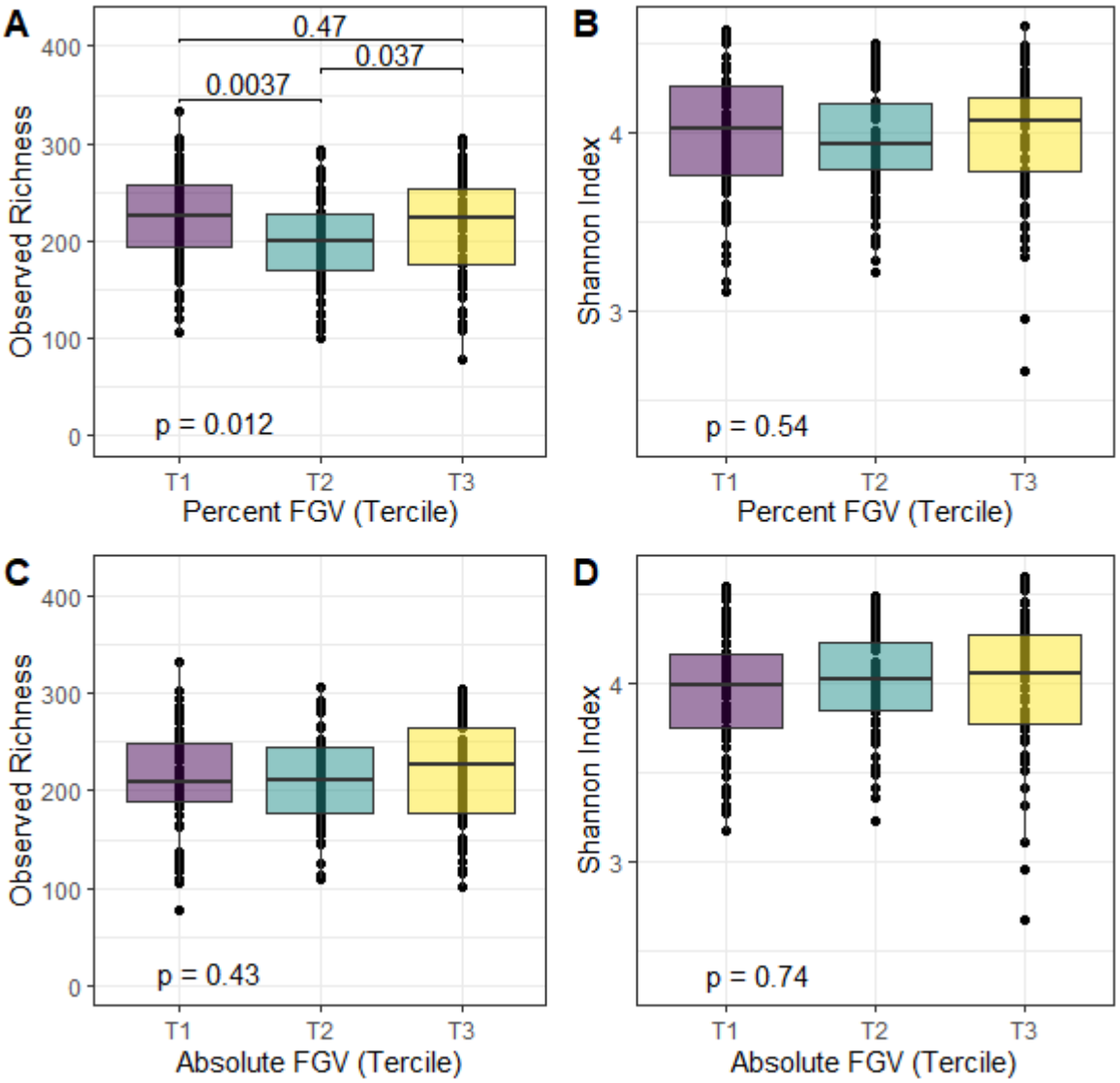


Figure 4. 3. Principal coordinate analysis (PCoA) plot of microbial composition derived from Bray-Curtis dissimilarity.

N= 218 girls in GOCS colored by %FGV (A) and aFGV (B) terciles. Ellipses are 95% confidence regions for each tercile.

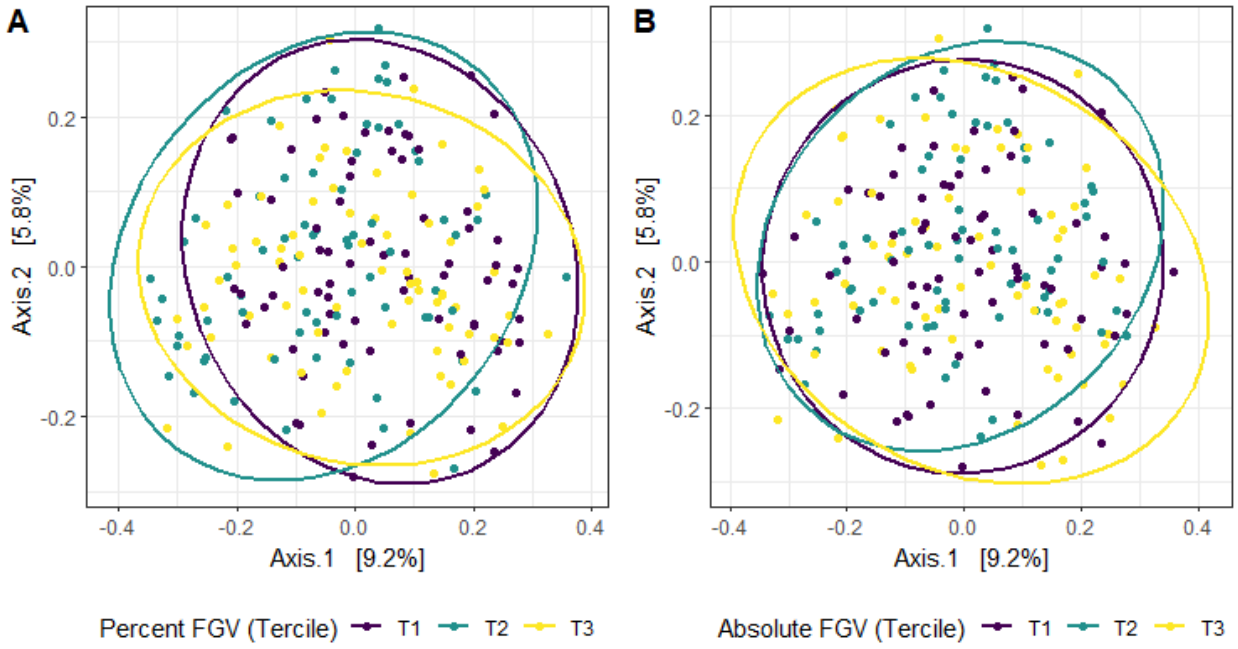


Table 4. 2. Multivariable PERMANOVA analyses to identify variation (R^2) in microbial beta diversity (Bray-Curtis dissimilarity) explained by study characteristics.

