Title
Use of Omics Technologies to Address the Links between Environmental Exposures, Epigenetics, and Risk of Pediatric Obesity in a Mexican-American Population

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Use of Omics Technologies to Address the Links between Environmental Exposures, Epigenetics, and Risk of Pediatric Obesity in a Mexican-American Population

By

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Abstract

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The alteration of gene expression mediated by epigenetic modifications has been proposed as a mechanism by which chemical and biological factors during gestation and childhood may influence health and adult disease onset. Changes in DNA methylation, the most commonly assessed epigenetic mechanism, have been linked to numerous exposures including diet, metals, and chemicals, such as phthalates. The majority of individuals, including pregnant women and children, have detectable levels of metabolites of phthalates, which are used in consumer products to increase flexibility of plastics and as solvents. Phthalate exposure during early life has been associated with poor birth and developmental health outcomes, which may be mediated by epigenetics. Increasing evidence in human and animal models has shown an association between exposure to phthalates and DNA methylation levels. However, there is limited research on the relationship between pregnancy phthalate exposure and imprinted gene methylation. Imprinted genes exhibit expression of one parental allele and many are involved in early growth and development.

Progress in the field of metabolomics has allowed for the assessment of thousands of metabolites in biological specimens. Metabolomic levels in humans have been associated with age and obesity; however, research of the effect of metabolites, especially the diverse classes of lipid metabolites, on DNA methylation is sparse. Animal studies have identified associations between maternal fatty acid dietary supplementation and offspring DNA methylation. The relationship between maternal lipid metabolites and DNA methylation of newborns has only been examined thus far in 40 mother-child dyads from a predominantly Caucasian study population.

Environmental exposures, in addition to their role in epigenetic regulation, have been shown to impact human health, such as obesity status. Despite advances in the identification of additional risk factors of obesity, the influence of maternal psychosocial factors on child obesity status and biomarkers has been poorly examined.

The aims of this dissertation are to address key knowledge gaps of the relationships of early life exposures, epigenetics, and childhood obesity. The analysis benefitted from numerous samples and longitudinal data accumulated since 1998 by the Center for the Health Assessment of Mothers and Children of Salinas (CHAMACOS) birth cohort study of hundreds of Mexican-American children and their mothers. The well-documented environmental exposures and the high prevalence of parental and child obesity in the CHAMACOS population make this an excellent study population to assess the role of early life exposure on epigenetic mechanisms and health. Results from the research in this dissertation can inform public health prevention strategies in the general population and more targeted approaches in Hispanic subpopulations, which are projected to comprise a greater portion of the United States population in the upcoming decades.

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DEDICATIONS

This dissertation is dedicated to my parents, who taught me the importance of devoting my work to the service of others; my siblings, who have supported me through the peaks and valleys of grad school; and to my wonderful niece, who is a constant ray of sunshine and motivates me to set a strong example as a woman in science.

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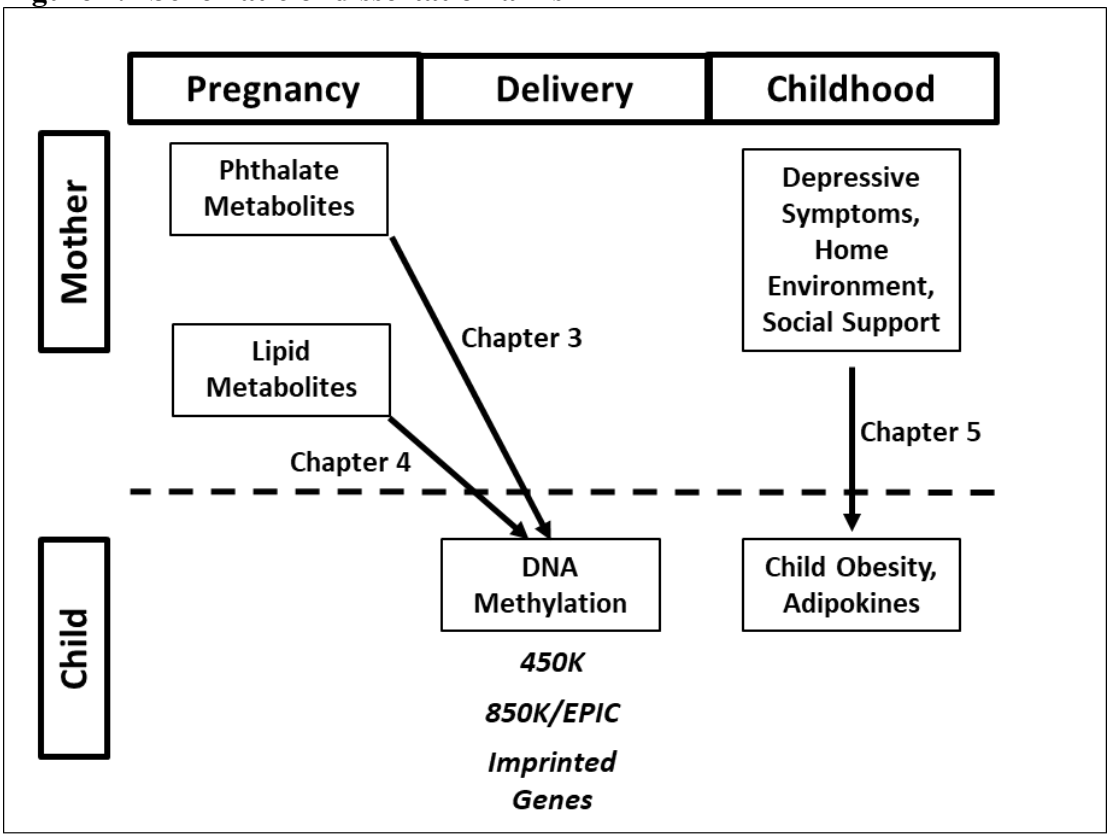
CHAPTER 1. Aims and Organization of Dissertation

Epigenetic modifications, specifically DNA methylation, have been proposed as a mechanism by which chemical, biological, and environmental factors during pregnancy and early development may influence disease etiology in adulthood. The research in this thesis examines the interplay between early life environmental exposure, newborn DNA methylation, and obesity status in the children from the CHAMACOS study. Pregnant mothers were enrolled in the study in 1998-2000, with the aim to assess the relationship between pesticide exposure and child development. Over the past 20 years, a wealth of demographic, exposure, and health data, as well as over 350,000 biological specimens, have been collected. This cohort, with a high prevalence of parental and child obesity and documented exposure to a wide array of environmental chemicals during pregnancy and early childhood, provides a unique opportunity to assess the effect of early life environmental exposures on DNA methylation, additional biomarkers, and child obesity status. The aims are as follows:

Figure 1.1 Schematic of dissertation aims



Specific Aims:

Aim 1 (Chapter 3): To determine whether DNA methylation of imprinted genes in newborns is associated with phthalate exposure during gestation. We will assess the influence of prenatal exposure to phthalates, known endocrine disruptors, on DNA methylation of imprinted genes in cord blood. We will compare significant findings across DNA methylation

platforms and validate hits using expression analysis. Hypothesis: Early life exposure to phthalates is associated with modifications of newborn DNA methylation levels in imprinted genes, which are involved in biological pathways relevant to growth.

Aim 2 (Chapter 4): To examine whether newborn genome-wide methylation levels are related to maternal lipidomic profiles during pregnancy. We will utilize data on 92 lipid metabolites measured in maternal plasma samples collected around 26 weeks gestation and determine if they are associated with methylation levels measured at over 450,000 CpG sites. Hypothesis: Maternal lipid metabolites, which are present in blood and can cross the placenta, are associated with newborn DNA methylation throughout the genome.

Aim 3 (Chapter 5): To assess whether child obesity status is influenced by protective or adverse home environment factors. We will utilize data collected throughout childhood on maternal depressive symptoms and perceived social support, as well as assessment of the home learning environment. We will examine how these factors during early life contribute to child growth at ages 5, 9, and 14. Hypothesis: Increased maternal depressive symptoms, low perceived social support, and poor home environment are associated with suboptimal profiles of adiponectin and leptin, biomarkers of obesity, as well as obesity status.

CHAPTER 2. Background and Significance

2.1 Environmental Epidemiology

The complexity of human diseases, including obesity, cannot be fully explained by genetic underpinnings, poor diet, or lifestyle factors. There is increasing interest in the role of environmental exposures in disease etiology, known as the field of environmental epidemiology[1]. An individual's environment can include external toxicants, internal exposures (i.e. hormones), maternal-fetal interactions, and psychosocial factors[1]. Epigenetic influence on gene expression has recently emerged as a critical pathway explaining the impact of environmental exposures on various health outcomes[2, 3]. Epigenetic mechanisms regulate gene expression without changes in DNA sequence and include DNA methylation, histone modifications, and non-coding RNAs[4]. Epigenetic mechanisms, which are important in nuclear organization, transcription, genome stability, and imprinting control, can be used as biomarkers of exposure or disease[1].

2.2 Epigenetics and Health

Changes to epigenetic profiles have been related to numerous health outcomes, including cardiovascular disease[5], autoimmune diseases[6, 7], mental health status[8-10], asthma[11], and cancer[12, 13]. Growing evidence in humans suggests that early life environmental exposures can impact epigenetic profiles and future disease risk[2, 14]. Specifically, the prenatal period has been identified as a particularly sensitive time for epigenetic disruption due to the two critical periods of establishment and modifications of epigenetic profiles accompanying fetal development[15]. The first period of epigenetic vulnerability occurs during the development of germline cells, which is accompanied by methylation re-programming and histone modifications[15]. The second takes place following fertilization, when the genome is demethylated and then remethylated[2]. Animal models have shown that environmental exposures can induce epigenetic changes, resulting in altered risk of diseases, such as obesity[16].

2.3 Child Obesity

Currently, development of obesity is primarily attributed to a caloric imbalance, where excess energy is stored as fat through adipogenesis, or inadequate physical activity. New evidence suggests that exposure to environmental factors, including chemical exposure, may play a role in the growing obesity epidemic[17-19]. Body mass index (BMI) is often used as an indicator of obesity. Children have obesity if their BMI is greater than the 95th percentile of the CDC sex-specific BMI-for-age growth charts[20]. Despite recognized limitations to using BMI to characterize obesity status (i.e. it does not capture abdominal fat), it is highly correlated with body fat measured by dual-energy X-ray absorptiometry, a more accurate but cost-prohibitive method in large study populations[21]. Childhood obesity been linked to asthma[22-25], hypertension[26, 27], and non-alcoholic fatty liver disease[28]. In addition, children diagnosed as obese have an increased likelihood of having obesity in adulthood, which is a risk factor for cardiovascular disease and type 2 diabetes[29-31].

2.4 DNA Methylation

DNA methylation is the epigenetic mechanism most commonly investigated and refers to the potential of a cytosine (C) base to be methylated at its 5th carbon if followed by a guanine (G) base in the DNA code, known as a CpG site. Roughly 30 million CpG sites are distributed

throughout the human genome, comprising the methylome, in regions known as CpG islands, shores, shelves, and gene bodies[32]. CpG islands refer to stretches of DNA with a high frequency of CpG dinucleotides, often located near the gene promoter, whereas shores and shelves are regions within 0-2 or 2-4 kb of CpG islands, respectively[32]. The genome also contains differentially methylated regions (DMRs), genomic regions where methylation patterns at several adjacent CpG sites differ[33]. DNA methylation patterns are reprogrammed during the prenatal period and may vary by tissue and cell type[34]. Gain of DNA methylation (hypermethylation) can result in gene silencing, while loss of methylation (hypomethylation) can lead to overexpression[35, 36].

2.5 Imprinted Genes and Epigenetic Regulation

Recent studies have established the presence of approximately 100 imprinted genes within the human genome[37]. Genomic imprinting involves expression dependent on the parental origin of the allele[38, 39]. Specifically, in imprinted genes, only the paternal or maternal allele is expressed[39]. The determination of active versus inactive allele is regulated by DNA methylation established during the process of epigenetic reprogramming that occurs in developing gametes[39]. In imprinted genes, the term DMR is commonly used to refer to genomic regions with several adjacent CpG sites whose methylation is parent-of-origin-dependent (iDMRs) [40].

2.6 Epigenetics and Environmental Exposures

The epigenome is simultaneously heritable, susceptible to environmental insult, and known to accumulate environmental effects throughout the life course[4]. A number of human studies have demonstrated the effects of environmental exposures on imprinted gene DNA methylation, including the influence of diet[41-44], cigarette smoke[45], maternal antibiotic use[46], endocrine disrupting compounds[47-49], metal exposure[50-53], and maternal stress[54, 55]. Animal studies have shown altered methylation of imprinted gene DMRs related to chemical exposures[56-60].

Limited but growing evidence indicates that exposure to phthalates, a group of endocrine disrupting chemicals (EDC), is associated with DNA methylation changes[14, 61, 62]. Phthalates are a family of chemicals often found in consumer products, resulting in common exposure in the United States[63, 64]. Phthalates are often added to plastics to increase flexibility or are used as solvents in personal care products such as perfumes and lotions[65]. Common routes of exposure to phthalates, which are not chemically bound to their substrates and leach into the environment, include ingestion, dermal absorption, and inhalation[65]. Phthalate esters undergo quick metabolism and some have half-lives less than 24 hours[66, 67]. A majority of U.S. residents, including pregnant women, now have detectable concentrations of phthalate metabolites in their urine, considered the "gold-standard" for measurement of phthalate exposure[63, 68]. It appears that exposures are both chronic and pervasive[66].

Human studies have demonstrated associations between phthalate exposure before or during pregnancy and adverse impacts on health outcomes in children, including altered birth weight[69-71], gestational age[72, 73], pre-term birth[74, 75], child growth and development[76-78], child behavior[79], respiratory health[80, 81], and thyroid function[82]. A growing body of mechanistic, animal, and human data shows a strong link between phthalates and the biological pathways that influence obesity[83].

Epigenetic effects of phthalates on both global and site specific methylation have been observed in rodent studies[62, 84, 85]. However, research on the effects of phthalate exposure on

imprinted genes in humans is limited. Only two human studies have assessed the association between phthalate exposure during pregnancy and placental imprinted gene methylation. LaRocca et al. found that first trimester urine concentrations of low molecular weight (LMW) phthalate metabolites, as well as a summary measure of 11 phthalate metabolites, were inversely associated with placental *H19* and *IGF2* DNA methylation[47]. A study by Zhao et al. found that third trimester DEHP phthalate metabolite concentrations were inversely associated with *IGF2* DNA methylation in placenta samples[49]. Another study by Goodrich et al. examined the relationship between prenatal and childhood exposure to endocrine disrupting chemicals, including phthalates, and DNA methylation of two imprinted genes (*H19*, *IGF2*) in peri-adolescents[48]. They found that third trimester phthalate metabolite levels of monobenzyl (MBzP) and mono-isobutyl (MiBP) phthalates in maternal urine samples were associated with *H19* hypermethylation in blood from 247 8-14 year old children, but this finding was attenuated to non-significance after adjustment for multiple testing. Currently, there is no data available on the relationship between cord blood imprinted gene methylation profiles and prenatal exposure to phthalates.

2.7 Epigenetics and Obesity

Maternal obesity is a significant predictor of obesity in children[86-88]. The strong relationship between maternal and child obesity suggests a role for genetics and/or shared environmental factors. Changes in DNA methylation can affect the expression of genes involved in adipogenesis and metabolism, and this epigenetic dysregulation can be a critical pathway for obesity development in children[89, 90]. Several studies have observed site-specific differences in DNA methylation of obese vs non-obese subjects; however, they have been limited by small sample sizes[91-94]. Imprinted genes, which are regulated by DNA methylation, are valuable targets for study with respect to the early origins of obesity because they function as critical mediators of fetal growth[95, 96]. Researchers at Duke University have shown that paternal obesity in the NEST birth cohort is associated with hypomethylation at four iDMRs in newborns, including the insulin-like growth factor 2 (*IGF2*) iDMR, and maternal obesity was linked with changes in methylation at two iDMRs[40, 97]. Although sample size was limited, the data suggest that parental obesity can alter methylation at iDMRs, which can impact fetal growth and child development.

2.8 Biological Markers of Obesity

Adiponectin and leptin are hormones that play an important role in the complex metabolic pathways related to obesity. The protein hormone adiponectin, which is primarily derived from adipose tissue, has anti-diabetic, anti-atherogenic, anti-inflammatory, and insulin-sensitizing properties that mediate the pathogenesis of metabolic and cardiovascular disease[98, 99]. Leptin, another adipokine, is a hormone predominantly secreted by adipose tissue and has also been found in other tissues such as ovaries, placenta, and the brain[100, 101]. Leptin can moderate energy homeostasis through interaction with central circuits in the hypothalamus[102]. Obese children have been shown to have lower circulating adiponectin and higher leptin[103].