	Percent FGV		Absolute FGV	
Characteristic	***R²***	**p-value**	***R²***	**p-value**
FGV	0.013	0.01	0.008	0.73
Body fat percentage	0.009	0.35	0.011	0.10
Age	0.005	0.18	0.005	0.21
Antibiotic use	0.006	0.08	0.006	0.08
Birth mode	0.004	0.79	0.004	0.83
Breast feeding	0.009	0.46	0.009	0.43
Daily caloric intake	0.013	0.61	0.013	0.58
Maternal education	0.005	0.30	0.005	0.29
Ethnicity	0.007	0.03	0.006	0.06
TV hours	0.003	1.00	0.003	1.00
Residuals	0.926		0.929	

Table 4. 3. Principal coordinate analysis (PCoA) plot of predicted MetaCyc pathway abundance derived from Bray-Curtis dissimilarity among fecal samples provided by 218 girls in GOCS colored by %FGV (A) and aFGV (B) terciles. Ellipses are 95% confidence regions for each tercile.

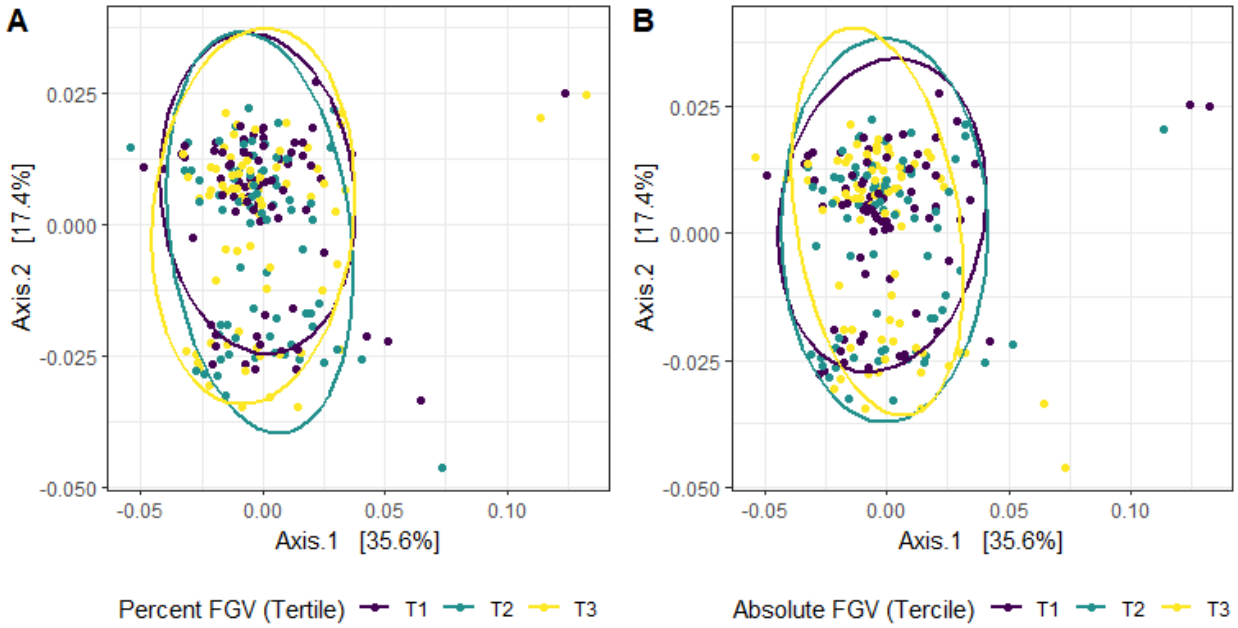


Table 4. 4. Multivariable PERMANOVA analyses to identify variation (R2) MetaCyc pathway beta diversity (Bray-Curtis dissimilarity) explained by study characteristics

	Percent FGV		Absolute FGV	
Characteristic	R^2	**p-value**	R^2	**p-value**
FGV	0.008	0.56	0.007	0.66
Body fat percentage	0.009	0.40	0.009	0.48
Age	0.004	0.55	0.004	0.57
Antibiotic use	0.006	0.20	0.006	0.20
Birth mode	0.003	0.64	0.004	0.46
Breast feeding	0.011	0.28	0.010	0.32
Daily caloric intake	0.016	0.26	0.016	0.25
Maternal education	0.007	0.19	0.007	0.15
Ethnicity	0.004	0.44	0.004	0.49
TV hours	0.002	0.85	0.003	0.77
Residuals	0.929		0.930	

Chapter 5. Conclusions and Public Health Relevance

The peripubertal period represents an important life stage during which environmental chemicals may influence the risk of breast cancer in later life. The role of the gut microbiome in this process remains unclear. As observed in this dissertation, endocrine disrupting chemicals (EDCs) measured at different time points throughout puberty can have varying impacts on the breast density in adolescence. Select EDCs measured at Tanner breast stage B4 (B4), including mono(2-ethyl-5-carboxypentyl) phthalate and di(2-ethylhexyl) phthalate metabolites were positively associated with absolute breast fibroglandular volume; others measured at 1-year post-menarche, including triclosan, were inversely associated percent breast fibroglandular volume. Other phthalates measured at 1-year post-menarche, including monobenzyl phthalate, were inversely associated with diversity of the gut microbiome. However, no association between the gut microbiome and breast density in adolescence was found.

By using the framework of lifecourse epidemiology, this dissertation contributes to a growing body of evidence evaluating the influence of environmental factors during puberty and addresses several methodological challenges in studying the relation to breast density. Significantly, chapter 2 found high variability in EDC biomarker concentrations across stages of puberty and identified potential windows of susceptibility during puberty for breast density. The use of longitudinal exposure data from a prospective cohort with high retention allowed for an innovative and unconventional application of generalized estimating equations. Additionally, this dissertation presents several exploratory hypotheses and methodologies related to a novel and potentially significant mechanism through which EDCs might act on breast density- the gut microbiome. Chapter 3 is among the first human studies to evaluate the relation of phenol, phthalate, and paraben EDCs to the gut microbiome; it is also the first to consider how a mixture of EDCs, rather than single chemical, might influence the gut microbiome. Chapter 4 is one of

three studies to examine the association between breast density and the gut microbiome, and the first to do so among adolescents.

Taken together, these studies point to the importance of the pubertal window of susceptibility to environmental factors in influencing breast cancer risk. This research contributes to an understanding of the variation in breast cancer risk associated with environmental exposures in childhood to develop early-life recommendations for breast cancer prevention. Future studies may build on the hypotheses generated and methodologies used in this dissertation to further investigate environmental influences on breast cancer risk.