2.9 Metabolomics

Technological advances in omics disciplines have allowed for more in-depth characterization of biological samples. For instance, metabolomics has emerged as a useful tool to examine the profiles of small molecule metabolites in biospecimens[104]. Untargeted metabolomics measures thousands of metabolites in a biological sample, while targeted methods

focuses on a subset of metabolites, generally categorized by their involvement in specific biological pathways[105]. Metabolite levels in humans have been associated with obesity[106], age[105], genetic variants[107], and epigenetics[108, 109].

2.10 CHAMACOS Cohort

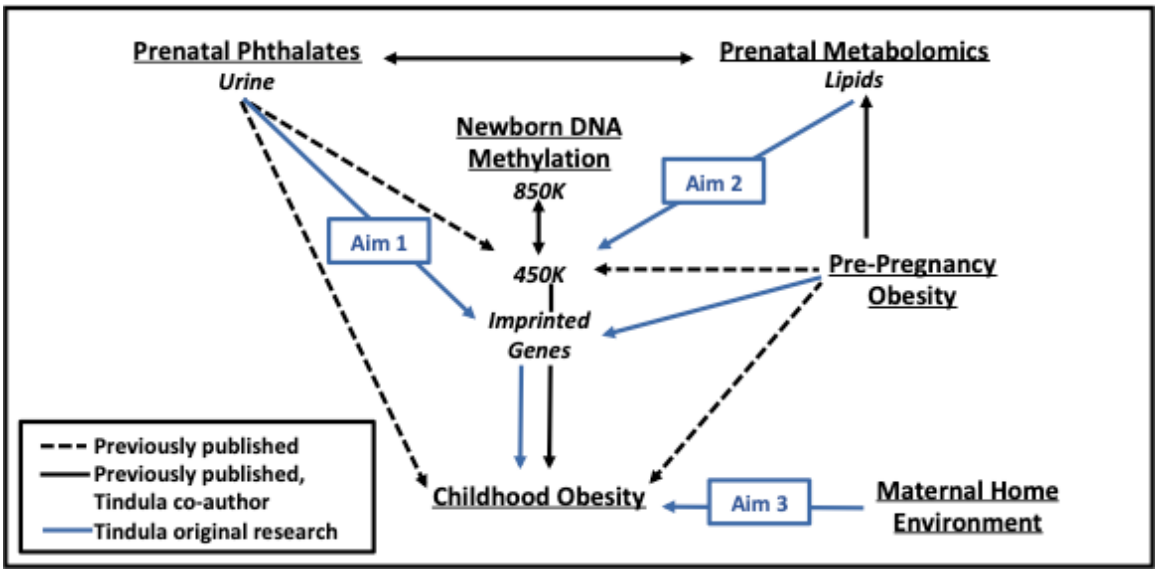


Figure 2.1 Previous and proposed research in the CHAMACOS cohort. Previously published or pending works with Gwen Tindula included as a co-author include: 1) Zhou et al. 2018[110], 2) Solomon et al. 2018[111], and an 3) upcoming publication on child BMI and newborn DNA methylation.

The CHAMACOS birth cohort is well-suited to study the interplay between early life exposure, DNA methylation, and child obesity status. A total of 601 pregnant women were enrolled in the CHAMACOS study, and 532 remained in the study at delivery. CHAMACOS mothers were primarily young (M=25.6±5.3 years), married, low-income, Mexican-born, Spanish-speaking women who were farm workers themselves or lived with farm workers at the time of enrollment. Assessments have been conducted at ~13 and 26 weeks gestation, after delivery, and when the children were between 6 months and 18 years of age.

Mothers and children in CHAMACOS have a high prevalence of obesity. Among mothers, 23% have pre-pregnancy BMI's greater than 30 kg/m^2. Previous assessment in the CHAMACOS study found strong associations between maternal and child obesity[86]. Specifically, having an obese mother significantly increased the odds of the child being overweight or obese (adjusted OR=2.4). Additionally, we examined the relationship between pre-pregnancy BMI and maternal metabolomics profiles, observing numerous associations of metabolomic markers involved in lipid and nucleic acid metabolism and the inflammatory response with maternal BMI[110]. The influence of maternal depressive symptoms on child obesity at age 7 was also examined in CHAMACOS. Children whose mothers consistently demonstrated depressive symptoms had 2.4 times the adjusted odds of overweight/obesity compared to those whose mothers never experienced symptoms of depression[112]. In addition, we explored the relationship of parental obesity and child growth trajectories with imprinted gene methylation. We did not observe any significant associations between parental obesity status and imprinted

gene DNA methylation, contrary to previous findings in the NEST cohort[40, 97]. We observed a negative relationship between *PEG3* methylation and average childhood adiponectin and a positive relationship between *PLAGL1* and log leptin levels, which were attenuated after adjusting p-values for multiple hypothesis testing.

In addition to the parental and child obesity research, we have also examined the effects of environmental exposures during pregnancy on newborn DNA methylation. We assessed the relationship between genome-wide DNA methylation patterns in 336 CHAMACOS newborns using the 450K platform and phthalate exposure at two time points in early and late pregnancy, adjusting for cord blood cell composition[113]. Site-specific analysis uncovered several significant CpG sites associated with phthalate metabolite concentrations during pregnancy that remained after adjustment for multiple comparisons (FDR). Using the bioinformatics tools *DMRcate* and *comb-p* to determine DMRs, characterized by multiple adjacent CpG sites with different methylation profiles based on phenotype, identified 27 regions that overlapped between the two methodologies that were significantly related to multiple phthalate metabolites. Many of the DMR hits were located in genes involved in development and metabolic health.

2.11 Significance

The first two chapters of this dissertation seek to address the gaps in our understanding of the relationship between *in utero* exposure and DNA methylation of Mexican-American newborns. The focus of Chapter 3 is to identify the potential role of phthalate exposure on imprinted genes. Phthalates are ubiquitous chemicals with numerous adverse impacts on human health. The research described in Chapter 3 is essential to understand how exposure to phthalates, known endocrine disrupting chemicals, can affect epigenetic regulation of imprinted genes, which are determinants of early childhood growth. Chapter 4 examines how maternal lipid metabolites during pregnancy are related to infant genome-wide DNA methylation. Epigenetic dysregulation in newborns could be one mechanism to explain previous studies identifying links between lipid metabolomic profiles in pregnant women and poor birth outcomes. Chapters 3 and 4 will add to the growing evidence of the potential role of *in utero* exposures on DNA methylation. Altered DNA methylation status is posited to mediate observed relationships between early life exposures, fetal development, and future health trajectories, a concept described by the Developmental Origins of Health and Disease.

Chapter 5 examines how postnatal home environment can influence obesity risk in adolescents. Much of the current research on obesity focuses on how excessive caloric intake and a sedentary lifestyle impact obesity status. The research described in this chapter seeks to understand the connection of maternal depressive symptoms, perceived social support and home learning environment with adipokines and obesity in youth as they age. This research is essential since childhood obesity is linked with other co-morbidities and can increase risk of obesity and disease in adulthood. Identifying additional contributing risk factors of obesity and potential avenues of intervention is critical to address this significant public health issue.

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CHAPTER 3. DNA Methylation of Imprinted Genes in Mexican-American Newborn Children with Prenatal Phthalate Exposure

3.1 Abstract

Background: Imprinted genes exhibit expression in a parent-of-origin-dependent manner and are critical for child development. Recent limited evidence suggests that prenatal exposure to phthalates, ubiquitous endocrine disruptors, can affect their epigenetic dysregulation. Methods: We quantified DNA methylation of nine imprinted gene differentially methylated regions by pyrosequencing in 296 cord blood DNA samples in a Mexican-American cohort. Fetal exposure was estimated by phthalate metabolite concentrations in maternal urine samples during pregnancy. Results: Several differentially methylated regions of imprinted genes were associated with high molecular weight phthalates. The most consistent, positive, and False Discovery Rate significant associations were observed for *MEG3*. Conclusions: Phthalate exposure *in utero* may affect methylation status of imprinted genes in newborn children.

3.2 Introduction

Epigenetic influences on gene expression have been implicated as a mediator of the relationship between environmental exposures and health status. Early life adverse environments can cause epigenetic shifts, establishing disease trajectories into adulthood, an idea described by the Developmental Origins of Health and Disease (DOHaD) hypothesis[1, 2]. Epigenetic modifications can alter gene expression without changing the underlying nucleotide sequence. DNA methylation is the epigenetic mechanism most often studied[1, 3, 4]. It is simultaneously heritable and susceptible to environmental insults, and is able to reveal cumulative effects of environmental exposures throughout the life course[5, 6].

Genomic imprinting involves the expression of only one allele of the gene and is dependent on the parental origin of the expressed allele[7]. The determination of active versus inactive allele is regulated by DNA methylation that is established during the process of epigenetic reprogramming that occurs in the developing gametes[8]. The majority of the known imprinted genes within the human genome have differentially methylated regions (DMRs) whose methylation is also parent-of-origin-dependent[9, 10]. Methylation patterns in the DMRs are remodeled and established prior to germ layer specification, and are maintained in somatic tissues throughout life[8]. A number of human studies have demonstrated the effects of environmental exposures on imprinted gene DNA methylation, including the influence of diet[11-14], cigarette smoke[15], maternal antibiotic use[16], metal exposure[17-22], and maternal stress[23, 24]. Differentially methylated regions of imprinted genes represent a unique opportunity to assess the effects of *in utero* exposure since they influence development and growth in early life[18].

Limited but growing evidence indicates that exposure to phthalates is associated with DNA methylation changes of imprinted genes[25-27]. Phthalates, diesters of phthalic acid, are a family of chemicals often found in consumer products, resulting in common exposure in the United States[28, 29]. Some phthalates, such as di-(2-ethylhexyl) phthalate (DEHP), are added to plastics to increase flexibility and can be found in toys, plastic containers, and medical supplies. Other phthalates, such as diethyl phthalate (DEP), are used as solvents in personal care products such as perfumes and lotions[30]. Common routes of exposure to phthalates, which are not chemically bound to their substrates and leach into the environment, include ingestion, dermal absorption, and inhalation. Previous studies in humans have demonstrated associations between phthalate exposure during or prior to pregnancy and adverse impacts on health outcomes in

children, including altered birth weight[31-33], gestational age[34, 35], pre-term birth[36, 37], child growth and development[38, 39], child behavior[40], and asthma[41].

Research in animals[42-45] has shown associations between phthalates and global and site-specific methylation; however, there is a paucity of information on imprinted gene DMR methylation in relation to phthalate exposure *in utero*. Only two human studies are available at this time that have examined the relationship between phthalate exposure during pregnancy and placental imprinted gene methylation (*H19* and *IGF2*)[25, 27]. Currently there is no data available on the relationship between cord blood imprinted gene methylation profiles and prenatal exposure to phthalates.

Previously, we reported on the prenatal phthalate metabolite concentrations in participants of the Center for the Health Assessment of Mothers and Children of Salinas (CHAMACOS) study and observed inverse associations of MEP concentrations with DNA methylation of repetitive elements in newborns and children[46]. We have also assessed the relationship between prenatal phthalate exposure in CHAMACOS mothers and both genome-wide site-specific and region DNA methylation as assessed by the 450K array in newborns, identifying 27 different regions within the human genome associated primarily with DEHP metabolites[47]. The objective of the present study is to assess the impact of prenatal phthalate exposure on DNA methylation of imprinted genes in CHAMACOS newborns.

3.3 Methods

Study Subjects

CHAMACOS is a longitudinal birth cohort study assessing neurological, developmental, and respiratory health effects of environmental exposure in pregnant Mexican-American women and their children residing in the agricultural region of Salinas Valley, CA[48]. From 1999-2000, a total of 601 pregnant women were enrolled in the study and 527 women delivered live, singleton newborns. At the time of enrollment, CHAMACOS mothers were at least 18 years of age, Spanish or English speaking, eligible for low income health insurance, and were receiving prenatal care at one of several participating clinics. During pregnancy, CHAMACOS mothers were interviewed twice (13.2 ± 5.1 and 26.0 ± 2.7 weeks gestation) by trained bilingual, bicultural staff regarding reproductive and medical history, sociodemographic factors, and pregnancy-specific lifestyle and environmental exposures[48, 49]. The pregnancy visits will subsequently be referred to as either the early or late pregnancy visits.

Immediately following delivery, whole cord blood was collected from the umbilical vein in BD vacutainers (Becton, Dickinson and Company, Franklin Lakes, NJ) without anticoagulant. Blood specimens were allowed to clot for at least 30 minutes, centrifuged at 1200 rpm for 10 minutes, divided into serum and blood clot aliquots, and stored at -80°C. The QIAamp Blood DNA Maxi kit (Qiagen, Inc., Santa Clarita, CA) was used to isolate genomic DNA from clots as previously described[50]. Methylation of nine imprinted gene DMRs was measured in 296 newborn children (148 girls and 148 boys) that had sufficient DNA available for analysis. The subset was not significantly different from the main CHAMACOS cohort in many demographic and exposure characteristics (child sex, poverty index, education, gestational age, and parity). However, mothers in this sample tended to be slightly younger and more obese, lived fewer years in the United States, and gave birth to children that were less likely to have a low birth weight. The number of CHAMACOS participants with pyrosequencing data is approximately double the subjects included in existing literature assessing the relationship between phthalate

exposure and imprinted genes[25, 27] and we anticipate we will have sufficient power to detect a minimum of a 0.05 linear correlation between phthalates and imprinted genes.

CHAMACOS study protocols were approved by the University of California, Berkeley Committee for Protection of Human Subjects. All mothers provided written informed consent at the time of enrollment.

Phthalate Metabolite Measurements

Maternal urine samples were collected during the early and late pregnancy visits. The urine samples were aliquoted, barcoded, and stored at -80°C in the UC Berkeley School of Public Health Biorepository. Eleven phthalate metabolites were quantified using online solid phase extraction coupled with isotope dilution high-performance liquid chromatography-electrospray ionization-tandem mass spectrometry, as previously described[46, 49, 51, 52]. The metabolites measured included three LMW metabolites (MEP, mono-n-butyl phthalate (MBP), mono-isobutyl phthalate (MiBP)), four DEHP metabolites (mono(2-ethylhexyl) phthalate (MEHP), mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), mono(2-ethyl-5-oxohexyl) phthalate (MEOHP), mono(2-ethyl-5-carboxypentyl) phthalate (MECPP)), and four high molecular weight (HMW) metabolites (monobenzyl phthalate (MBzP), mono(3-carboxypropyl) phthalate (MCPP), monocarboxyoctyl phthalate (MCOP), monocarboxynonyl phthalate (MCNP)). Quality control procedures comprised incorporation of laboratory and field blanks, calibration standards, and spiked controls with low and high concentrations into the experimental runs.

The limits of detection (LOD) for all the phthalate metabolites have been previously described[46, 51, 53]. The instrumental reading values were used when the measured phthalate metabolite concentrations fell below the LOD. In instances where the measured concentrations were below the LOD and instrumental signals were unavailable, the "fill-in" method, described in Lubin *et al*.[54] was used to impute phthalate metabolite concentrations from a log-normal distribution. Summary measurements of the DEHP metabolites in units of micrograms per liter were generated as described previously[55]. Specifically, molar concentrations of each DEHP metabolite were calculated by dividing the concentration of the metabolite by its molecular weight. The molar concentrations for each DEHP phthalate metabolite were summed and then multiplied by the average molecular weight of the metabolites. All phthalate metabolites, with the exception of the DEHP metabolites, were analyzed individually and HMW and LMW sum variables were not generated since values are largely driven by DEHP and DEP metabolites, respectively. Phthalate metabolite concentrations (micrograms per liter) were divided by creatinine concentrations (grams per liter), measured using a commercially available diagnostic enzyme method (Vitros CREA slides; Ortho Clinical Diagnostics, Raritan, NJ) concurrently with the phthalate metabolites, to generate values (micrograms per gram creatinine) adjusted for urinary dilution as previously described[49].

Since we anticipate that average phthalate metabolite concentrations during pregnancy will be a more stable indicator of pregnancy exposure, we assessed *in utero* phthalate exposure as the average of the $\log_{10}$ transformed concentrations of creatinine-corrected phthalate metabolites from the two prenatal visits[52]. Participants with measurements greater or less than three times the interquartile range for a particular phthalate were removed from analyses. All descriptive analyses, figures, and regression models included creatinine adjusted phthalate metabolite concentrations averaged across pregnancy. Additionally, we performed a sensitivity analysis that included adjusting for urinary dilution using specific gravity instead of creatinine,

and we observed that it did not affect the findings. Specific gravity was quantified with a refractometer (National Instrument Company, Inc., Baltimore, MD), and urinary phthalate metabolites were adjusted for specific gravity as previously described[46]. The results of the principal component and mean methylation analyses of the imprinted gene and average phthalate exposure during pregnancy when adjusting for specific gravity were similar to the creatinine adjusted models.

DNA Methylation Analyses

The EZ DNA Methylation kit (Zymo Research, Irvine, CA, USA) was used for bisulfite conversion of 800 ng of DNA, following the manufacturer's instructions. Treating DNA with sodium bisulfite converts unmethylated cytosines to uracils and leaves methylated cytosines unchanged.

The PyroMark PCR Kit (Qiagen, Valencia, CA, USA) was used to prepare bisulfite-treated DNA for PCR amplification, using published primer sequences for nine imprinted gene DMRs and assays previously developed and validated at Duke University[8, 56]. These imprinted genes were selected based on their biological significance and availability of reliable assays[25, 57-61]. Pyrosequencing of the amplified and bisulfite converted DNA was achieved using the Pyromark Q96 MD system (Qiagen), yielding percent methylation estimates for the CpG sites within the sequence analyzed for each DMR. Methylation was measured for the following imprinted genes: *H19*, *Insulin-like Growth Factor 2* (*IGF2*), *Maternally Expressed Gene 3* (*MEG3* and *MEG3-IG*), *Mesoderm Specific Transcrip*t (*MEST*), *Neuronatin* (*NNAT*), *Pleomorphic Adenoma Gene-Like 1* (*PLAGL1*), *Paternally Expressed Gene 3* (*PEG3*), and *Epsilon Sarcoglycan/ Paternally Expressed Gene 10* (*SGCE/PEG10*). Specifically, methylation was measured at the DMRs upstream of *IGF2* exon 3 (chr 11p15.5; 3 CpG sites), upstream of the *H19* gene (chr 11p15.5; 4 CpG sites), two DMRs involved in regulating the *DLK1/MEG3* imprinted domain (chr 14q32.2; *MEG3-IG*: 4 CpG sites; *MEG3*: 7 CpG sites), the *MEST* promoter (chr 7q32.2; 4 CpG sites), the *NNAT* locus (chr 20q11.23; 3 CpG sites), the *PEG3* promoter (chr 19q13.43; 10 CpG sites), the *PLAGL1* locus (chr 6q24.2; 6 CpG sites), and the *SGCE/PEG10* promoter (chr 7q21.3; 6 CpG sites). The paternal allele is expressed for the genes *IGF2*, *DLK1*, *MEST*, *NNAT*, *PEG3*, *PLAGL1*, and *SGCE*; whereas the maternal allele is expressed in *MEG3* and *H19*. The selected imprinted genes are critical in the regulation of early growth[61], and alterations of DNA methylation by environmental insults, such as phthalate exposure, could have implications for imprinted gene expression and downstream effects on growth trajectories. In the CHAMACOS cohort, we have observed that *in utero* exposure to MEP is associated with increased odds of being overweight or obese that persists from age 5 to 12[52]. Quality assurance measures involved inclusion of technical repeats and positive and negative controls. Samples whose methylation values exceeded two standard deviations from the mean for the plate were reanalyzed. The coefficients of variation (CV) of intra-plate repeat measures ranged from 1-3%. Using mixtures of DNA with fully methylated and unmethylated sequences[8, 61], we have previously shown that pyrosequencing allows for the detection of 0.5-5% methylation differences.

Three approaches were used to account for cell composition in the analysis based on cytological differential cell counts[62] and 450K-array estimates[47]. The methods included adjusting for 1) cytological differential cell counts (DCC) in a subset of cord blood samples[46, 62] and 2) estimating cell type proportions from CHAMACOS 450K cord blood DNA methylation data[47]

using adult[63] and cord blood[64] flow sorted reference panels. The cord blood reference panel includes estimates of nucleated red blood cells (nRBCs).

Gene Expression Analysis

Validation of significant hits of the relationship between average phthalate metabolites during pregnancy and imprinted gene methylation in newborns was performed using a two-step RT-PCR in a subset of the CHAMACOS participants with imprinted gene data and available isolated RNA. Specifically, we assessed the overall expression of *MEG3* in 119 CHAMACOS children with imprinted gene data, since methylation changes due to environment exposure could lead to alteration of gene expression. As a negative control, we also examined overall expression patterns in *DLK1* and its relationship with *MEG3-IG* methylation, an imprinted gene DMR where we did not expect to see changes in gene expression since the mean methylation across the DMR was not significantly associated with phthalate exposure. Expression of the reference gene *GAPDH* was used as a control. SuperScript™ IV VILO™ Master Mix was used to convert RNA to cDNA. Predesigned TaqMan Gene Primer Assays for *MEG3, MEG3-IG,* and *GAPDH*, as well as, TaqMan Fast Advanced Master Mix were used in the RT-PCR analysis according to the manufacturer's protocols. Reactions were performed on a Rotor-Gene 6000 (Qiagen, formerly Corbett Life Science). Amplicon specificity was verified by reviewing melt curves. Ct values were calculated for each gene, and ΔCt values, calculated as the difference between the imprinted genes (*MEG3* or *MEG3-IG*) and *GAPDH* Ct values, were used in statistical analysis. Interplate replicates and negative and positive controls were included in the expression analysis for quality control.

Statistical Analyses

Covariates included in the regression models were selected from factors related to phthalate metabolite levels in the CHAMACOS cohort, after performing bivariate analyses (i.e. linear regressions, Student's *T*-test, ANOVA), and important covariates identified in previous studies examining the relationship between imprinted genes and prenatal phthalate exposure. The relevant covariates include pre-pregnancy BMI, years in the US, parity, and child sex. Pre-pregnancy BMI and years in the US were coded as continuous variables, while parity and sex were coded as shown in Table 3.1. Additionally, a plate (batch) variable was included in the regression models to adjust for technical effects.

Prior to performing regression analyses, we logit-transformed the methylation fractions to 'M-values' to reduce dependence of their variance on their mean levels. Methylation fractions have more intuitive biological interpretation, but the M-value is more statistically valid for the differential analysis of methylation levels[65, 66]. Methylation outliers, specifically M-values that were below the 25th percentile minus 3 times the interquartile range and values greater than the 75th percentile plus 3 times the interquartile range, were designated as not available (NA) in the data set, resulting in one to four substitutions for all imprinted genes, with the exception of the *MEST* and *PLAGL1* DMRs. As a result of the correlation between CpG sites within a DMR, we decided to conduct DMR-level principal components (PCs) analyses of the data to determine the independent M-value methylation signals, represented as linear combinations of CpGs that explain at least 95% of variability at the locus. DMR-level principal components (PC) analyses yielded a total of 26 PCs across the nine investigated imprinted gene DMRs (number of PCs: *H19*=2, *IGF2*=2, *MEG3*=1, *MEG3-IG*=3, *MEST*=2, *NNAT*=2, *PLAGL1*=4, *PEG3*=6, and *SGCE/PEG10*=4). The first PCs for all imprinted gene DMRs demonstrated similar contributions

for each CpG to the variance explained by PC1. We fit separate regression models for each combination of the 26 PCs and 12 phthalate metabolite variables (11 phthalate metabolites and the DEHP summary variable) averaged across pregnancy, with the PCs as the response and the average phthalate metabolite concentrations and confounder variables (mentioned above) as covariates. Since the analysis with the PCs does not provide information regarding direction of association, we also performed separate regression analyses for each imprinted gene DMR using the average M-value methylation across the CpGs within the DMR as the outcome. We confirmed regression findings of the relationship between average phthalate metabolites and mean imprinted gene methylation by implementing a bootstrap analysis, sampling individuals with replacement for 1,000 iterations.

Since many imprinted genes impact early growth patterns and *MEG3* has been associated previously with growth outcomes at birth, we performed additional analyses to examine the relationship between average methylation of the *MEG3* DMR and birth weight. A dichotomous birth weight variable was generated at a cutoff of 2,500 grams to distinguish between low and normal/high birth weight newborns. We performed a two sample t-test to compare mean methylation values in the *MEG3* DMR in the two birth weight groups. We also ran regressions to test the association between *MEG3* mean methylation and birth weight, coded as a continuous or binary variable, adjusting for infant sex, maternal pre-pregnancy BMI, methylation plate, route of delivery, and gestational age.

We controlled for the false discovery rate (FDR). The FDR can be defined as the average number of false rejections of the null hypothesis divided by the total number of rejections[67]. All statistical analyses were performed in R Version 3.2.3 (R Foundation for Statistical Computing, Vienna, Austria, 2013). P-values less than 0.05 were considered significant, and two-sided statistical tests were used.

3.4 Results

CHAMACOS Participants

Demographic characteristics of CHAMACOS mothers and children are presented in Table 3.1. Most mothers were young, living below the poverty level, had previously delivered children, had low levels of education, and were overweight or obese (BMI $\geq$ 25 kg/m^2) prior to pregnancy. Approximately half of the women resided in the US for over 5 years at the time of delivery (mean = 6.9 years, SD = 6.9 years). The majority of the children, equally represented by boys and girls, were born term (93.2%) and with a normal birth weight (96.6%).

Phthalate Exposure

Table 3.2 shows the distributions of average phthalate metabolite concentrations across pregnancy for the CHAMACOS mothers included in this study. Detection frequencies of phthalate metabolites during pregnancy were 90-100%. Compared to ten other phthalate metabolites, MEP had the highest urinary concentrations during pregnancy (IQR: 90.7-444.7 μg/g creatinine) in the CHAMACOS cohort[49, 52], consistent with trends observed in the general U.S. population of adults[28]. Levels of urinary MEHHP (IQR: 9.5-29.2), MEOHP (IQR: 7.0-20.7), and MECPP (IQR: 17.7-45.3) contributed most to the DEHP sum concentration.

DNA Methylation of Imprinted Genes

Distributions of DNA methylation values at individual CpG sites within each DMR and average methylation for each of the nine imprinted gene DMRs are shown in Figure 3.1. The

percentage of DNA methylation varies between imprinted genes and within the individual CpG sites within DMRs. *MEG3* exhibited the highest (73±5%) and *PEG3* had the lowest (35±1%) average methylation compared to the other seven imprinted genes DMRs, which were closer to the expected methylation level of 50% and ranged from 44 to 55%. Moreover, whereas some of the imprinted genes (*H19*, *MEST*, *PLAGL1, SGCE*) have relatively similar CpG methylation values across each DMR, the *MEG3-IG* and *NNAT* DMRs demonstrate a wide range of methylation in CpGs from different individuals (*MEG3-IG*: 36-71%; *NNAT*: 42-75%).

Phthalates and DMR Methylation Principal Component Analyses

Significant results of the regression analyses of associations between PCs of imprinted gene DMR methylation in cord blood DNA and phthalate metabolite concentrations during pregnancy are shown in Table 3.3. After adjusting for years in the United States at the time of pregnancy, parity, pre-pregnancy BMI, child sex, and batch, HMW phthalate metabolites were related to PCs of the *MEG3* DMRs and PC 3 of *PEG3*. All DEHP metabolites assessed were significantly associated with PC1 of the *MEG3* DMR (MEHP: p=0.03; MEHHP: p=0.002; MEOHP: p=0.0003; MECPP: p=0.0001). MBzP was also related to PC1 of the *MEG3* DMR (p=0.01). MCPP was associated with PC2 of the *MEG3-IG* DMR (p=0.01). Average pregnancy levels of MCOP (p=0.02) and MCNP (p=0.03) were associated with the third principal component of *PEG3*. Of the observed associations between the average phthalate metabolites during pregnancy and the *MEG3* DMRs, only the associations between *MEG3* and certain DEHP metabolites (MEOHP, MECPP, Σ DEHP) remained significant after FDR adjustment (all p<0.04).

Phthalates and Imprinted Gene Average DMR Methylation

In order to determine directions of association, we analyzed the relationship between phthalate exposure and DNA methylation averaged across all CpG sites within each imprinted gene DMR (Figure 3.2, Table 3.4). In crude models without adjustment for confounders, we found a positive association between average pregnancy DEHP metabolites and mean methylation percent at the *MEG3* DMR (Figure 3.2, all p<0.05). In models adjusting for covariates, *MEG3-IG* and *PEG3*, which were significantly associated with some of the phthalate metabolites in the PC analysis, were no longer significant in the analysis with mean M-value methylation as the outcome (*MEG3-IG* and MCPP; *PEG3* and MCOP; *PEG3* and MCNP; all p-values>0.05). MBzP (β=0.17, p=0.01) and DEHP metabolites (MEHP: β=0.12, p=0.03; MEHHP: β=0.21, p=0.002; MEOHP: β=0.23, p=0.0003; MECPP: β=0.31, p=0.00007) were positively associated with mean methylation across the *MEG3* DMR. All of the observed relationships remained significant after FDR adjustment (all p<0.05), with the exception of the associations with MEHP and MBzP. Bootstrap analysis confirmed the findings; specifically, the significant and positive association between HMW phthalates and mean methylation across CpG sites within the *MEG3* DMR.

Adjustment for Cell Composition

Figure 3.3 presents the regression estimates for the significant relationships of the mean *MEG3* methylation and prenatal phthalate metabolite concentrations adjusting for cell type composition using three different methodologies as described in the methods section. The array-based estimates of cell composition[47, 64] produced estimates of the regression coefficients similar to the model without cell type adjustment (unadjusted). Coefficients from models adjusting for

cytological DCC[62] showed even stronger relationships between phthalate exposure and *MEG3* methylation in the same direction. Adjusting for white blood cell composition estimates using CHAMACOS 450K array data and the cord blood flow sorted reference panel replicated the crude models of the imprinted gene mean methylation and average pregnancy phthalate metabolites, suggesting limited bias due to cell type composition.

Gene Expression Validation

Following identification of significant hits for the association between DEHP and BzBP phthalate metabolites and *MEG3* methylation, we assessed gene expression of the *MEG3* gene in a subset of 119 CHAMACOS participants. Ct values for *MEG3* ranged from 22 to 36, with a mean of 27.6. As expected based on previous relationships between methylation and expression[6], *MEG3* M-value methylation was inversely correlated with *MEG3* expression relative to the housekeeping gene *GAPDH* (Pearson r=-0.10, p=0.29). As a control, we also assessed the relationship between *DLK1* expression and *MEG3-IG* mean methylation, an imprinted gene DMR that was not significantly associated with phthalate exposure. As expected, we did not observe a significant relationship (Pearson r=-0.05, p=0.58). Additionally, we tested the relationships between phthalate metabolite concentrations and *MEG3* expression, adjusting for the covariates identified in the methylation analysis. We observed consistent negative associations between DEHP metabolites and *MEG3* expression (MEHP: β=-0.44, MEHHP: β= -1.14, MEOHP: β=-0.63, MECPP: β=-1.21); however, none of the associations reached statistical significance.

Imprinted Gene Methylation and Birth Weight

We found a significant difference in mean *MEG3* percent methylation levels comparing low birth weight newborns and newborns with normal to high birth weights (t=-2.35, p=0.04), with lower average methylation values in the low birth weight group. However, the regression analyses did not show a significant relationship between *MEG3* methylation and continuous and binary birth weight in the CHAMACOS newborns.

3.5 Discussion

We examined the association of prenatal phthalate exposure and imprinted gene methylation profiles in newborn children using DNA isolated from cord blood. Previous analysis in the CHAMACOS cohort of repetitive element methylation and array-based DMRs uncovered significant associations of DNA methylation in cord blood with MEP and DEHP metabolites, respectively[46, 47]. In the current study of nine imprinted genes, the most consistent findings were the associations between DEHP and BzBP phthalate metabolites and DNA methylation of the *MEG3* DMR, located within an intron, downstream of the *MEG3* promoter[68]. DNA methylation profiles in the CHAMACOS cohort were similar to values reported for imprinted genes in other cohorts with DNA isolated from cord blood samples[68].

To the best of our knowledge, our study is the first to demonstrate the relationship between *MEG3* DNA methylation and prenatal phthalate exposure. In CHAMACOS newborns, prenatal exposure to several DEHP metabolites was positively and significantly associated with DNA methylation at the *MEG3* DMR. Validation analysis demonstrated an inverse relationship between *MEG3* methylation and expression, which is consistent with expected effects of hypermethylation. However, this result did not quite reach statistical significance. Relatively weak relationships between differential DNA methylation and expression of the same genes is

common, as regulatory mechanisms may also include non-coding RNAs and chromatin modifications[1].

Hypomethylation or increased expression of *MEG3* in humans have previously been associated with higher birth weight and large for gestational age status, respectively[69, 70]. Conversely, decreased expression of *MEG3* has been observed in human Intrauterine Growth Restriction placenta samples[71]. In the current study, we did not find a significant relationship between *MEG3* methylation and continuous birth weight, which corroborates a previous report by Vidal et al. (2013)[16]. However, we did observe a significant difference when we contrasted *MEG3* methylation in low birth weight newborns and normal weight ones. In addition to its relationship with birth outcomes, hypermethylation of the *MEG3* gene in human tumor samples and cell lines has previously been associated with tumorigenesis[57, 58, 72-76]. Additionally, epigenetic and genetic studies have also uncovered links between *MEG3* variants and methylation patterns and diabetes[77]. Specifically, Kameswaram *et al*.[78] found decreased expression of *MEG3* in human islets from T2D organ donors, which was highly correlated with hypermethylation of *MEG3*. Studies in mice have demonstrated that decreased expression of the *Meg3* gene can impact glucose tolerance and reduce insulin secretion[79]. Modification of epigenetic profiles in *MEG3* following environmental exposure could lead to shifts in hormonal and metabolic markers, as well as early growth.