Appendix 1. Supplemental content for Chapter 2

Table A1. 1. Comparison of characteristics for girls in the Growth and Obesity Cohort Study with breast assessments (n=366) and without breast assessments (n=159)

Characteristic	**Included in the analysis* (n=366)**			**Excluded from the analysis (n=159)**		
	Study Time Point			**Study Time Point**		
	Tanner Stage B1	Tanner Stage B4	1-Year Post-Menarche	Tanner Stage B1	Tanner Stage B4	1-Year Post-Menarche
	(n=293)	(n=333)	(n=232)	(n=122)	(n=115)	(n=34)
Urine sample provided						
B1	293 (100.0)	261 (78.4)	197 (84.9)	122 (100.0)	79 (68.7)	28 (82.4)
B4	261 (89.1)	333 (100.0)	219 (94.4)	79 (64.8)	115 (100.0)	28 (82.4)
1Y PM	197 (67.2)	219 (65.8)	232 (100.0)	28 (23.0)	28 (24.3)	34 (100.0)
Age, years (mean (SD))	7.87 (0.45)	11.38 (0.88)	13.40 (0.82)	7.92 (0.50)	11.24 (0.98)	14.01 (1.18)
Age at menarche, years (mean (SD))	12.09 (0.91)	12.04 (0.89)	12.42 (0.76)	12.14 (1.08)	11.86 (1.09)	12.88 (1.09)
BMI Z-score (mean (SD))	0.85 (1.10)	0.88 (1.10)	0.86 (1.11)	0.86 (1.17)	0.96 (1.07)	0.67 (1.08)
Body fat percentage (mean (SD))	25.58 (4.41)	26.93 (5.11)	30.62 (5.61)	25.91 (5.02)	26.64 (5.40)	29.75 (5.85)
Body fat percentage (count (%))						
Underfat/Normal	155 (54.4)	198 (62.3)	55 (40.1)	69 (57.5)	74 (67.3)	11 (47.8)
Overfat	82 (28.8)	68 (21.4)	39 (28.5)	28 (23.3)	20 (18.2)	2 (8.7)
Obese	48 (16.8)	52 (16.4)	43 (31.4)	23 (19.2)	16 (14.5)	10 (43.5)
Maternal education (%)						
Secondary education or less	236 (80.5)	271 (81.4)	192 (82.8)	96 (78.7)	85 (73.9)	22 (64.7)
Greater than secondary education	57 (19.5)	62 (18.6)	40 (17.2)	26 (21.3)	30 (26.1)	12 (35.3)
Duration of breast feeding (count (%))						
< 3 months	90 (30.7)	107 (32.1)	74 (31.9)	35 (28.7)	31 (27.0)	13 (38.2)
3-6 months	171 (58.4)	188 (56.5)	134 (57.8)	77 (63.1)	69 (60.0)	17 (50.0)
> 6 months	32 (10.9)	38 (11.4)	24 (10.3)	10 (8.2)	15 (13.0)	4 (11.8)
Birth mode (%)						
Caesarean	85 (29.0)	91 (27.3)	73 (31.5)	50 (41.0)	32 (27.8)	7 (20.6)
Vaginal	208 (71.0)	242 (72.7)	159 (68.5)	72 (59.0)	83 (72.2)	27 (79.4)
Average caloric intake, kCal (mean (SD))	1878.81 (490.73)	1873.00 (457.64)	1745.07 (446.37)	1906.21 (544.87)	1871.09 (465.24)	1759.18 (530.93)

* Only girls with breast assessments at 2-years post-menarche were included in the analysis

Figure A1. 1. Distribution of BPA concentration (log10-transformed and creatinine-adjusted) at B1, B4, or 1-year post-menarche for girls with breast composition assessments and those without breast composition assessments.

Only girls with breast composition assessments were included in the analysis. P-values were produced from non-parametric t-test for two-sample comparison of means.

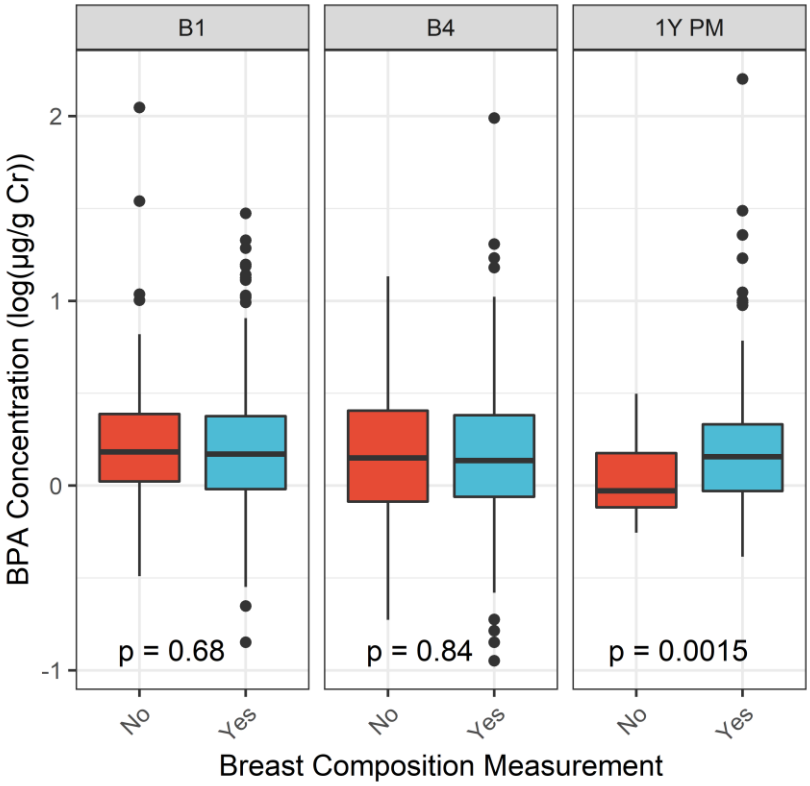


Figure A1. 2. Plot of Spearman correlation for EDC biomarker concentrations Tanner Stage B1 among 293 girls participating in the Growth and Obesity Cohort Study in Santiago, Chile.

Acronyms: Bbenzophenone-3 (bp3), bisphenol-A (bpa), bisphenol S (bps), triclosan (tcs), ethylparaben (etpb), methylparaben (mepb), propylparaben (prpb), mono(2-ethyl-5-carboxypentyl phthalate (mecpp), mono(2-ethyl-5-hydroxyhexyl) phthalate (mehhp), mono(2-ethylhexyl) phthalate (mehp), mono(2-ethyl-5-oxohexyl) phthalate (meohp), monoethyl phthalate (mep), mono-isobutyl phthalate (mibp), mono-n-butyl phthalate (mbp), mono-3-carboxypropyl phthalate (mcpp), oxidative di(2-ethylhexyl) phthalates (dehp), high-molecular weight phthalates (hiphth), low-molecular weight phthalates (lophth), parabens (parbf), phenols (phenf).