Only two studies have assessed the relationship between pregnancy phthalate exposure and imprinted gene methylation, both in placenta samples. Zhao *et al*.[27] measured urinary phthalate concentrations of 5 metabolites (2 LMW and 3 HMW) and DNA methylation of *IGF2* (2 CpGs) in 181 placenta samples collected in China. The researchers observed that DEHP metabolites were significantly and negatively associated with *IGF2* DNA methylation. Additionally, LaRocca *et al*.[25] examined the association between phthalate metabolite concentrations and DNA methylation of the *H19* and *IGF2* imprinted genes in 179 placenta samples from two cohorts, the Harvard Epigenetic Birth Cohort (HEBC) and the Predictors of Preeclampsia Study (POPS). The authors found a significant inverse relationship between *H19* methylation in placenta and the sum of the LMW phthalates metabolite concentrations and the sum of all the 11 phthalate metabolites measured. A similar pattern was observed for the *IGF2 DMR0*. They also observed an inverse association between DEHP metabolites and *IGF2 DMR0*, similar to the results seen in the study by Zhao *et al*. However, both of these studies did not analyze *MEG3* methylation in respect to phthalates.

The number of CpG sites in the studies assessing placenta DNA methylation and the cord blood analysis in CHAMACOS differ between the studies. Additionally, only two imprinted genes were assessed in the placenta studies, while our study involved eight imprinted genes with nine DMRs. We are also mindful that the difference between the tissues used (placenta vs. cord blood) may also be the reason for observed differences in methylation results with phthalate exposure.

A recent study by Goodrich *et al*.[26] examined the relationship between prenatal and childhood exposure to heavy metals and endocrine disrupting chemicals, including phthalates, and DNA methylation of differentially methylated regions of two imprinted genes (*H19*: 4 CpG sites, *IGF2*: 7 CpG sites) in peri-adolescents. They found that phthalate metabolite levels (MBzP and MIBP) in maternal urine samples were associated with *H19* hypermethylation in blood from 247 8-14 year old children, but this finding was attenuated to non-significance after adjustment for multiple testing. Overall, the CHAMACOS results as well as the findings by LaRocca *et al*.[25], Zhao *et al*.[27], and Goodrich *et al*.[26] suggest potential epigenetic modifications in imprinted

genes involved in fetal growth, in association with prenatal phthalate exposure from the time of birth that may persist into adolescence.

A hypothesized mechanism by which exposure to environmental chemicals may induce epigenetic shifts involves altered transcription factor occupancy. Martin and Fry[80] performed an analysis across 11 studies that included altered cord blood and placental methylation patterns of CpG sites within 341 genes related to five environmental contaminants (lead, arsenic, cadmium, tobacco smoke, and mercury). They found that 56 transcription factor binding sites were enriched in the promoter regions of the genes, which could impact the access of DNA methyltransferase to CpG sites, inhibiting the process of DNA methylation and leading to hypomethylation of the gene. Additionally, the authors provided suggestive evidence that glucocorticoid-receptor dependent signaling may mediate the relationship between environmental chemical exposure and transcription factor activity, since the promoter regions of 38 of the 56 genes encoding the transcription factors contained glucocorticoid-responsive-related sequence elements. It is possible that prenatal exposure to phthalates could initiate a similar response of altered transcription factor activity in the current study in the *MEG3* DMR, located downstream of the promoter region, resulting in methylation shifts. Future studies should further explore the mechanism proposed by Martin and Fry in additional environmental contaminants, including phthalates.

One of the notable strengths of our study involves the novel investigation of DNA methylation profiles of multiple imprinted genes in newborns in relation to prenatal phthalates, a ubiquitous endocrine disruptor. Two previous studies[25, 27] assessed the role of pregnancy phthalate levels on DNA methylation in imprinted genes; however, they used placenta samples and only for three or fewer imprinted regions. In addition to the advantages of assessing a greater number of imprinted genes in our study, our analyses also benefited from a larger sample size.

There were also several limitations in our study. In the umbilical cord, nRBCs and leukocytes mixed in the clots may contribute to DNA methylation levels[64]. However, we were able to account for variable leukocyte composition typical in umbilical cord blood in sensitivity analyses using cytological differential cell count[62], as well as a cord blood reference panel including nRBCs[47, 64]. The results for the models that did and did not adjust for cell counts were very similar. Another potential limitation involves the applicability of the findings to other populations, given that the CHAMACOS participants are Mexican-Americans from families with low socioeconomic status. Since observed exposure levels to phthalates in CHAMACOS women were similar to the general population of U.S. adults, the data are likely relevant to many other populations. In this study, we have focused on nine imprinted gene DMRs that have been previously established and validated, and we intend to explore other regions with parental-origin-dependent differential methylation in the future.

3.6 Acknowledgements

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3.7 Publication

This work was published in:

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3.8 Tables

Table 3.1 Demographic characteristics of CHAMACOS mothers and children (1999-2000)

Characteristic	CHAMACOS mothers and their children with imprinted gene DMR methylation data (N=296) n (%)
Child sex	
Boy	148 (50.0)
Girl	148 (50.0)
Child gestational age at birth	
34-36 weeks	20 (6.8)
≥ 37 weeks	276 (93.2)
Child birthweight	
Low birthweight (< 2500 g)	10 (3.4)
Normal birthweight (≥ 2500 g)	286 (96.6)
Maternal age at pregnancy	
18-24	137 (46.3)
25-29	101 (34.1)
30-34	46 (15.5)
35-45	12 (4.1)
Number of years mother lived in US at pregnancy	
Less than 1 year	55 (18.6)
1-5 years	102 (34.5)
6-10 years	70 (23.6)
11 or more years	69 (23.3)
Maternal pre-pregnancy BMI[a]	
Underweight (< 18.5 kg/m^2)	2 (0.7)
Normal (18.5-24.9 kg/m^2)	118 (40.7)
Overweight (25-29.9 kg/m^2)	115 (39.7)
Obese (≥ 30 kg/m^2)	55 (19.0)
Parity	
0	106 (35.8)
≥ 1	190 (64.2)
Education	
≤ 6th grade	125 (42.2)
7-12th grade	114 (38.5)
≥ High school graduate	57 (19.3)
Poverty Status	
≤ Poverty level	184 (62.2)
> Poverty level	112 (37.8)

[a]Total number of observations for maternal pre-pregnancy BMI varies due to missing data
Abbreviations: BMI, body mass index; CHAMACOS, Center for the Health Assessment of Mothers and Children of Salinas; DMR, differentially methylated region

Table 3.2 Distribution of phthalate metabolite concentrations averaged across pregnancy

Phthalate Metabolite	Pregnancy Average (N=265)			
	Median	**IQR**	**Minimum**	**Maximum**
MEP	214.2	(90.7, 444.7)	7.2	6607.7
MBP	24.4	(14.2, 45.3)	3.2	228.6
MiBP	2.7	(1.6, 5.3)	0.1	192.0
MEHP	3.9	(2.2, 7.0)	0.1	101.6
MEHHP	16.1	(9.5, 29.2)	1.4	478.6
MEOHP	12.1	(7.0, 20.7)	0.9	353.4
MECPP	26.7	(17.7, 45.3)	5.3	665.2
ΣDEHP	60.9	(37.1, 98.5)	8.0	1524.5
MBzP	8.2	(4.7, 13.8)	0.5	97.4
MCPP	2.0	(1.3, 2.9)	0.1	28.3
MCOP	3.4	(2.1, 4.7)	0.5	82.7
MCNP	1.9	(1.3, 2.6)	0.3	17.1

All units are in μg/g creatinine.

Abbreviations: IQR, interquartile range; MEP, monoethyl phthalate; MBP, mono-n-butyl phthalate; MiBP, mono-isobutyl phthalate; MEHP, mono(2-ethylhexyl) phthalate; MEHHP, mono(2-ethyl-5-hydroxyhexyl) phthalate; MEOHP, mono(2-ethyl-5-oxohexyl) phthalate; MECPP, mono(2-ethyl-5-carboxypentyl) phthalate; DEHP, di-2-ethylhexyl phthalate; MBzP, monobenzyl phthalate; MCPP, mono(3-carboxypropyl) phthalate; MCOP, monocarboxyoctyl phthalate; MCNP, monocarboxynonyl phthalate.

Table 3.3 Regression models of average maternal phthalate metabolite concentrations during pregnancy with principal components (PCs) of child imprinted gene DMR M-value methylation at delivery

Imprinted Gene DMR	PC #	Significant Exposure	β[a]	95% CI	P-Value
MEG3	PC 1	MEHP	-0.321	(-0.602, -0.039)	0.03
	PC 1	MEHHP	-0.544	(-0.885, -0.203)	2E-03
	PC 1	MEOHP	-0.615	(-0.944, -0.286)	3E-04*
	PC 1	MECPP	-0.812	(-1.211, -0.412)	9E-05*
	PC 1	Σ DEHP	-0.719	(-1.099, -0.338)	3E-04*
	PC 1	MBzP	-0.448	(-0.765, -0.132)	0.01
MEG3-IG	PC 2	MCPP	0.050	(0.013, 0.087)	0.01
PEG3	PC 3	MCOP	-0.051	(-0.095, -0.007)	0.02
	PC 3	MCNP	-0.055	(-0.104, -0.006)	0.03

Covariates in each regression model included batch, years in the USA, parity, pre-pregnancy BMI, and child sex. Models included $\log_{10}$ transformed concentrations of creatinine-corrected phthalate metabolites.

[a]β for PCs are not interpretable; for direction of association, refer to mean methylation analysis

*Significant after FDR adjustment (all $p<0.04$). $p<0.05$ statistically significant.

Abbreviations: PC, principal component; MEHP, mono(2-ethylhexyl) phthalate; MEHHP, mono(2-ethyl-5-hydroxyhexyl) phthalate; MEOHP, mono(2-ethyl-5-oxohexyl) phthalate; MECPP, mono(2-ethyl-5-carboxypentyl) phthalate; DEHP, di-2-ethylhexyl phthalate; MBzP, monobenzyl phthalate; MCPP, mono(3-carboxypropyl) phthalate; MCOP, monocarboxyoctyl phthalate; MCNP, monocarboxynonyl phthalate.

Table 3.4 Regression models of average maternal phthalate metabolite concentrations during pregnancy with mean child imprinted gene DMR M-value methylation at delivery

Imprinted Gene DMR	Significant Exposure	β	95% CI	P-Value
MEG3	MEHP	0.120	(0.015, 0.225)	0.03
	MEHHP	0.206	(0.078, 0.333)	2E-03*
	MEOHP	0.232	(0.110, 0.335)	3E-04*
	MECPP	0.306	(0.157, 0.455)	7E-05*
	Σ DEHP	0.271	(0.130, 0.413)	2E-04*
	MBzP	0.168	(0.050, 0.286)	0.01

Covariates in each regression model included batch, years in the USA, parity, pre-pregnancy BMI, and child sex. Models included $\log_{10}$ transformed concentrations of creatinine-corrected phthalate metabolites.

*Significant after FDR adjustment (all $p<0.05$). $p<0.05$ statistically significant.

Abbreviations: FDR, false discovery rate; MEHP, mono(2-ethylhexyl) phthalate; MEHHP, mono(2-ethyl-5-hydroxyhexyl) phthalate; MEOHP, mono(2-ethyl-5-oxohexyl) phthalate; MECPP, mono(2-ethyl-5-carboxypentyl) phthalate; DEHP, di-2-ethylhexyl phthalate; MBzP, monobenzyl phthalate.

3.9 Figures

Figure 3.1 Distribution of average and CpG specific imprinted gene differentially methylated region percent methylation in CHAMACOS newborns. The white box plots represent distributions of the % methylation of individual CpGs in the nine imprinted gene DMRs in 296 newborns. The leftmost box plot for each imprinted gene DMR represents the first CpG assessed in the DMR. The gray box plots to the right of each group of white boxes represent the average % methylation across all CpG sites within an IG DMR. The total number of observations for each imprinted gene varies slightly due to missing data.

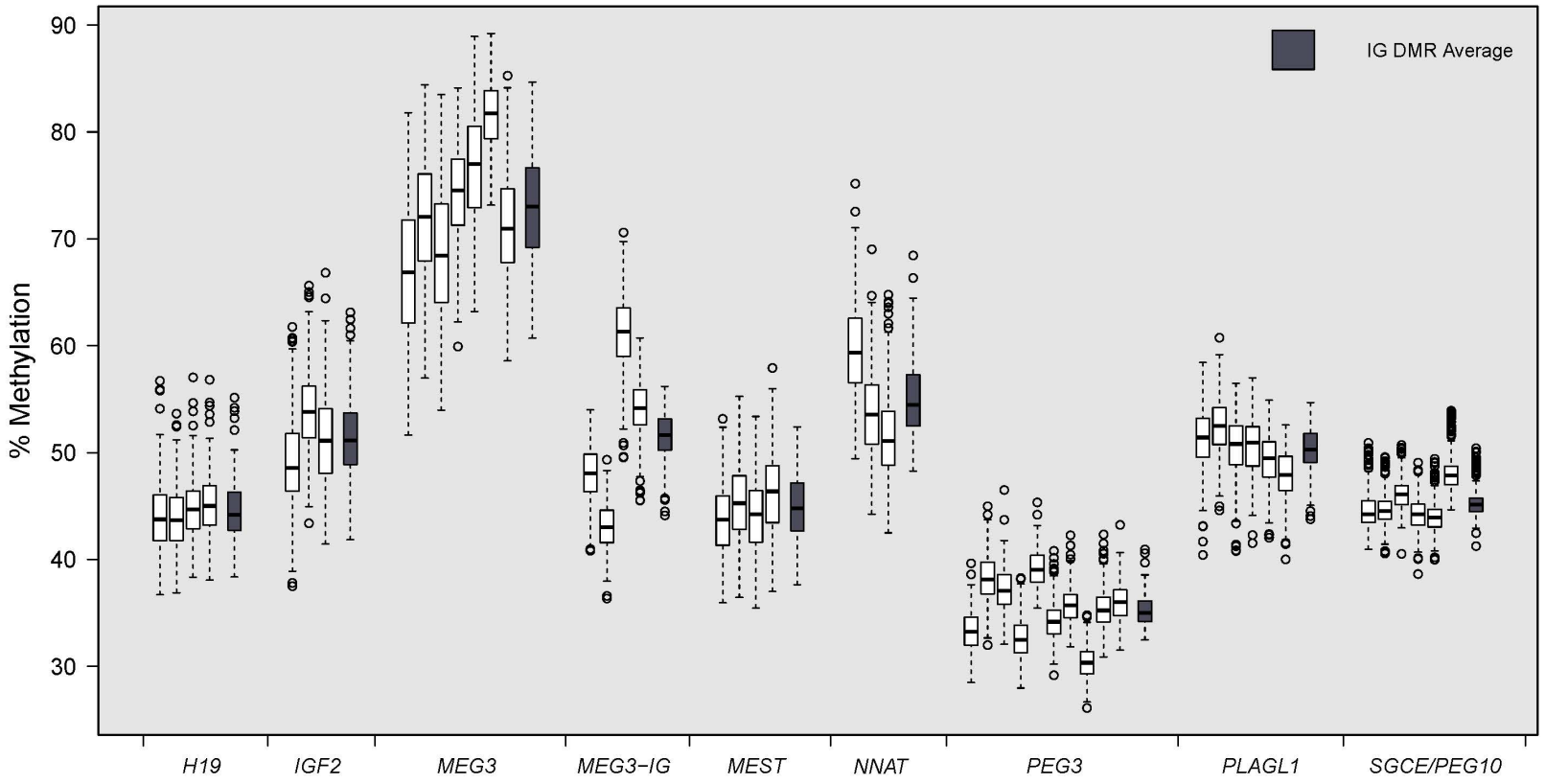


Figure 3.2 Scatter plots of the crude relationships between prenatal DEHP phthalate metabolites and mean percent methylation of the *MEG3* differentially methylated region.
MEHP, mono(2-ethylhexyl) phthalate; MEHHP, mono(2-ethyl-5-hydroxyhexyl) phthalate; MEOHP, mono(2-ethyl-5-oxohexyl) phthalate; MECPP, mono(2-ethyl-5-carboxypentyl) phthalate.

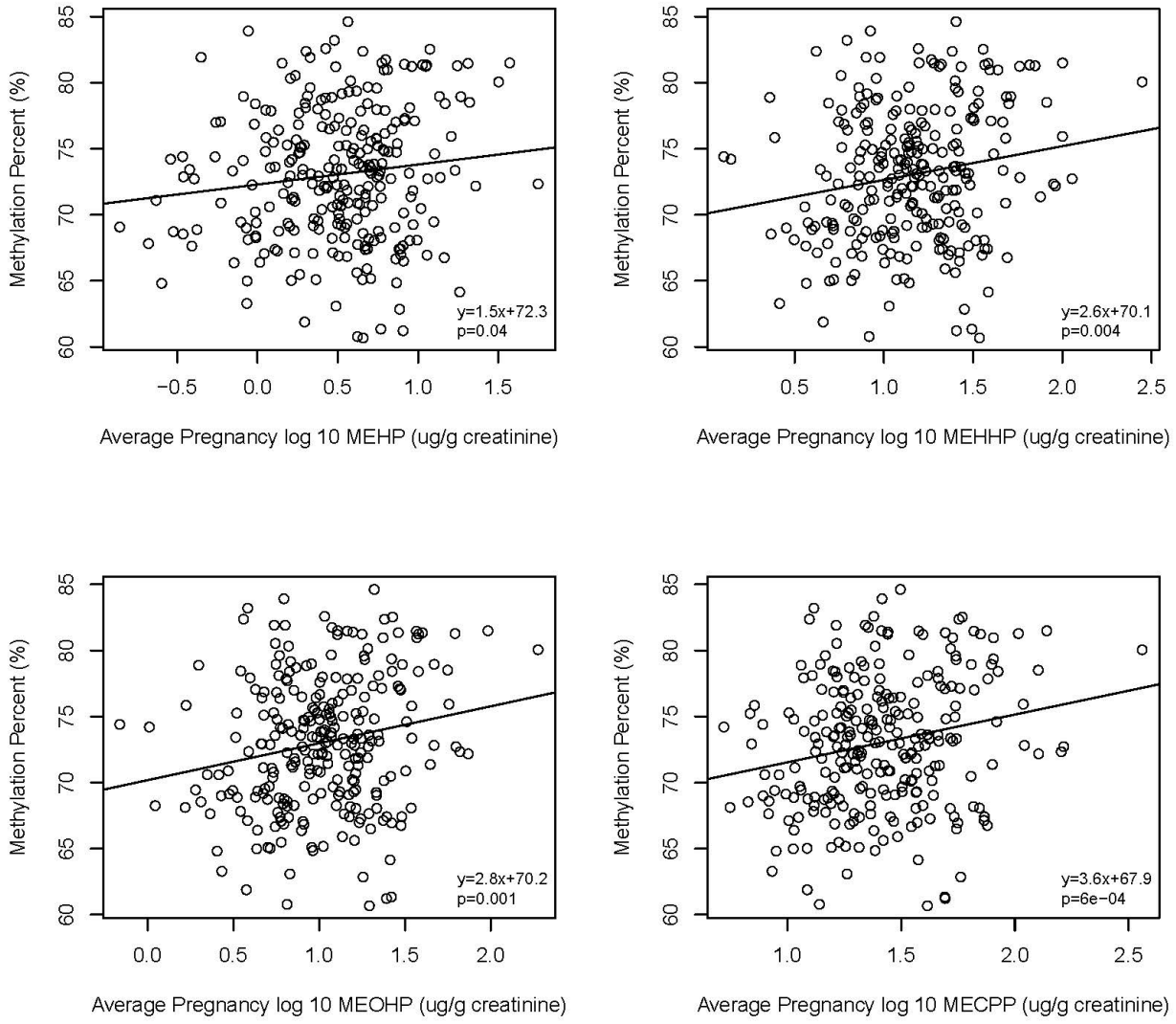
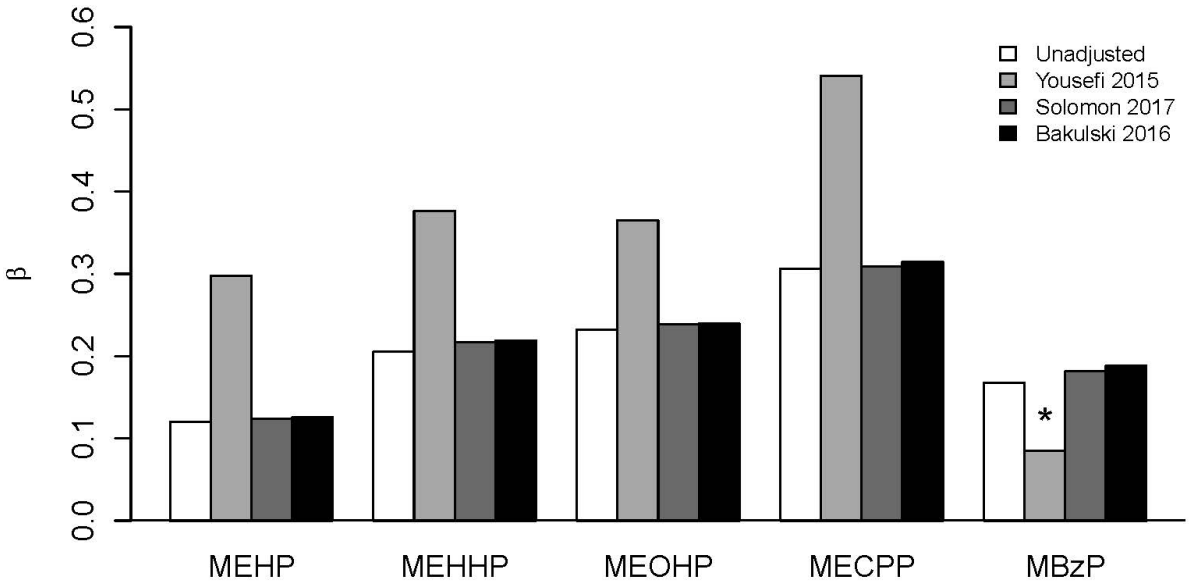


Figure 3.3 Regression coefficients of the relationships between prenatal DEHP phthalate metabolites and mean methylation of the *MEG3* DMR with and without cell type adjustment. The methods included adjusting for cytological differential cell counts in a subset of cord blood samples (Yousefi 2015) and estimating cell type proportions from CHAMACOS 450K cord blood DNA methylation data using adult (Solomon 2017) and cord blood (Bakulski 2016) flow sorted reference panels. The cord blood reference panel includes estimates of nucleated red blood cells (nRBCs).
*Not statistically significant at $p<0.05$; all other presented relationships were significant at $p<0.05$.
MEHP, mono(2-ethylhexyl) phthalate; MEHHP, mono(2-ethyl-5-hydroxyhexyl) phthalate; MEOHP, mono(2-ethyl-5-oxohexyl) phthalate; MECPP, mono(2-ethyl-5-carboxypentyl) phthalate; MBzP, monobenzyl phthalate.



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CHAPTER 4. Pregnancy Lipidomic Profiles and DNA Methylation in Newborns from the CHAMACOS Cohort

4.1 Abstract

Background: Lipids play a role in many biological functions and the newly emerging field of lipidomics aims to characterize the varying classes of lipid molecules present in biological specimens. Animal models have shown associations between maternal dietary supplementation with fatty acids during pregnancy and epigenetic changes in their offspring, demonstrating a mechanism through which prenatal environment can affect outcomes in children; however, data on maternal lipid metabolite levels during pregnancy and newborn DNA methylation in humans are sparse. Methods: In this study, we assessed the relationship of maternal lipid metabolites measured in the blood from pregnant women with newborn DNA methylation profiles in the CHAMACOS cohort. Targeted metabolomics was performed by selected reaction monitoring liquid chromatography and triple quadrupole mass spectrometry to measure 92 metabolites in plasma samples of pregnant women at approximately 26 weeks gestation. DNA methylation was assessed using the Infinium HumanMethylation 450K BeadChip adjusting for cord blood cell composition. Results: We uncovered numerous false discovery rate (FDR) significant associations between maternal metabolite levels, particularly phospholipid and lysolipid metabolites, and newborn methylation. The majority of the observed relationships were negative, suggesting that higher lipid metabolites during pregnancy are associated with lower methylation levels at genes related to fetal development. Conclusion: These results further elucidate the complex relationship between early life exposures, maternal lipid metabolites, and infant epigenetic status.

4.2 Introduction

Metabolomics has emerged as a useful tool to examine the profiles of small molecule metabolites in biospecimens[1]. Factors that have been associated with metabolite levels include age[2], diseases such as obesity[3], genetic variants throughout the human genome[4], and epigenetics[5, 6]. Both untargeted and targeted methodologies are commonly used in metabolomic research, with the former measuring thousands of metabolites in a biological sample and the latter focusing on a subset of metabolites, often involved in specific biological pathways[2]. For instance, lipidomics evaluates the function and distribution of lipids, which have a greater diversity of molecular species compared to the other classes of biological molecules in the body, such as carbohydrates and amino acids[7].

Epigenetic mechanisms regulate gene expression without changes in DNA sequence and include DNA methylation, histone modifications, and non-coding RNAs[8]. DNA methylation is the most commonly examined epigenetic mechanism and refers to the addition of a methyl group to the C5 position of the cytosine ring in a CpG dinucleotide by DNA methyltransferases[9]. DNA methylation patterns are retained following somatic cell division[10]. In the CHAMACOS cohort and as part of the Pregnancy and Childhood Epigenetics (PACE) consortium, we have explored the relationship of infant DNA methylation with early life environmental exposure of mothers to chemicals during pregnancy, including endocrine disrupting chemicals[11-13] and smoking[14]. Since the epigenome undergoes remodeling and rapid cell division in the prenatal period[8], this life stage is considered particularly sensitive to environmental insults and can have implications for disease trajectories[15].

There remains a paucity of information on the relationship between metabolite profiles, in general and specific to the lipid species, and DNA methylation, particularly the link between maternal lipid metabolites during pregnancy and offspring epigenetics, which could be a mechanism through which the *in utero* environment exerts an effect on epigenetic mechanisms that could impact offspring health. Most of the existing research on this topic has been conducted in animal models and has focused on the relationship between maternal nutrition, including dietary supplementation with fatty acids, and offspring methylation status[16, 17]. Only one study to date has examined the association between *in utero* metabolic traits, including lipid metabolites, and DNA methylation profiles in newborns[5]. The authors found significant relationships between maternal fatty acid levels and newborn methylation globally and at target genes related to early growth.

Previous research in the Center for the Health Assessment of Mothers and Children of Salinas (CHAMACOS) cohort examined the association between maternal prenatal phthalate urine metabolite levels and targeted metabolomic compounds present in plasma and urine, collected at 26 weeks gestation[18]. Additionally, we assessed the relationship between pre-pregnancy body mass index (BMI) and maternal metabolomics profiles. We observed numerous associations of metabolomic markers involved in lipid and nucleic acid metabolism and the inflammatory response with both urinary phthalate metabolites and maternal BMI. The current study aims to determine the association between maternal metabolomic profiles in pregnancy and child DNA methylation at birth, emphasizing metabolites enriched for lipid pathways that were confirmed as relevant in previous work in CHAMACOS[18]. This study will contribute to the growing body of evidence of the influence of the early life environment on epigenetic mechanisms.

4.3 Methods

Study population

Study participants included women and children from the CHAMACOS study, a longitudinal birth cohort originally aimed at examining the relationship of pesticide and environmental chemical exposure with the health and development of Mexican-American children from Salinas Valley, CA. A more in depth discussion of the study population has been characterized previously[19]. From 1999-2000, a total of 601 pregnant women were enrolled, resulting in the delivery of 527 liveborn singletons. Two pregnancy interviews were conducted at an average of 13.4 and 26.0 weeks gestation, with an additional visit after delivery. This study includes the 81 mother–child pairs with both metabolomic data from blood collected at the second pregnancy visit and with newborn Illumina HumanMethlyation 450K BeadChip data. The University of California, Berkeley Committee for Protection of Human Subjects approved all study protocols and written informed consent was obtained from the CHAMACOS mothers.

Pregnancy Metabolite Measurements

Biological samples collected from 115 CHAMACOS women around 26 weeks gestation (mean=26.4, SD=3.2) were analyzed to characterize maternal metabolomics profiles during pregnancy. Detailed descriptions of the metabolomics analyses in the CHAMACOS cohort have been previously described[18]. Briefly, blood from the women was analyzed in duplicates by selected reaction monitoring (SRM) liquid chromatography (LC) and triple quadrupole mass spectrometry (MS), a method previously validated by the Nomura research group[20]. SRM was used to identify metabolites, which were measured using the area under the curve. C_{12} MAGE,

Pentadecanoic acid, and D^3N^{15} Serine were utilized in the normalization of plasma nonpolar positive, plasma nonpolar negative, and plasma polar metabolites, respectively. Laboratory and field blanks, as well as internal standards, were included to ensure quality, and repeat samples had good reproducibility (CVs ≤ 3–15%). For this study, we focused on the plasma metabolites involved in lipid pathways since lipids are involved in diverse biological activities including metabolic and structural functions, inflammation, signaling, and endocrine regulation[21].

Cord blood collection and processing

At delivery, hospital staff collected cord blood in two types of vacutainers, one coated in heparin (green top) and the other without the anticoagulant (red top). Blood clots were aliquoted from the nonheparinized vacutainers and were stored at −80 °C until use in DNA isolation.

DNA preparation

DNA isolation of anticoagulant-free umbilical cord blood clot samples was performed using QIAamp DNA Blood Maxi Kits (Qiagen, Valencia, CA) following the manufacturer's protocol, with the exception of small modifications that were previously described[22].

450K BeadChip DNA methylation analysis

DNA samples of 1ug were bisulfite converted using Zymo Bisulfite Conversion Kits (Zymo Research, Irvine, CA), whole genome amplified, enzymatically fragmented, purified, and applied to Illumina Infinium HumanMethylation450 BeadChips (Illumina, San Diego, CA) following the manufacturer's instructions[23]. Samples were randomized across assay wells, chips and plates to prevent batch bias. Robotics handled the 450K BeadChips, which were analyzed using the Illumina Hi-Scan system. DNA methylation was assessed at 485,512 CpG sites.

Probe signal intensities were extracted by Illumina GenomeStudio software (version XXV2011.1, Methylation Module 1.9) methylation module and background subtracted. A variety of quality assurance and control measures were implemented and have been previously described[24], such as determination of assay repeatability and batch effects. The All Sample Mean Normalization (ASMN) algorithm[24], adjusted for color channel bias, batch effects and differences in Infinium chemistry. Additionally, Beta Mixture Quantile (BMIQ) normalization was used to account for differences between the two Illumina probes[25]. Samples were retained if 95% of sites assayed had detection P-values greater than 0.01. Criteria for removal included: 1) sites with annotated probe SNPs (n = 65) and with common SNPs (minor allele frequency >5%) within 50bp of the target identified in the MXL (Mexican ancestry in Los Angeles, California) HapMap population (n = 49,748); 2) sites mapped to the X (n = 10,708) and Y (n = 95) chromosomes[14]; 3) cross-reactive probes identified by Chen et al.[26] (n = 26,950); and 4) probes where 95% of samples had detection P-values greater than 0.01 (n= 460). A total of 398,483 CpG sites remained for analysis. Methylation beta values across sites were logit transformed to the *M*-value scale to more accurately adhere to modeling assumptions[27]. Methylation observations greater or less than three times the interquartile range for a given CpG site were removed prior to regression analyses to reduce the influence of methylation outliers.

Statistical analysis

To examine the distributions of individual metabolites in maternal blood during pregnancy, we computed descriptive statistics. We used Student's T-tests to compare methylation profiles between CHAMACOS newborns with methylation data that were included

(N=81) and excluded (N=288) in the current analyses, with results indicating no significant differences in methylation between the two groups. There was no difference between the subset of the participants included in this study and the rest of the CHAMACOS cohort.

To determine the relationship between maternal pregnancy metabolomics profiles as the exposure and 450K DNA methylation of their newborn children as the outcome, we fit *limma* linear models with empirical Bayes variance shrinkage[28]. In order to reduce the influence of metabolite outliers, all 92 maternal lipid metabolites were log(1+x) transformed. We adjusted for sex and batch in each of the models. Since DNA methylation has been observed to vary by cell type[29], we also accounted for white blood cell type proportions in statistical models. To estimate cord blood proportions of 7 white blood cell types, including nucleated red blood cells, we utilized a cord blood reference dataset from Johns Hopkins[30]. We adjusted p-values for multiple hypotheses testing using the Benjamini-Hochberg (BH) false discovery rate (FDR) threshold for significance of 0.05[31]. We also identified CpGs that were significant based on the more conservative Bonferroni correction (uncorrected p value $< 1.36 \times 10^{-9}$ to account for 36,660,436 tests). This analysis was performed using R statistical computing software (v3.5.1)[32].