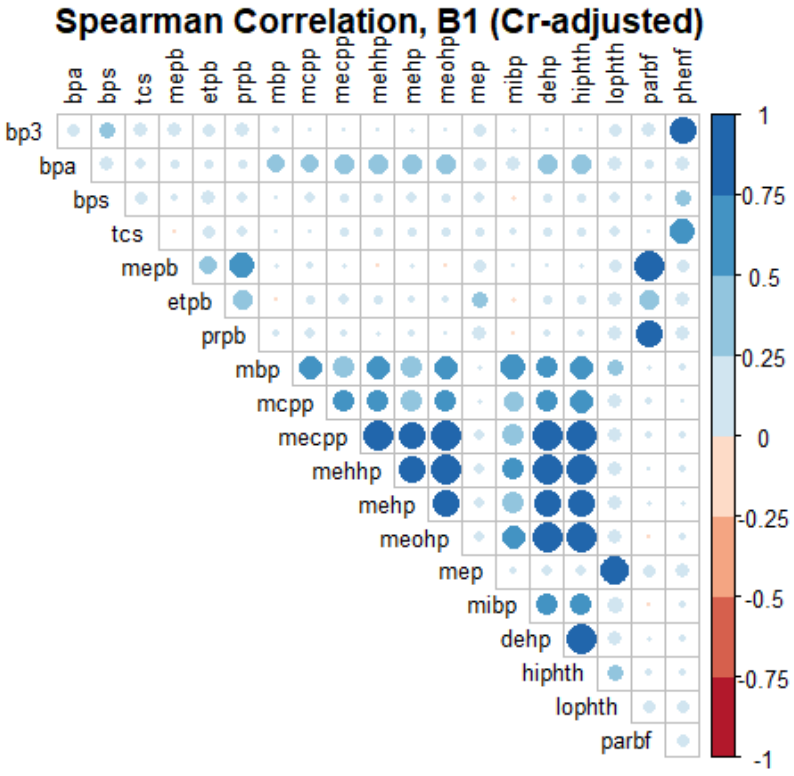


Figure A1. 3. Plot of Spearman correlation for EDC biomarker concentrations Tanner Stage B4 among 333 girls participating in the Growth and Obesity Cohort Study in Santiago, Chile.

Acronyms: Bbenzophenone-3 (bp3), bisphenol-A (bpa), bisphenol S (bps), triclosan (tcs), ethylparaben (etpb), methylparaben (mepb), propylparaben (prpb), mono(2-ethyl-5-carboxypentyl phthalate (mecpp), mono(2-ethyl-5-hydroxyhexyl) phthalate (mehhp), mono(2-ethylhexyl) phthalate (mehp), mono(2-ethyl-5-oxohexyl) phthalate (meohp), monoethyl phthalate (mep), mono-isobutyl phthalate (mibp), mono-n-butyl phthalate (mbp), mono-3-carboxypropyl phthalate (mcpp), oxidative di(2-ethylhexyl) phthalates (dehp), high-molecular weight phthalates (hiphth), low-molecular weight phthalates (lophth), parabens (parbf), phenols (phenf)

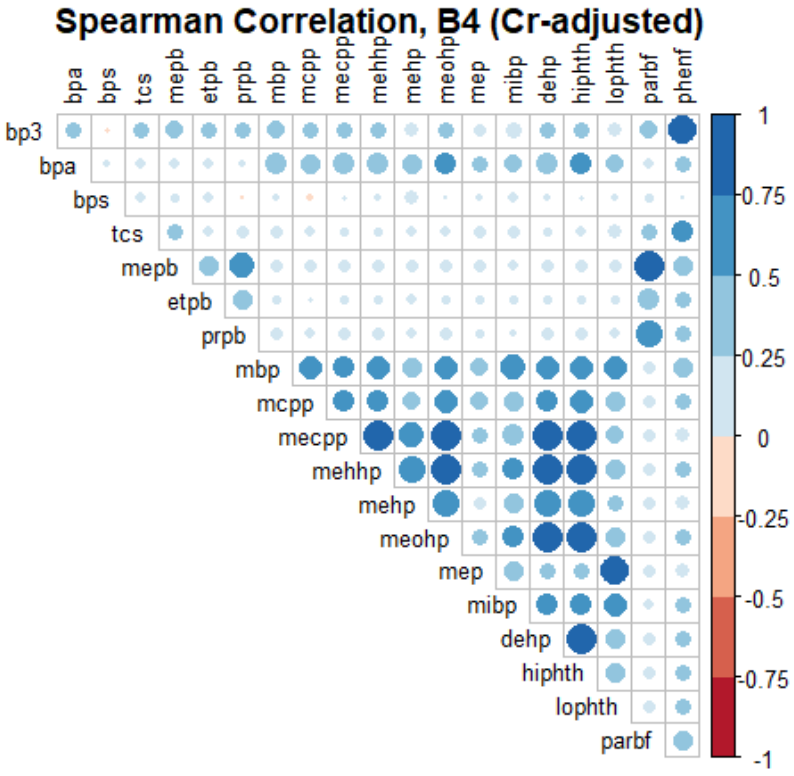
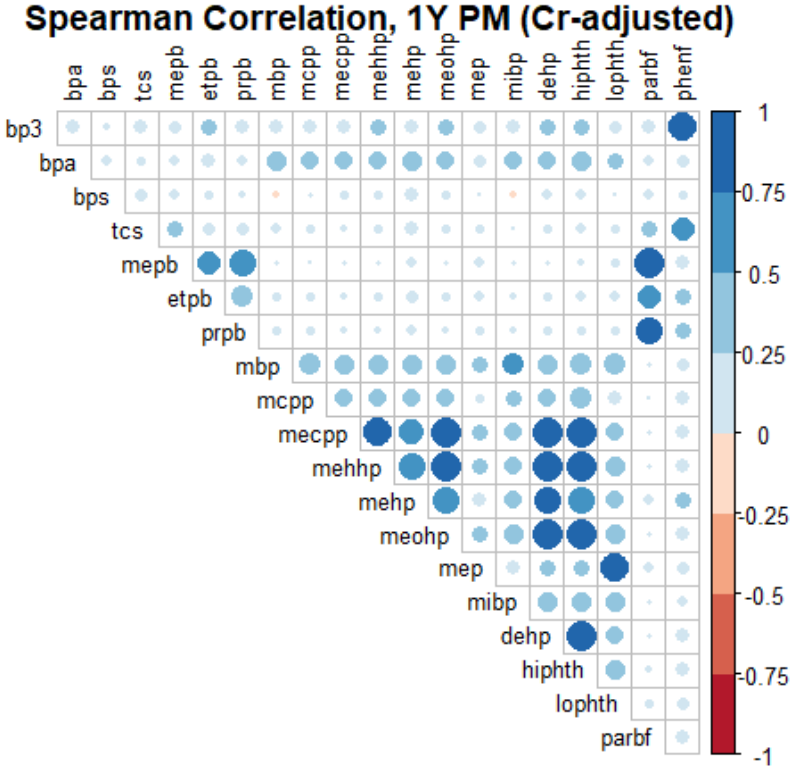


Figure A1. 4. Plot of Spearman correlation for creatinine-adjusted EDC biomarker concentrations 1-year post-menarche among 232 girls participating in the Growth and Obesity Cohort Study in Santiago, Chile.