Gene ontology analysis

We used the online tool PANTHER (protein annotation through evolutionary relationship)[33] to classify the function and relevant pathways of genes with FDR significant CpG sites related to lipid metabolites. Briefly, the list of genes was entered into the "Gene List Analysis" tool in PANTHER and the functional classification for each gene was generated. Gene ontology related to molecular function, biological process, cellular component, protein class, and pathway was available for most of the genes identified in the regression results.

4.4 Results

Study Population

Characteristics of CHAMACOS mothers and children included in the study are presented in Table 4.1. Additional data on CHAMACOS demographic parameters have been previously described[19]. Most mothers were overweight or obese (BMI $\geq$ 25 kg/m^2) prior to pregnancy. Roughly equal portions of children were boys and girls, 5% were of low birth weight (<2500 g) and 7% born were premature (<37 weeks).

Maternal Prenatal Metabolomic Profiles

In this study, we focused on maternal metabolites previously identified to be relevant to lipid biosynthesis, arachidonate enrichment and release, and inflammatory signaling[18]. The 92 plasma metabolites in the study included fatty acids, lysolipids, phospholipids, sphingolipids, monoacylglycerols, diacylglycerols, and triacylglycerols (Table 4.2, Figures 4.1 and 4.2). Example distributions of specific fatty acid, lysolipid, and phospholipid metabolites are shown in Figure 4.1. Median lipid metabolite counts ranged from 0.01 (C16:0e/C18:1 PSe) to 44,132.92 (C16:0/C18:1 PC) (Table 4.2). In Figure 4.2, we plotted the median values for the metabolites within each of the four quartiles based on the ranges in the metabolite distributions. The phosphatidylcholine (PC) phospholipids had the highest median values and a broad range across subjects. All metabolites were log(1+x) transformed in analyses in order to reduce the influence of outliers.

Maternal Metabolomics and Newborn DNA Methylation Analyses

Numerous maternal prenatal metabolites were significantly associated, after correcting for multiple hypotheses testing, with DNA methylation of CHAMACOS newborns (Table 4.3). The metabolites that were related to newborn DNA methylation after adjusting for relevant covariates, indicated by an asterisk above the bar plots in Figure 4.2, were distributed across the four quartiles based on metabolite ranges, with four of the nine significant metabolites found in the lowest quartile of ranges (Figure 4.2A). After adjusting for child sex, batch, and white blood cell type estimates, 4 phospholipids (C16:0/C20:4 PS, C16:0e/C18:1 PSe, C18:0/C20:4 alkyl PA, C18:0e/C18:1 PEe), 4 lysolipids (C16:0 alkyl LPA, C18:1 alkyl LPA, C18:1 LPI, C20:4 LPS), and the fatty acid C18:0 FFA were among the significant metabolites related to newborn methylation. The number of CpG sites significantly associated with a specific metabolite ranged from 1 to 6. The phospholipid C18:0/C20:4 alkyl PA, shown in Figure 4.3, was one of two phospholipid metabolites associated with methylation at 6 different CpG sites, shown by the data points above the red genome-wide significance threshold. Prior to adjustment of p values for multiple hypotheses testing, this metabolite was associated with 10,338 CpG sites throughout the genome. Four of the FDR significant CpG sites associated with C18:0/C20:4 alkyl PA were also related to C18:1 LPI.

Several CpG sites had significant relationships with one phospholipid (C18:0/C20:4 alkyl PA) and/or two lysolipid (C16:0 alkyl LPA, C18:1 LPI) metabolites (lysolipids are phospholipid breakdown products). For instance, cg12106728 was negatively associated with C18:0/C20:4 alkyl PA (β=-1.014, FDR p=0.005) and C18:1 LPI (β=-1.667, FDR p=0.003). A CpG site that maps to *CTDSP2* and *MIR26A2*, cg17169243, was inversely related to C18:0/C20:4 alkyl PA (β=-0.454, FDR p=0.019), C18:1 LPI (β=-0.807, FDR p=0.002), and C16:0 alkyl LPA (β= -0.366, FDR p=0.021). cg19220754, located in the body of *TNPO1*, was hypomethylated with increased C18:0/C20:4 alkyl PA (β=-1.843, FDR p=0.019) and C18:1 LPI (β=-2.962, FDR p=0.019). cg24175823 was negatively associated with C18:0/C20:4 alkyl PA (β=-1.341, FDR p=0.002), C18:1 LPI (β=-2.039, FDR p=0.018), and C16:0 alkyl LPA (β=-1.024, FDR p=0.018). Out of the 28 total significant relationships between metabolites and CpG sites, 22 had negative regression coefficients, suggesting that increased lipidomic levels in maternal blood are generally associated with lower DNA methylation in their newborn children.

Although most of the significant associations were negative, some of the positive relationships observed between maternal metabolite levels and infant DNA methylation had the largest effect sizes. A CpG site that maps to *SEPT2* and *HDLBP*, cg16787284, was positively related to C16:0e/C18:1 PSe (β=25.723, FDR p=0.026) and C18:0e/C18:1 PEe (β=1.154, FDR p=0.021). cg22539279, located in *NEK11* and *ASTE1*, was also hypermethylated with increased C16:0e/C18:1 PSe (β=23.200, FDR p=0.021).

In addition, seven of the FDR-significant relationships remained after using the more stringent Bonferroni adjustment. These included the associations of cg10874881 and cg16597728 with the phospholipid C16:0/C20:4 PS, cg12106728 and cg24175823 with the phospholipid C18:0/C20:4 alkyl PA, cg21883754 with the phospholipid ether C16:0e/C18:1 PSe, and cg12106728 and cg17169243 with the lysolipid C18:1 LPI.

Pathway Analysis of Gene Hits

The maternal metabolites significantly associated with newborn methylation included complex lipids and products of lipid degradation, side products generated from choline and fatty

acid release for the developing fetus (Figure 4.4A and 4.4B). These lipids provide a methyl source for DNA methylation and drive growth, providing lipid building blocks and energy (Figure 4.4B). In order to determine the biological relevance of the metabolomic and methylation findings, we ran pathway analysis of the genes for which the significant CpG hits were mapped (Table 4.4). The most common molecular functions relevant to the genes included binding and catalytic activity. Additionally, the genes were involved in a range of biological processes at the cellular and metabolic levels and participated in biological regulation, response to stimulus, and localization. The genes *BDNF*, *PC*, and *SEPT2* have been related to a variety of pathways, including Huntington disease, pyruvate metabolism, and Parkinson disease, respectively.

4.5 Discussion

In this study, we characterized the lipidomic profiles of pregnant women from the CHAMACOS cohort, building upon previous research that identified associations of metabolomic data with phthalates, endocrine disrupting chemicals, and maternal pre-pregnancy BMI[18]. We focused on maternal metabolite levels assessed around 26 weeks gestation, rather than at child delivery, due to the availability of existing metabolomic data and more importantly, to better capture exposures relevant during fetal development that could relate to newborn epigenetic profiles. The 92 lipid metabolites assessed had broad ranges of levels in the women's plasma. We further examined the relationship between maternal metabolite counts and offspring DNA methylation. Out of the lipid metabolites assessed, we identified significant and predominantly negative associations among four phospholipids, four lysolipids, and a fatty acid with newborn methylation. Additionally, several of the significant metabolites were related to multiple CpG sites. For example, the phospholipids C16:0/C20:4 PS and C18:0/C20:4 alkyl PA were both associated with 6 CpG sites. These results strengthen the evidence that maternal metabolites, particularly lysolipids and phospholipids, are related to reduced offspring DNA methylation at CpG sites in genes involved in a variety of biological processes, including catalytic and binding activities.

The wide range in lipid metabolite levels observed in CHAMACOS is similar to a study conducted in 40 mother-child dyads from a clinical birth cohort in Michigan[5]. Marchlewicz et al. examined acylcarnitine, free fatty acid, and amino acid metabolite levels of mothers during the first trimester and at delivery, as well as of their children at birth. They observed wide distributions of free fatty acid levels similar to CHAMACOS, with medians ranging from 0.00 to 152.70 nmol/mL and 0.00 to 247.90 nmol/mL for the maternal first trimester and delivery metabolites, respectively. In CHAMACOS mothers, median free fatty acid metabolite counts ranged from 1.12 to 4337.56. Higher levels in the CHAMACOS cohort could be attributed to differences in study populations; since CHAMACOS participants have a high prevalence of obesity and are Mexican-American as compared to the population in the Marchlewicz et al. study, which was mostly Caucasian; and the species of free fatty acid metabolites assessed in each cohort.

In addition to determining the distributions of lipid metabolites in maternal plasma, another critical objective of the current study was to assess the relationship between maternal blood lipid levels and offspring DNA methylation at birth. Using a targeted approach, previous research in animal models has demonstrated associations between maternal fatty acid diet and increased DNA methylation of the fatty acid desaturase 2 (*Fads2*) gene in the liver of offspring, which encodes for Δ6 desaturase, a rate limiting enzyme in polyunsaturated fatty acid synthesis[16, 17]. A similar study in humans of the effect of fatty acid supplementation showed no

significant differences when comparing cord blood promoter methylation profiles of offspring of pregnant women receiving DHA or those in the placebo group[34]. Additionally, Sphingosine 1-phosphate, a sphingolipid, has been linked at the cellular level to another epigenetic mechanism, histone acetylation[35].

Only a couple of studies have examined the association between blood lipid metabolite profiles and DNA methylation in humans. Petersen et al.[6] assessed concurrent metabolomics profiles, including lipid metabolites, and DNA methylation levels in 1814 adults in Germany. Using methylation data measured by the Infinium HumanMethylation450 BeadChip platform, as used in the current study, and utilizing genome-wide SNP data, they were able to identify a significant and positive relationship, which also showed a strong genetic effect, between the phospholipid PC aa C38:4 and cg11250194. Additionally, several CpG site and metabolite associations were discovered after correcting for genetic effects, including a positive relationship between cg17901584 and the phospholipid PC ae C36:5. We also observed significant associations with phospholipid metabolite levels in the CHAMACOS cohort. Specifically, maternal levels of C16:0/C20:4 PS, C16:0e/C18:1 PSe, C18:0e/C18:1 PEe, and C18:0/C20:4 alkyl PA were all related to newborn DNA methylation.

In CHAMACOS, we observed predominantly negative relationships between maternal second trimester metabolite counts and newborn DNA methylation. Marchlewicz et al.[5] conducted a similar study in 40 predominantly white (85%) mothers and their children from Michigan. They examined whether maternal metabolomic profiles are associated with cord blood methylation globally, measured by *LINE-1* and *LUMA*, and at genes relevant for growth, including the imprinted genes *H19* and *IGF2*. They observed that maternal fatty acids measured during the first trimester were significantly and positively correlated with infant methylation at *LINE-1, IGF2*, *ESR1*, and *PPARα,* and negatively correlated with *H19* and *LUMA*. Additional correlations were found for maternal delivery and cord blood metabolite levels with newborn methylation, with similar directions of association. Although we were not able to replicate the results observed in the Marchelwizc et al. study since they used a candidate gene approach, we were also able to observe a significant relationship between maternal lipid levels and imprinted gene methylation in newborns. Specifically, the relationship between cg27492749, a CpG site within another imprinted gene (*SGCE)*, and the lipid metabolite C18:0/C20:4 alkyl PA. Taken together, these studies provide preliminary evidence of associations between maternal metabolomic environments during pregnancy and DNA methylation status of their offspring.

Several of the genes with significant CpG sites related to maternal metabolite levels in the CHAMACOS cohort are involved in biological pathways associated with early life development. For instance, the *BDNF* gene encodes for the growth factor, brain-derived neurotrophic factor, which is important in the development of the central and peripheral nervous systems of infants[36]. Human septins, including the protein encoded by *SEPT2*, can assemble into higher-order cytoskeletal structures, such as filaments and bundles, which are necessary to the growing fetus[37]. Additionally, the *PC* gene encodes for the enzyme pyruvate carboxylase, which fuels the tricarboxylic acid cycle, the major pathway that drives the biosynthesis of amino acids[38].

The statistically significant lipids in the current study can be linked with DNA methylation in several ways. First, the process of lipid degradation releases fatty acids and lysolipids, as well as choline, which is transported to the developing infant. Choline serves as a major methyl source for DNA methylation and is also necessary for the synthesis of new biological membranes for rapidly dividing cells. The observed associations between DNA

methylation and lipid degradation products may be possible because the metabolic flux is relatively slow or their abundance is low. In contrast, we did not find associations for the phosphatidylcholine (PC) or triacylglycerol (TG) metabolites, which serve as major sources of either fatty acids or choline[39, 40]. The metabolic flux through PC and TG metabolism and their relative abundance is relatively high (Figure 4.2), which could obscure the association. Second, fatty acids provide a major energy source which drives growth in the developing fetus. Thirdly, ether lipids regulate cell differentiation and cellular signaling[41]. Ether lipids are an integral component of lipid membranes, provide enhanced integrity at lipid raft microdomains, and are important for membrane fusion and trafficking.

A limitation of the present study includes the relatively small sample size, which could limit the power to detect significant differences based on the large number of tests performed. However, our study has more power than the most comparable study[5], which has half the number of mother-child dyads with metabolomic and DNA methylation data. Another limitation is the absence of data on child metabolomic profiles at birth. However, given that maternal lipid metabolites can be transferred to the developing fetus via the placenta and that the prenatal period is a time of epigenetic remodeling, we feel that exploring the relationship between maternal metabolite levels during pregnancy, a sensitive time for epigenetic changes, is more relevant. Additionally, although DNA methylation is the most commonly studied epigenetic mechanism, maternal lipid metabolites could also be related to other epigenetic marks in children, including histone modifications.

The main strengths of the current study include the use of multiple 'omics' data from the well-characterized CHAMACOS cohort, including the assessment of numerous classes of lipids, which expanded upon the fatty acids that have been previously assessed in relation to infant methylation data. This allowed for the discovery of novel associations between maternal metabolite levels, including phospholipids and lysolipids, and newborn DNA methylation, which could have implications on child health. Previous studies[42, 43] in pregnant women with poor birth outcomes compared to controls revealed differences in metabolomic profiles of lipid metabolites. Additionally, lower levels of lysophospholipids, phospholipids, and monoacylglycerols during early pregnancy have been associated with macrosomia[44]. Epigenetics could be one mechanism whereby *in utero* exposure to maternal metabolites could impact health in early life. Future research should further explore the potential role of maternal metabolomic profiles on offspring DNA methylation and its role in child health.

4.6 Acknowledgements

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4.7 Publication

This work was published in:

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4.8 Tables

Table 4.1 Characteristics of CHAMACOS children and mothers (N=81 pairs) with newborn 450K data and assessment of maternal metabolomic profiles at 26 weeks gestation

Characteristics	
	N (%)
Maternal age at delivery	
18-24	32 (39.5)
25-29	36 (44.4)
30-34	11 (13.6)
35-45	2 (2.5)
Newborn sex	
Boy	39 (48.1)
Girl	42 (51.9)
	Mean (SD)
Maternal pre-pregnancy BMI (kg/m^2)	25.5 (4.3)
Newborn gestational age (weeks)	38.8 (1.7)
Newborn birth weight (grams)	3413.1 (558.0)
Newborn white blood cell count (%)	
Granulocytes	46.4 (10.3)
CD4+ T	15.5 (6.7)
CD8+ T	13.2 (3.8)
B cells	9.5 (3.2)
nRBC	10.4 (6.5)
Monocytes	8.1 (1.9)
NK cells	0.4 (1.2)

Table 4.2 Distribution of lipid metabolites in plasma of CHAMACOS mothers (N=81)

Metabolite	Pathway	Median	IQR
C16:0/C18:1 DAG	Diacylglycerol	21.56	[14.44, 30.06]
C16:0/C20:4 DAG	Diacylglycerol	0.91	[0.57, 1.35]
C18:0/C18:1 DAG	Diacylglycerol	6.22	[3.83, 9.53]
C18:0/C20:4 DAG	Diacylglycerol	0.07	[0.04, 0.12]
C12:0 AC	Fatty acid	1.12	[0.94, 1.41]
C16:0 AC	Fatty acid	25.18	[14.61, 40.10]
C16:0 FFA	Fatty acid	1660.35	[1282.00, 2199.70]
C18:0 AC	Fatty acid	8.19	[4.08, 11.94]
C18:0 FFA	Fatty acid	817.78	[664.32, 1159.16]
C18:1 FFA	Fatty acid	1109.79	[732.70, 1765.65]
C20:4 FFA	Fatty acid	2.96	[2.04, 3.78]
DHA	Fatty acid	232.88	[39.63, 752.88]
Phytanic Acid	Fatty acid	4337.56	[201.02, 10241.48]
C16:0 alkyl LPA	Lysolipid	0.46	[0.20, 0.85]
C16:0 LPA	Lysolipid	50.51	[40.01, 59.68]
C16:0 LPE	Lysolipid	0.28	[0.15, 0.61]
C16:0 LPS	Lysolipid	3.61	[2.37, 4.75]
C16:0e LPCe	Lysolipid	59.15	[34.00, 85.07]
C16:0e LPCe (lysoPAF)	Lysolipid	122.48	[83.12, 171.97]
C16:0e LPEe	Lysolipid	3.51	[2.56, 4.35]
C18:0 LPA	Lysolipid	9.77	[7.42, 13.39]
C18:0 LPE	Lysolipid	1.47	[0.91, 2.80]
C18:0 LPS	Lysolipid	0.11	[0.07, 0.17]
C18:0e LPEe	Lysolipid	15.59	[2.80, 24.27]
C18:0e LPGe	Lysolipid	0.25	[0.02, 1.12]
C18:1 alkyl LPA	Lysolipid	0.54	[0.37, 0.73]
C18:1 alkyl LPI	Lysolipid	1.3	[0.62, 2.88]
C18:1 LPE	Lysolipid	0.43	[0.21, 1.08]
C18:1 LPI	Lysolipid	0.12	[0.08, 0.17]
C18:1 LPS	Lysolipid	0.41	[0.19, 0.57]
C18:1e LPCe (lysoPAF)	Lysolipid	15.01	[2.96, 24.26]
C18:1e LPEe	Lysolipid	0.11	[0.07, 0.20]
C20:0 LPC	Lysolipid	13.84	[6.64, 19.75]
C20:4 LPA	Lysolipid	2.35	[1.68, 2.91]
C20:4 LPC	Lysolipid	292.84	[196.19, 367.39]
C20:4 LPS	Lysolipid	4.08	[3.60, 4.64]
C20:4e LPSe	Lysolipid	0.02	[0.01, 0.05]
C16:0e MAGE	Monoacylglycerol	255.78	[206.49, 327.70]
C18:0 MAG	Monoacylglycerol	154.63	[72.42, 199.03]
C18:1 MAG	Monoacylglycerol	35.7	[21.99, 64.30]
C18:2 MAG	Monoacylglycerol	6.14	[1.27, 12.82]
C20:4 MAG	Monoacylglycerol	0.47	[0.24, 0.88]
C22:6 MAG	Monoacylglycerol	0.05	[0.03, 0.09]

Metabolite	Pathway	Median	IQR
C16:0/C16:0 PI	Phospholipid	1.9	[1.03, 3.60]
C16:0/C18:1 PA	Phospholipid	11.3	[10.35, 13.37]
C16:0/C18:1 PC	Phospholipid	44132.92	[37269.07, 52132.60]
C16:0/C18:1 PI	Phospholipid	12.26	[7.23, 20.11]
C16:0/C20:4 PC	Phospholipid	28780.3	[24703.64, 34127.57]
C16:0/C20:4 PI	Phospholipid	7.28	[4.30, 12.53]
C16:0/C20:4 PS	Phospholipid	0.04	[0.02, 0.07]
C16:0e/C18:1 PCe	Phospholipid	2154.21	[1889.12, 2520.64]
C16:0e/C18:1 PEe	Phospholipid	0.75	[0.30, 1.02]
C16:0e/C18:1 PSe	Phospholipid	0.01	[0.01, 0.02]
C16:0e/C20:4 PCe	Phospholipid	2833.01	[2487.41, 3331.83]
C16:0p/C20:4 PCp	Phospholipid	1441.64	[1198.07, 1707.04]
C18:0/C18:1 alkyl PA	Phospholipid	4.04	[3.38, 4.59]
C18:0/C18:1 alkyl PI	Phospholipid	0.51	[0.30, 0.88]
C18:0/C18:1 PA	Phospholipid	35.8	[32.05, 42.42]
C18:0/C18:1 PC	Phospholipid	26515.68	[22344.95, 30784.37]
C18:0/C18:1 PE	Phospholipid	0.05	[0.03, 0.09]
C18:0/C18:1 PI	Phospholipid	16.47	[9.57, 28.92]
C18:0/C20:4 alkyl PA	Phospholipid	0.82	[0.68, 1.05]
C18:0/C20:4 alkyl PI	Phospholipid	0.48	[0.30, 0.89]
C18:0/C20:4 PA	Phospholipid	3.23	[2.75, 3.96]
C18:0/C20:4 PC	Phospholipid	25368.63	[22167.33, 28643.58]
C18:0/C20:4 PI	Phospholipid	36.05	[21.71, 52.25]
C18:0/C20:4 PS	Phospholipid	0.61	[0.18, 0.91]
C18:0e/C18:1 PCe	Phospholipid	2440.81	[1981.18, 2796.77]
C18:0e/C18:1 PEe	Phospholipid	0.7	[0.44, 1.18]
C18:0e/C18:1 PGe	Phospholipid	0.45	[0.03, 2.34]
C18:0e/C20:4 PCe	Phospholipid	4815.13	[4010.52, 5666.14]
C18:0e/C20:4 PEe	Phospholipid	0.09	[0.03, 0.16]
C18:0p/C20:4 PCp	Phospholipid	4569.48	[3835.01, 5374.80]
C18:0p/C20:4 PEp	Phospholipid	0.1	[0.04, 0.16]
Cardiolipin C18:1/18:1/18:1/18:1 T2	Phospholipid	1.04	[0.80, 1.31]
C16:0 Ceramide	Sphingolipid	12.11	[9.50, 13.92]
C16:0 SM	Sphingolipid	2178.44	[1830.64, 2544.65]
C18:0 Ceramide	Sphingolipid	1.79	[1.37, 2.36]
C18:0 SM	Sphingolipid	1997.51	[1135.78, 2943.23]
C18:0/C16:0 ceramide-1-phosphate	Sphingolipid	8.26	[7.32, 10.12]
C18:1 SM	Sphingolipid	2325.2	[2009.34, 2810.72]
C18:1/C16:0 ceramide-1-phosphate	Sphingolipid	92.51	[78.65, 109.94]
C20:4 SM	Sphingolipid	1400.77	[881.66, 1765.40]
Lactosylceramide C18:1/C18:0	Sphingolipid	0.5	[0.28, 0.77]
Sphinganine	Sphingolipid	3.64	[1.38, 11.14]
Sphingosine	Sphingolipid	36.95	[19.04, 51.62]
C16:0/C16:0/C16:0 TAG	Triacylglycerol	1690.96	[958.05, 2779.60]

Metabolite	Pathway	Median	IQR
C16:0/C18:1/C16:0 TAG	Triacylglycerol	10433.19	[7397.58, 13658.95]
C16:0/C20:4/C16:0 TAG	Triacylglycerol	3969.7	[2889.16, 5224.91]
C18:0/C18:0/C18:0 TAG	Triacylglycerol	216.26	[121.32, 356.69]
C18:0/C18:1/C18:0 TAG	Triacylglycerol	868.13	[430.36, 1782.46]
C18:0/C20:4/C18:0 TAG	Triacylglycerol	2.72	[1.61, 9.12]

Table 4.3 Significant results of the relationship between prenatal maternal metabolite levels in plasma and newborn cord blood DNA methylation

CpG sites	Chr	N	β[a]	SE	FDR P-Value	Significant Exposures	Genes	Gene Location	Relation to CpG Island
cg02496111	16	81	-3.172	0.467	0.012	C16:0/C20:4 PS	-	-	Island
cg03168497	17	81	-2.118	0.330	0.021	C18:1 alkyl LPA	*MYCBPAP*	Body	Island
cg04108939	1	80	1.169	0.187	0.039	C20:4 LPS	*BEST4*	Body	Island
cg06494167	2	80	-1.428	0.214	0.018	C18:1 LPI	*RMND5A*	TSS1500	Island
cg07370087	1	81	0.469	0.076	0.044	C18:0 FFA	-	-	-
cg08838610	17	81	-3.662	0.589	0.040	C16:0/C20:4 PS	*RPAIN, NUP88*	TSS200, 1st Exon	Island
cg09931872	10	80	-2.409	0.365	0.018	C16:0/C20:4 PS	*WDFY4*	5'UTR	-
cg10528455	2	81	0.492	0.076	0.019	C18:0/C20:4 alkyl PA	-	-	-
cg10874881	7	80	-2.546	0.346	**0.002**	C16:0/C20:4 PS	*POU6F2*	Body	-
cg12106728	12	79	-1.014	0.144	**0.005**	C18:0/C20:4 alkyl PA	-	-	Island
		79	-1.667	0.229	**0.003**	C18:1 LPI			
cg14630692	9	81	-0.305	0.047	0.019	C16:0 alkyl LPA	*URM1*	Body	-
cg14897096	11	78	-1.911	0.286	0.018	C16:0/C20:4 PS	*PC*	5'UTR	N_Shelf
cg15710245	11	79	-0.731	0.114	0.024	C18:0e/C18:1 PEe	*BDNF*	TSS200, TSS1500, Body, 5'UTR	Island
cg16597728	20	79	-2.063	0.293	**0.005**	C16:0/C20:4 PS	*APCDD1L*	Body	S_Shore
cg16787284	2	81	25.723	4.056	0.026	C16:0e/C18:1 PSe	*SEPT2, HDLBP*	TSS200, 5'UTR	Island
		81	1.154	0.180	0.021	C18:0e/C18:1 PEe			
cg17169243	12	79	-0.366	0.057	0.021	C16:0 alkyl LPA	*CTDSP2, MIR26A2*	Body, TSS1500	-
		79	-0.454	0.070	0.019	C18:0/C20:4 alkyl PA			
		79	-0.807	0.105	**0.002**	C18:1 LPI			
cg19220754	5	81	-1.843	0.281	0.019	C18:0/C20:4 alkyl PA	*TNPO1*	Body	Island
		81	-2.962	0.456	0.019	C18:1 LPI			
cg21883754	19	79	-21.976	2.924	**0.002**	C16:0e/C18:1 PSe	*TTYH1*	TSS200	N_Shore
cg22539279	3	81	23.200	3.607	0.021	C16:0e/C18:1 PSe	*NEK11, ASTE1*	TSS200	Island
cg24175823	10	77	-1.024	0.154	0.018	C16:0 alkyl LPA	-	-	Island
		77	-1.341	0.180	**0.002**	C18:0/C20:4 alkyl PA			
		77	-2.039	0.306	0.018	C18:1 LPI			
cg27492749	7	76	-1.548	0.235	0.019	C18:0/C20:4 alkyl PA	*SGCE, PEG10*	Body, TSS1500	Island

Bolded FDR p-values indicate CpGs that were also significant based on the more conservative Bonferroni correction (uncorrected p value < 1.36×10^{-9}). Abbreviations: Chr, chtomosome.

[a]Regression coefficients of the relationship between log(1+x) transformed maternal prenatal metabolites and newborn DNA methylation M values, adjusting for child sex, batch, and white blood cell composition.

Table 4.4 Pathway information for genes with CpG sites significantly associated with maternal metabolite levels. *APCDD1L*, *MYCBPAP*, *POU6F2*, *URM1*, and *WDFY4* did not map to any pathway data in PANTHER.

Gene	Molecular Function	Biological Process	Cellular Component	Protein Class	Pathway
BDNF	Binding; Molecular Transducer Activity	Biological Regulation; Cellular Component Organization or Biogenesis; Cellular Process; Multicellular Organismal Process; Response to Stimulus; Signaling	Cell; Extracellular Region; Membrane; Organelle; Synapse	Signaling Molecule	Huntington Disease; Metabotropic Glutamate Receptor Group II Pathway
BEST4				Transporter	
CTDSP2	Catalytic Activity				
NEK11		Cellular Process	Cell; Organelle	Transferase	
NUP88		Localization	Cell; Organelle; Protein-Containing Complex	Transporter	
PC	Catalytic Activity	Metabolic Process	Cell	Ligase	Pyruvate Metabolism
RMND5A	Catalytic Activity	Metabolic Process	Cell; Organelle; Protein-Containing Complex		
SEPT2	Binding; Catalytic Activity	Cellular Component Organization or Biogenesis; Cellular Process; Localization	Cell; Membrane; Protein-Containing Complex	Cytoskeletal Protein; Enzyme Modulator	Parkinson Disease
SGCE	Binding	Developmental Process		Calcium-Binding Protein	
TNPO1	Binding; Transporter Activity	Localization	Cell; Membrane; Organelle	Transfer/Carrier Protein; Transporter	
TTYH1	Transporter Activity		Cell	Transporter	

4.9 Figures

Figure 4.1 Representative histograms of three lipid metabolite classes: A) a fatty acid, C18:0 FFA; B) a lysolipid, C18:1 alkyl LPA; and C) a phospholipid, C18:0e/C18:1 PEe

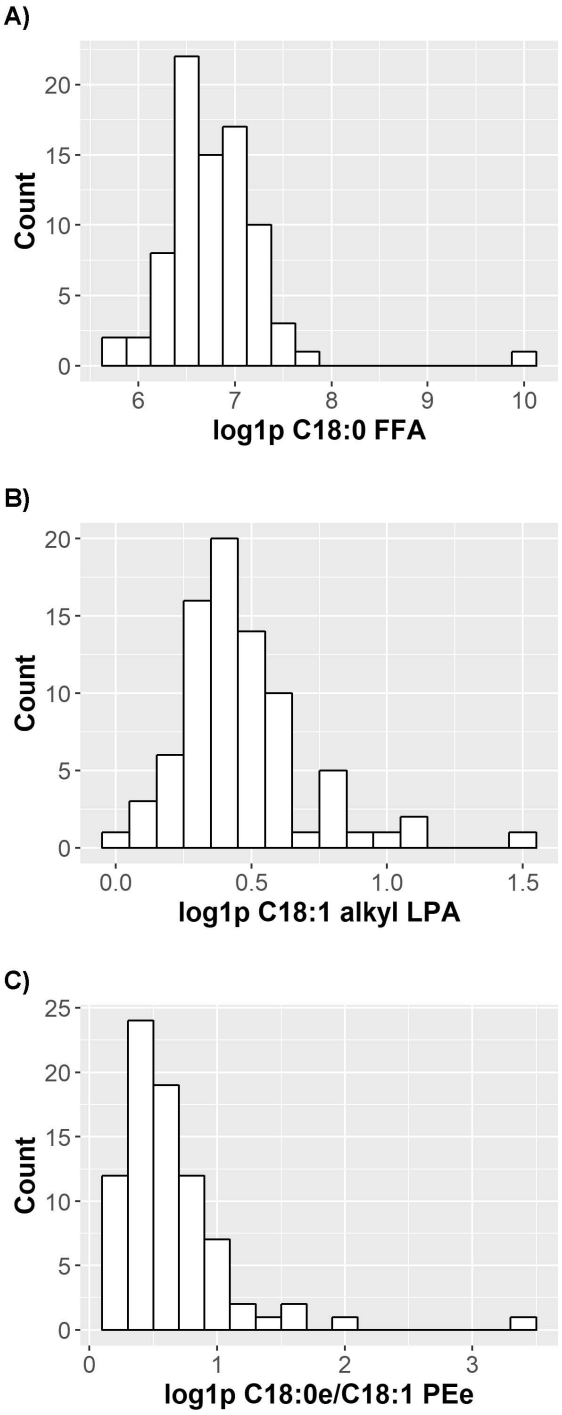


Figure 4.2 Median levels of metabolites with ranges in the A) first (lowest), B) second, C) third, and D) fourth (highest) quartiles. An asterisk (*) above a metabolite name represents metabolites that were significantly associated with DNA methylation in newborns.