Acronyms: Bbenzophenone-3 (bp3), bisphenol-A (bpa), bisphenol S (bps), triclosan (tcs), ethylparaben (etpb), methylparaben (mepb), propylparaben (prpb), mono(2-ethyl-5-carboxypentyl phthalate (mecpp), mono(2-ethyl-5-hydroxyhexyl) phthalate (mehhp), mono(2-ethylhexyl) phthalate (mehp), mono(2-ethyl-5-oxohexyl) phthalate (meohp), monoethyl phthalate (mep), mono-isobutyl phthalate (mibp), mono-n-butyl phthalate (mbp), mono-3-carboxypropyl phthalate (mcpp), oxidative di(2-ethylhexyl) phthalates (dehp), high-molecular weight phthalates (hiphth), low-molecular weight phthalates (lophth), parabens (parbf), phenols (phenf)



Appendix 2. Supplemental content for Chapter 3

Table A2. 1. List of Urinary EDC biomarkers and the lab-specific limit of detection

Biomarker		Source Lab	% Detect	LOD	ICC	% Invalid Replicates with both samples <LOD
Benzophenone-3	BP3	Mount Sinai	100	1	0.87	NA
		CDC	100	0.4		
Bisphenol-A	BPA	Mount Sinai	95	0.1	0.74	50
		CDC	97.5	0.2		
Bisphenol-F	BPF*	Mount Sinai	55	0.25	0.18	39
		CDC	30	0.2		
Bisphenol-S	BPS	Mount Sinai	35	0.25	0.95	37
		CDC	62.5	0.1		
Butyl paraben	BUPB*	Mount Sinai	57.5	0.1	0.81	62
		CDC	57.5	0.1		
Ethyl paraben	ETPB	Mount Sinai	85	0.2	0.82	21
		CDC	42.5	1		
Methyl paraben	MEPB	Mount Sinai	100	0.25	0.84	NA
		CDC	100	1		
Propyl paraben	PRPB	Mount Sinai	90	0.1	0.87	0
		CDC	100	0.1		
Triclorocarban	TCC*	Mount Sinai	15	0.1	0.83	53
		CDC	47.5	0.1		
Triclosan	TCS	Mount Sinai	97.5	1	0.94	0
		CDC	90	1.7		
Mono-benzyl phthalate	MBZP	Mount Sinai	97.5	0.1	0.93	0
		CDC	95	0.3		
Mono-(3-carboxypropyl) phthalate	MCPP	Mount Sinai	100	0.05	0.9	0
		CDC	97.5	0.2		
Mono- (2-ethyl-5-carboxypentyl) phthalate	MECPP	Mount Sinai	100	0.1	0.91	NA
		CDC	100	0.2		
Mono(2-ethyl-5-hydroxyhexyl)	MEHHP	Mount Sinai	100	0.1	0.9	NA
		CDC	100	0.2		
Mono-2-ethylhexyl phthalate	MEHP	Mount Sinai	97.5	0.3	0.85	50
		CDC	95	0.5		
Mono-(2-ethyl-5-oxohexyl) phthalate	MEOHP	Mount Sinai	100	0.1	0.83	NA
		CDC	100	0.2		
Mono-ethyl phthalate	MEP	Mount Sinai	100	0.1	0.94	NA
		CDC	100	0.6		
Mono-isobutyl phthalate	MIBP	Mount Sinai	100	0.1	0.91	0
		CDC	100	0.2		
Mono-n-butyl phthalate	MNBP	Mount Sinai	100	0.1	0.92	0
		CDC	97.5	0.4		

* BPF and TCC were excluded from the analysis based ICC <0.75 from overlapping CDC/Mount Sinai samples (n=40). BUPB was excluded from single-chemical analyses at B4 due to % detected (>50% invalid).

Figure A2. 1. Boxplot of log10-transformed phenol [A], paraben [A], and phthalate [B] concentrations by study time point.

Abbreviations: B1= Tanner breast stage 1, B4 = Tanner breast stage 4, 1Y PM = 1-year post-menarche

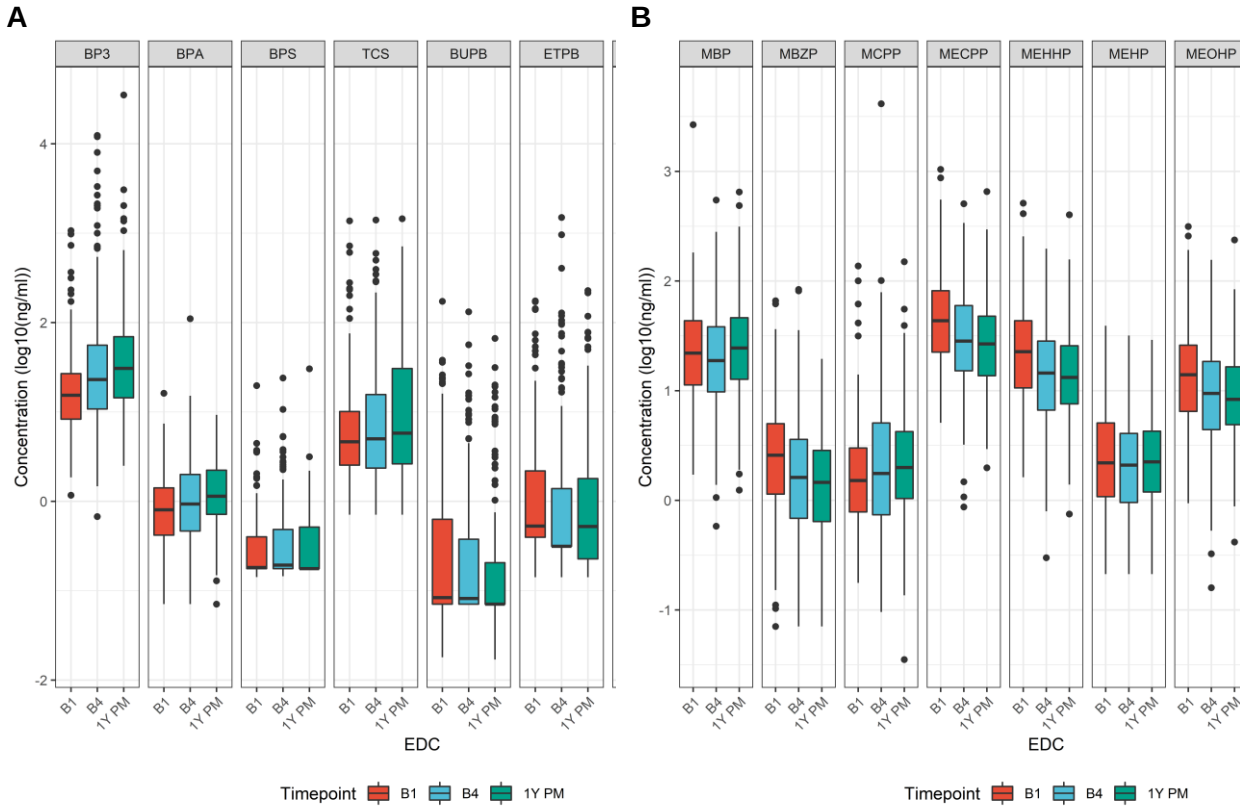


Table A2. 2. Relative abundance of microbial taxa by phylum, class, and genus in the GOCS cohort (N=257)

Taxonomic classification	Mean Relative Abundance
Phylum	
Firmicutes	65.9%
Bacteroidetes	18.1%
Actinobacteria	10.3%
Verrucomicrobia	2.6%
Proteobacteria	1.5%
Euryarchaeota	1.1%
Tenericutes	0.3%
Cyanobacteria	0.03%
Patescibacteria	0.02%
Fusobacteria	0.01%
Class	
Clostridia	57.9%
Bacteroidia	18.3%
Actinobacteria	6.9%
Erysipelotrichia	5.0%
Coriobacteriia	3.5%
Verrucomicrobiae	2.7%
Negativicutes	2.3%
Bacilli	1.5%
Gammaproteobacteria	1.3%
Mollicutes	0.3%
Genus	
Bacteroides	12.2%
Bifidobacterium	7.9%
[Eubacterium] coprostanoligenes group	6.0%
Tyzzerella 4	5.6%
Faecalibacterium	4.6%
Blautia	4.4%
Subdoligranulum	4.1%
Ruminococcaceae UCG-014	3.9%
Akkermansia	3.1%
[Eubacterium] hallii group	3.0%

Table A2. 3. Results from linear regression of single-chemical EDC concentration (log10-transformed, quartiled) on Shannon diversity by study time point, excluding girls with antibiotic use in the 6 months prior to stool sample (N = 241).

	Tanner Stage B1 (n=179)	**Tanner Stage B4 (n=209)**	**1 Year Post-Menarche (n=147)**
	β a (95%CI)	**β[a] (95%CI)**	**β[a] (95%CI)**
Log10-transformed EDC concentration			
BP3	-0.06 (-0.19, 0.05)	-0.02 (-0.1, 0.05)	0.04 (-0.05, 0.14)
BPA	0.14 (-0.03, 0.3)	0.08 (-0.05, 0.19)	-0.01 (-0.19, 0.17)
BPS	0.03 (-0.11, 0.18)	-0.03 (-0.15, 0.09)	-0.14 (-0.3, 0.01)**
BUPB	0 (-0.07, 0.07)	-0.02 (-0.09, 0.05)	0.04 (-0.04, 0.13)
ETPB	-0.01 (-0.07, 0.07)	-0.03 (-0.09, 0.04)	0.03 (-0.04, 0.11)
MBP	-0.12 (-0.27, 0.05)	-0.04 (-0.17, 0.09)	-0.17 (-0.34, -0.01)*
MBZP	-0.01 (-0.13, 0.12)	-0.06 (-0.16, 0.05)	-0.07 (-0.21, 0.06)
MCPP	-0.02 (-0.15, 0.12)	0 (-0.09, 0.08)	-0.06 (-0.18, 0.07)
MECPP	-0.01 (-0.17, 0.16)	0.06 (-0.1, 0.2)	-0.14 (-0.31, 0.03)**
MEHHP	-0.05 (-0.2, 0.13)	0.05 (-0.11, 0.19)	-0.12 (-0.29, 0.04)
MEHP	-0.01 (-0.14, 0.13)	0.09 (-0.04, 0.21)	-0.07 (-0.22, 0.09)
MEOHP	-0.07 (-0.22, 0.1)	0.05 (-0.1, 0.19)	-0.12 (-0.29, 0.04)
MEP	-0.02 (-0.13, 0.09)	-0.04 (-0.13, 0.05)	-0.09 (-0.2, 0.02)**
MEPB	0 (-0.08, 0.08)	-0.01 (-0.07, 0.06)	0.01 (-0.06, 0.08)
MIBP	-0.18 (-0.33, -0.03)*	-0.01 (-0.14, 0.14)	-0.15 (-0.34, 0.03)
PRPB	0 (-0.06, 0.06)	-0.01 (-0.06, 0.04)	0.02 (-0.04, 0.08)
TCS	0.03 (-0.06, 0.12)	0.03 (-0.05, 0.11)	-0.02 (-0.1, 0.06)
Quartiled EDC concentration			
BP3	-0.03 (-0.08, 0.02)	0 (-0.05, 0.04)	0.01 (-0.05, 0.06)
BPA	0.05 (-0.01, 0.11)**	0.03 (-0.02, 0.08)	0.02 (-0.04, 0.08)
BPS	0.01 (-0.04, 0.05)	-0.01 (-0.05, 0.03)	-0.03 (-0.1, 0.03)
BUPB	-0.02 (-0.07, 0.02)	-0.03 (-0.07, 0.02)	0.03 (-0.04, 0.1)
ETPB	-0.01 (-0.06, 0.04)	-0.04 (-0.09, 0.01)	0.01 (-0.05, 0.06)
MBP	-0.07 (-0.13, -0.01)*	-0.02 (-0.08, 0.03)	-0.05 (-0.12, 0.01)
MBZP	-0.02 (-0.08, 0.03)	-0.05 (-0.1, 0.01)**	-0.01 (-0.07, 0.04)
MCPP	-0.01 (-0.07, 0.05)	-0.01 (-0.06, 0.05)	-0.02 (-0.07, 0.04)
MECPP	-0.02 (-0.08, 0.04)	0.03 (-0.03, 0.08)	-0.06 (-0.12, 0.01)**
MEHHP	-0.02 (-0.08, 0.04)	0.02 (-0.04, 0.08)	-0.05 (-0.11, 0.01)
MEHP	-0.01 (-0.06, 0.05)	0.04 (-0.02, 0.09)	-0.03 (-0.09, 0.03)
MEOHP	-0.03 (-0.09, 0.03)	0.03 (-0.03, 0.09)	-0.05 (-0.12, 0.01)

MEP	-0.02 (-0.06, 0.03)	-0.02 (-0.07, 0.03)	-0.04 (-0.09, 0.01)
MEPB	0 (-0.05, 0.05)	0 (-0.05, 0.04)	0.03 (-0.02, 0.08)
MIBP	-0.06 (-0.12, 0)*	-0.02 (-0.07, 0.03)	-0.04 (-0.11, 0.03)
PRPB	0 (-0.05, 0.04)	-0.01 (-0.05, 0.04)	0 (-0.05, 0.06)
TCS	0 (-0.05, 0.05)	0.01 (-0.04, 0.06)	-0.01 (-0.06, 0.04)

a: Adjusted for age at clinic visit, maternal education (secondary education or less, more than secondary education), body fat percentage, (vaginal, caesarean), duration of predominant breast-feeding (<3 months, 3-6 months, >6 months), antibiotic use in the 6 months prior to stool sample (no, unknown), average caloric intake (g/day), and lab (CDC, Mount Sinai)

b: Adjusted for the same set of covariates but excluding lab

*: P-value <=0.05

**: P-value <=0.10

Table A2. 4. Results from linear regression of single-chemical EDC concentration (log10-transformed, quartiled) on Shannon diversity by study time point (N = 257) with Benjamini-Hochberg FDR adjusted values

	Tanner Stage B1 (n=197)			Tanner Stage B4 (n=233)			1 Year Post-Menarche (n=167)		
	Estimate[a]	P-value	q-value	Estimate[a]	P-value	q-value	Estimate[a]	P-value	q-value
Log10-transformed EDC concentration									
BP3	-0.05	0.34	0.98	-0.04	0.31	0.77	0.02	0.72	0.77
BPA	0.17	0.02	0.23[b]	0.07	0.24	0.77	-0.03	0.73	0.77
BPS	0.04	0.54	0.98	-0.03	0.6	0.77	-0.14	0.07	0.23[b]
BUPB	0	0.92	0.98	-0.02	0.6	0.77	0.02	0.71	0.77
ETPB	0	0.94	0.98	-0.02	0.51	0.77	0.02	0.61	0.77
MBP	-0.11	0.15	0.93	-0.03	0.57	0.77	-0.19	0.02	0.23[b]
MBZP	-0.01	0.87	0.98	-0.04	0.46	0.77	-0.1	0.11	0.25
MCPP	-0.01	0.9	0.98	-0.01	0.87	0.97	-0.08	0.17	0.3
MECPP	-0.01	0.92	0.98	0.05	0.5	0.77	-0.15	0.06	0.23
MEHHP	-0.04	0.63	0.98	0.04	0.53	0.77	-0.14	0.09	0.23
MEHP	0	0.96	0.98	0.07	0.25	0.77	-0.09	0.21	0.34
MEOHP	-0.07	0.38	0.98	0.04	0.51	0.77	-0.14	0.09	0.23[b]
MEP	-0.03	0.56	0.98	-0.04	0.34	0.77	-0.09	0.08	0.23[b]
MEPB	0.01	0.86	0.98	0	0.97	0.97	0	1	1
MIBP	-0.17	0.03	0.23[b]	0	0.96	0.97	-0.13	0.14	0.28
PRPB	0	0.98	0.98	-0.01	0.71	0.86	0.02	0.41	0.61
TCS	0.01	0.84	0.98	0.03	0.41	0.77	-0.03	0.48	0.66
Quartiled EDC concentration									
BP3	-0.03	0.26	0.92	-0.02	0.5	0.69	-0.01	0.84	0.94
BPA	0.06	0.04	0.42	0.03	0.27	0.69	0.01	0.78	0.94
BPS	0.01	0.65	0.92	-0.01	0.56	0.7	-0.04	0.25	0.49
BUPB	-0.02	0.43	0.92	-0.03	0.23	0.69	0.02	0.62	0.86
ETPB	0	0.85	0.92	-0.03	0.23	0.69	0	0.9	0.94
MBP	-0.05	0.07	0.42	-0.02	0.36	0.69	-0.07	0.04	0.2
MBZP	-0.02	0.51	0.92	-0.04	0.15	0.69	-0.02	0.38	0.62
MCPP	0	0.85	0.92	0	0.91	0.91	-0.02	0.43	0.65
MECPP	-0.02	0.55	0.92	0.02	0.43	0.69	-0.07	0.03	0.2
MEHHP	-0.01	0.82	0.92	0.02	0.48	0.69	-0.06	0.06	0.2
MEHP	-0.01	0.82	0.92	0.03	0.21	0.69	-0.04	0.11	0.33
MEOHP	-0.03	0.37	0.92	0.02	0.44	0.69	-0.07	0.04	0.2
MEP	-0.02	0.39	0.92	-0.03	0.25	0.69	-0.03	0.19	0.42
MEPB	0	0.87	0.92	-0.01	0.8	0.87	0.02	0.33	0.6
MIBP	-0.05	0.07	0.42	-0.02	0.48	0.69	-0.05	0.14	0.37
PRPB	0	0.99	0.99	0	0.82	0.87	0	0.94	0.94

TCS	-0.01	0.71	0.92	0.01	0.58	0.7	0	0.87	0.94

a: Models for time points B1 and B4 are adjusted for age at clinic visit, maternal education (secondary education or less, more than secondary education), body fat percentage, (vaginal, caesarean), duration of predominant breast-feeding (<3 months, 3-6 months, >6 months), antibiotic use in the 6 months prior to stool sample (yes, no, unknown), average caloric intake (g/day), and lab (CDC, Mount Sinai). Models for time point 1-year post-menarche did not include lab as a covariate.

b: FDR <0.25

Appendix 3. Supplemental content for Chapter 4

Figure A3. 1. Relative abundance of bacterial phyla across %FGV terciles.

Global and pairwise p-values are presented from Kruskal-Wallis and t-tests, respectively. Ns = non-significant at an alpha level of 0.05; **: p<= 0.05; *: p<= 0.10

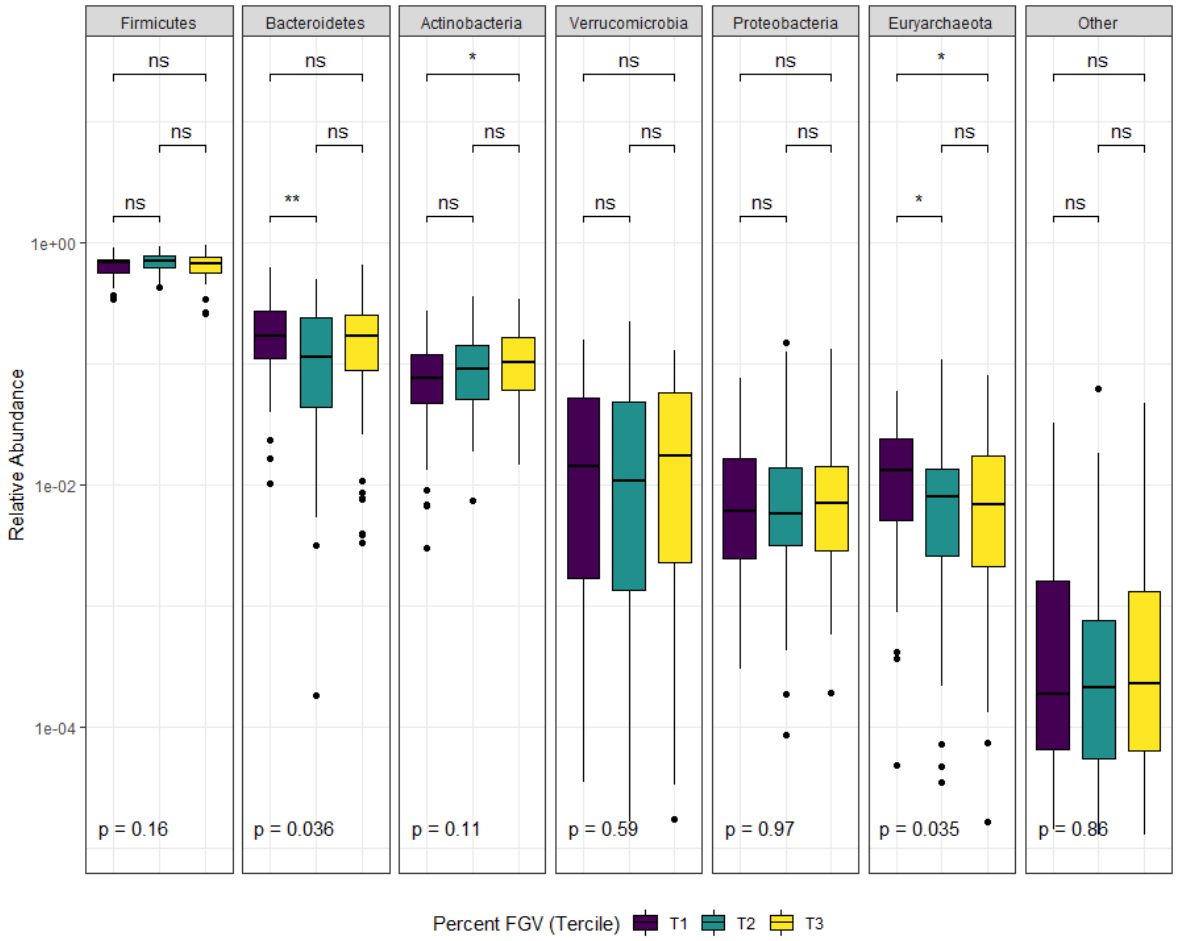
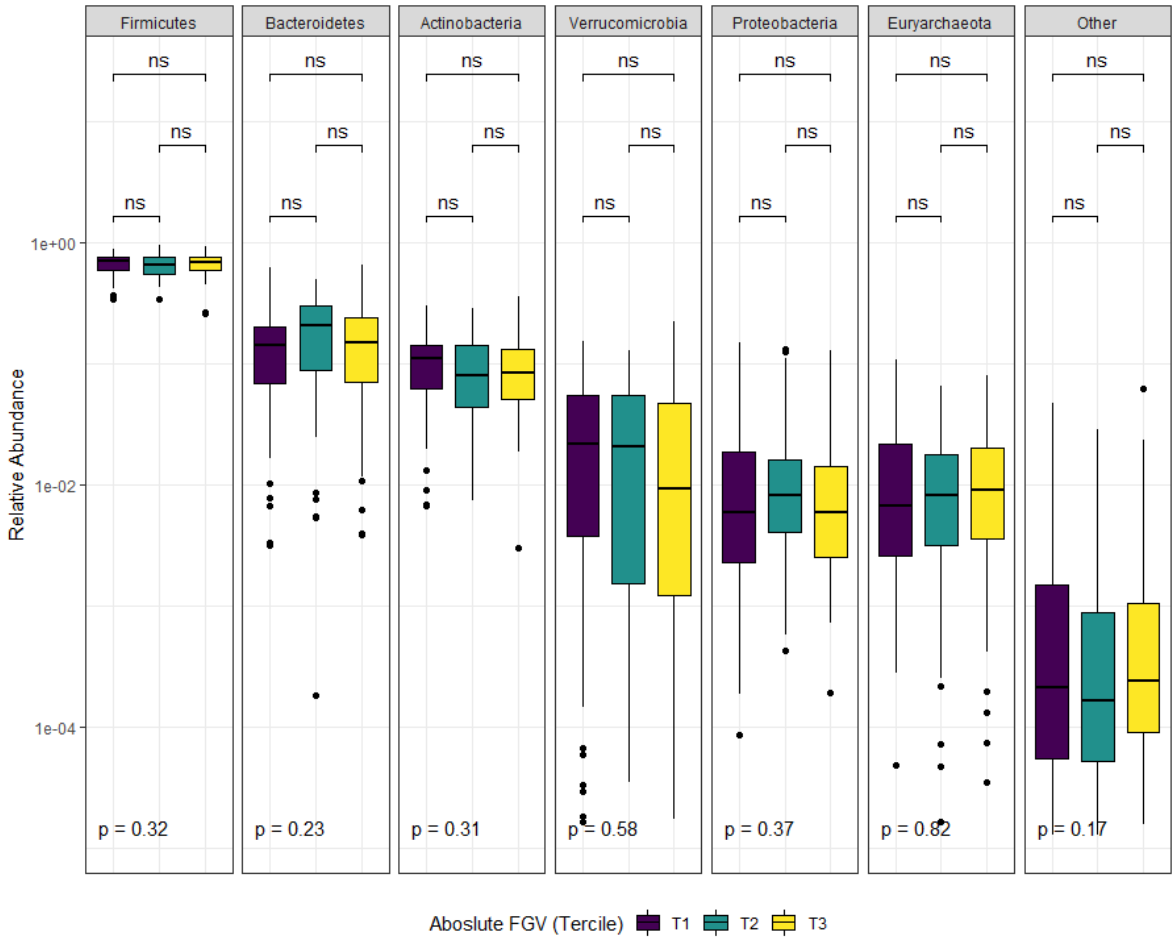


Figure A3. 2. Relative abundance of bacterial phyla across aFGV terciles.

Global and pairwise p-values are presented from Kruskal-Wallis and t-tests, respectively. Ns = non-significant at an alpha level of 0.05; **: p<= 0.05; *: p<= 0.10



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