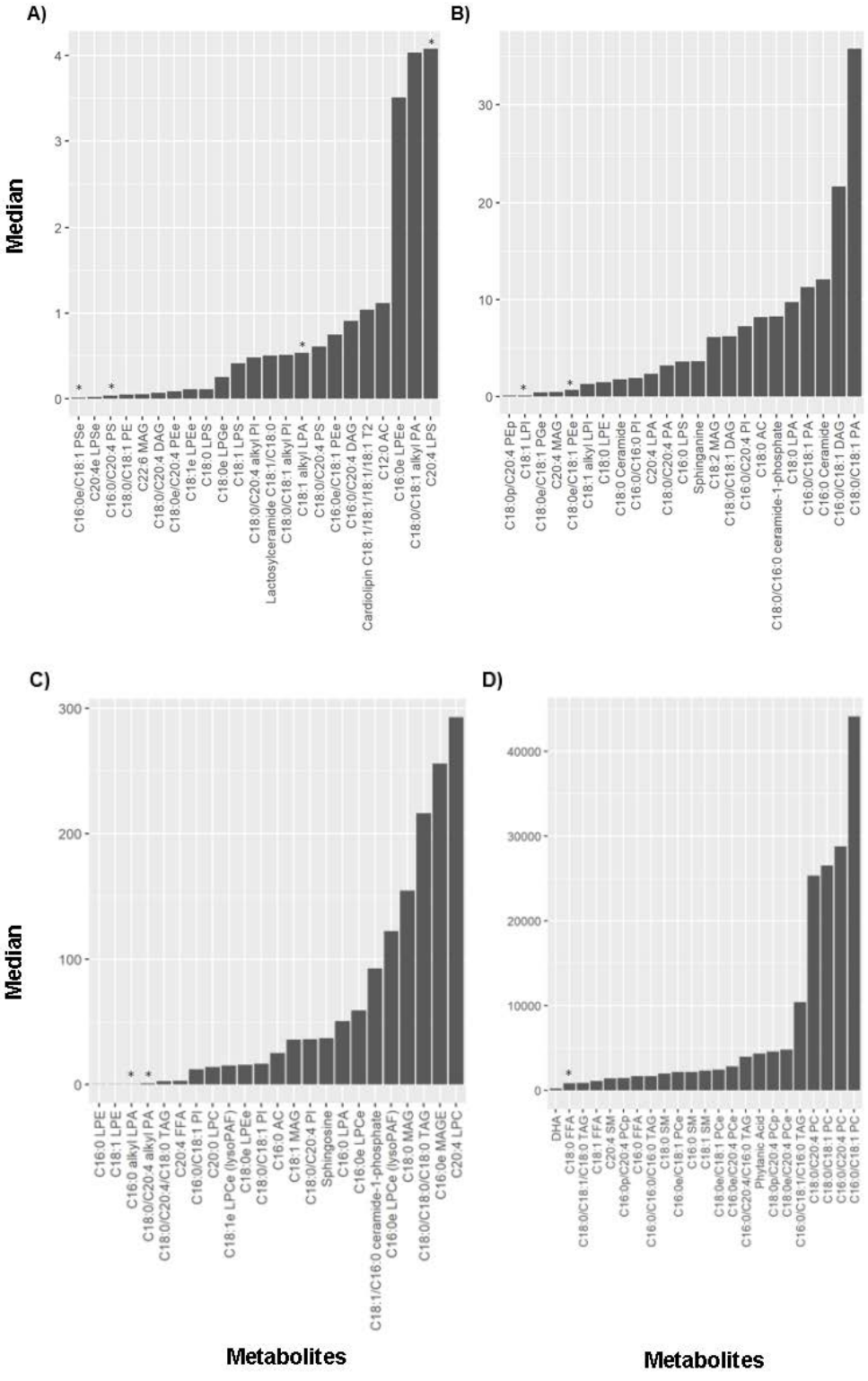


Figure 4.3 Manhattan plot of CpG sites of CHAMACOS newborns associated with C18:0/C20:4 alkyl PA levels in the blood of their mothers during pregnancy. Red line represents genome-wide significance threshold of $-\log_{10}(5.00E\text{-}08)$, while the blue line corresponds to the suggestive threshold of $-\log_{10}(1.00E\text{-}05)$.

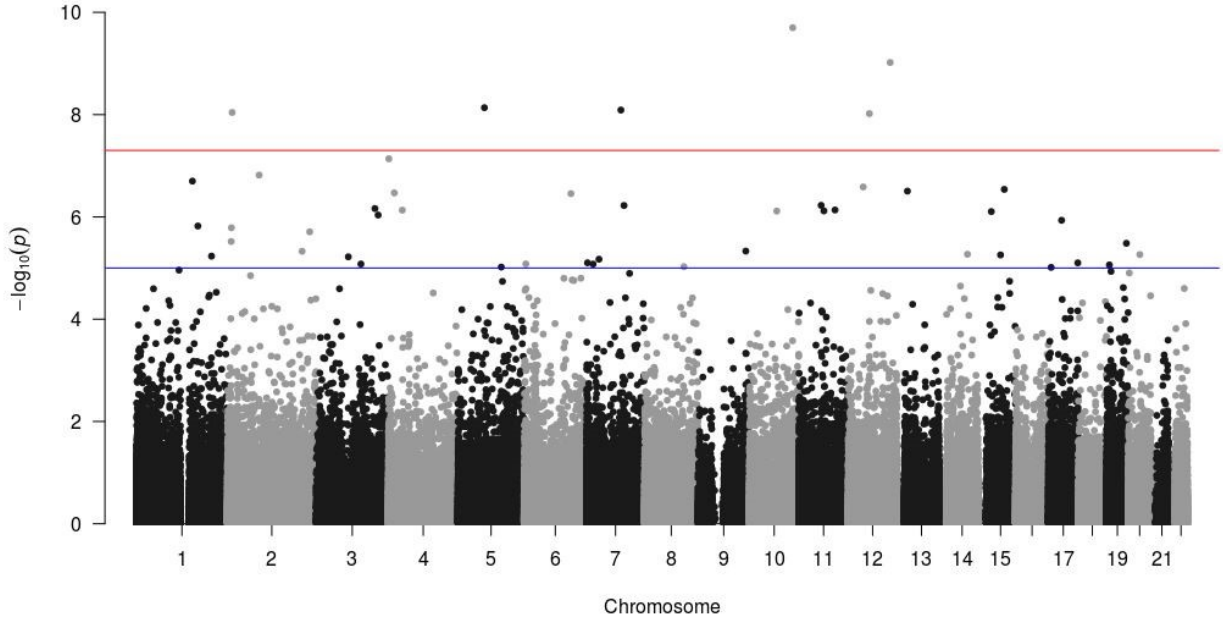
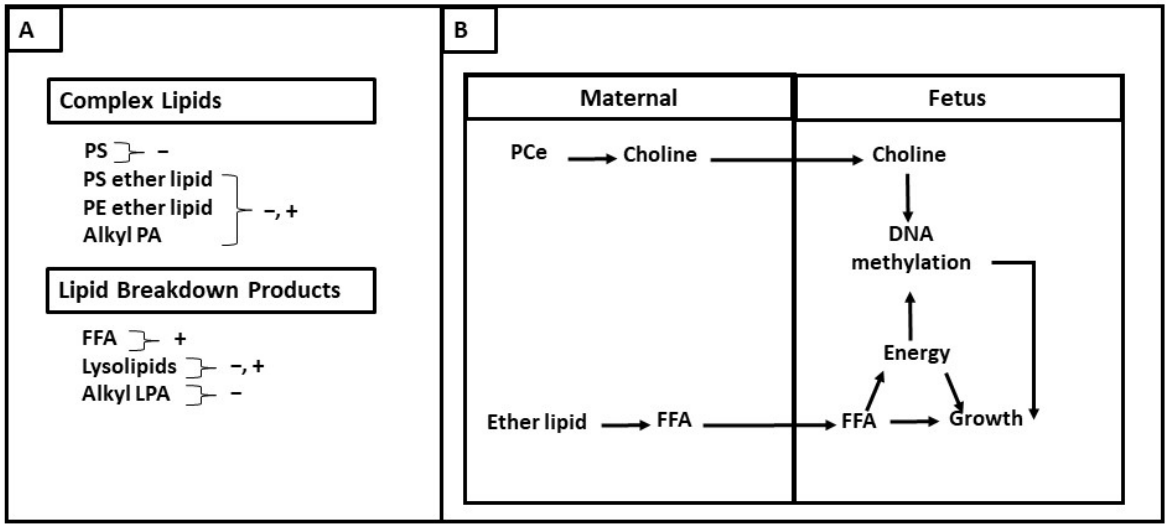


Figure 4.4 Diagram of the significant positive (+) and negative (-) associations observed between maternal metabolites during pregnancy and newborn DNA methylation (A) and the biological pathways whereby maternal lipid levels could impact DNA methylation of their children (B).



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CHAPTER 5. Early Life Home Environment and Obesity in a Mexican-American Birth Cohort: The CHAMACOS Study

5.1 Abstract

Background: Little is known about the impact of the home environment on biomarkers of obesity, such as adipokines, in children. In this study, we examined the relationship of maternal depressive symptoms and potentially protective social factors, including maternal support and the home learning environment, with BMI and adipokines. Methods: Data were obtained from 326 Mexican American participants from the Center for the Health Assessment of Mothers and Children of Salinas cohort. Plasma adipokine levels were assessed in 326 children by enzyme-linked immunoassay at birth or ages 5, 9, or 14. Maternal depressive symptoms were evaluated using the Center for Epidemiological Studies Depression Scale when children were 1, 3.5, 7, and 9 years old; social support was assessed by the Duke-UNC Questionnaire at ages 1 and 5; and home learning environment by the Home Observation for the Measurement of the Environment (H.O.M.E.) at 6 months and 1, 2, 3.5, 7, 9, and 10.5 years. Results: Age was significantly associated with adiponectin (β=-5.0, standard error=0.2) and leptin (β=0.01, standard error=0.003) levels. Individual time point analyses identified significant positive associations of H.O.M.E. scores in childhood with adiponectin at ages 9 (H.O.M.E. score; age 3.5: β=0.9, p=0.04) and 14 (H.O.M.E. score; age 7: β=0.6, p=0.02, age 9: β=0.6, p=0.05, age 10.5: β=0.5, p=0.04). We observed significant relationships of maternal depressive symptoms at age 9 with adiponectin and BMI z-score at age 14 (β=-0.2, p=0.003 and β=0.02, p=0.002, resp.), which were confirmed in longitudinal models. Conclusions: This study adds new evidence that adverse and protective aspects of the home environment could lead to altered obesity status in children.

5.2 Introduction

Obesity is influenced by lifestyle, genetic, and environmental risk factors[1] and remains a critical public health issue nationwide[2, 3]. The burden of the obesity epidemic falls disproportionately on minorities; specifically, Hispanic and non-Hispanic black youth have a higher odds of obesity compared to non-Hispanic white and Asian youth[3]. Children diagnosed as obese, based on body mass index (BMI) at or above the 95th percentile, have an increased risk of developing cardiovascular disease and diabetes later in life and to become obese as adults[4, 5].

Increasing evidence suggests that early life measures of the home environment, including family stability, may influence risk of obesity[6-8]. Aspects of the home environment have also been associated with the production of biologically-active markers of obesity secreted by adipose tissue, including the adipokines: leptin and adiponectin[9-11]. The protein hormone adiponectin, which is primarily derived from adipose tissue, has anti-diabetic, anti-atherogenic, anti-inflammatory, and insulin-sensitizing properties that mediate the pathogenesis of metabolic and cardiovascular disease[12, 13]. Studies in adults have shown inverse relationships between self-reported early life measures of a poor home environment, including abuse, neglect, and household dysfunction, with adiponectin levels assessed in adulthood[9, 11, 14]. Additional research has reported lower adiponectin levels in adult subjects with depressive symptoms in comparison to controls[15-17].

Leptin, another adipokine, is a hormone predominantly secreted by adipose tissue and has also been found in other tissues such as ovaries, placenta, and the brain[18, 19]. Leptin can moderate energy homeostasis, which can be altered during depression, through interaction with central circuits in the hypothalamus[16, 20]. Similar to adiponectin, a number of studies in adults have

reported associations between leptin levels and early life family or home dysfunction[9, 10, 14] and depressive symptoms in adulthood[21]. In contrast to adiponectin, the amount of leptin secreted into circulation is higher in adults who have experienced poor home environments or mental illness. However, the only prospective study to date in children[22], utilizing data from 170 12-year old children from Great Britain, reported suggestive, yet insignificant lower levels of leptin in children exposed to early life maltreatment.

In the Center for the Health Assessment of Mothers and Children of Salinas (CHAMACOS) cohort, we previously reported that 7-year old children whose mothers consistently demonstrated depressive symptoms had 2.4 times the adjusted odds of overweight/obesity compared to those whose mothers never experienced symptoms of depression[23]. Adipokine trajectories from birth to age 9 have been characterized in a subset of CHAMACOS participants, and have shown three distinct risk groups for each adipokine with complex age dynamics through childhood[24]. Thus far, studies on aspects of the home and family environment and adipokines have focused primarily on adult populations, are cross-sectional in nature, are predominantly conducted in African-American and White populations, and primarily are focused on symptoms of mental health and family dysfunction, rather than protective home environment factors.

In the current study, we examine the relationship of maternal depressive symptoms, as well as the positive influences of social support and a healthy home environment, with BMI and adipokines in children from the CHAMACOS cohort, a Mexican-American birth cohort with high levels of childhood obesity[25]. We explored these specific psychosocial factors based on previous research that has provided preliminary evidence of how early life quality of the home environment[26-28], maternal depression[23, 29], and offspring social support[30] can impact adiposity from infancy to adulthood, making use of available data measured at multiple time points in early childhood in the CHAMACOS cohort. We hypothesize that children who were raised in a better home environment with less stress and adversity, and whose mothers had better social support and exhibited fewer symptoms of depression, would have increased adiponectin levels and lower BMI and leptin levels through decreased activation of the hypothalamic-pituitary-adrenal (HPA) axis, which can alter levels of hormones that impact obesity risk. We anticipate that recent and previous exposures to home environment factors will influence adipokines and obesity status, given the increasing awareness of how early life exposures can alter future disease risk. Furthermore, we expand our previous analysis of adipokines to a larger number of participants (n=326) and extend follow up from age 9 through age 14.

5.3 Methods

Study population

CHAMACOS is a longitudinal cohort established to assess the health effects of environmental exposures, such as pesticides, on child growth and development[31]. A total of 601 pregnant women were enrolled in the study between October 1999 and October 2000. Mothers enrolled in the CHAMACOS study were >18 years of age, <20 weeks gestation at enrollment, eligible for low income health insurance (Medi-Cal eligible), Spanish or English speaking, and intended to deliver at the county hospital. Of the 536 live births, the number of children with adipokine measurements at different time points were as follows: delivery (adiponectin, leptin; N=217, N=211), age 5 (N=227, N=201), age 9 (N=250, N=244), and age 14 (N=233, N=228). The total number of children included in this study was N=326, and most had adipokine data for more than three time points (Figure 5.1).

All study procedures were approved by the Committee for the Protection of Human Subjects at the University of California, Berkeley. Informed consent was obtained from all mothers; oral assent was obtained from children from age 7 to 11 years, and written assent was obtained starting at 12 years.

Procedures

Women were interviewed at ~13 and ~26 weeks gestation, following delivery, and during the developmental assessments of the children at 6 months, 1, 2, 3.5, 5, 7, 9,10.5, and 14 years of age. To accommodate the study participants, questionnaires and assessments were administered in either English or Spanish by bilingual, bicultural interviewers. Information gathered at each interview included sociodemographic factors, health status, diet, and an assessment of exposure risk factors.

Maternal depressive symptoms and protective factors, including social support and H.O.M.E scores, were measured throughout childhood and early adolescence in CHAMACOS participants. Maternal depressive symptoms were assessed using the Center for Epidemiological Studies Depression Scale (CES-D)[32] when the children were 1, 3.5, 7, and 9 years old (Figure 5.1). The recommended cutoff score of 16[33] was used to identify mothers exhibiting symptoms of depression and the continuous CES-D scores (max range of 0-60, with a score of 60 representing the worst possible score) were used in regression analyses. Social support was characterized in mothers at child age 1 and 5 years using the Duke-University of North Carolina Functional Social Support Questionnaire, with scores representing an average of responses on a 1-5 scale to a validated eight-item instrument designed to measure functional social support. Higher scores indicate increased social support[34]. Characteristics of the home environment conducive to child development were determined at 6 months and 1, 2, 3.5, 7, 9, and 10.5 years of age using the Home Observation for the Measurement of the Environment (H.O.M.E.)[35]. For earlier visits (ages 6m – 2y), we used the Infant-Toddler H.O.M.E. inventory that combines observations of the home environment and mother child interactions by trained bilingual interviewers with questions about toys, books and the overall home learning environment that were asked of the mothers. The total H.O.M.E. scores are a combination of the responsivity, avoidance, learning and involvement subscales with a range of 16 – 45 points. At later time points (3.5y – 10.5y), we used a short-form of the H.O.M.E. inventory that was based only on maternal questionnaires that was comprised of emotional and cognitive subscales with a range of 5 – 24 points. Higher social support and H.O.M.E. scores indicate more protective environments, whereas increased depression scale scores refer to more severe maternal depressive symptoms. Cronbach's α, a measure of internal consistency, was 0.77, 0.57, and 0.79 for maternal depressive symptoms, social support, and H.O.M.E. scores, respectively, measured throughout childhood in the CHAMACOS cohort.

Children were weighed and measured by trained research staff at ages 5, 9 and 14 years. We measured the children's barefoot standing height to the nearest 0.1 cm using a stadiometer. Standing weight was measured to the nearest 0.1 kg using an electronic scale at age 5 (Tanita 1582) and a foot-to-foot bioimpedence scale at ages 9 and 14 (Tanita TBF-300A Body Composition Analyzer). BMI was calculated as kg/m^2. The CDC age- and sex-specific percentiles were used to categorize children as underweight, normal weight, overweight, or obese and BMI z-scores were used in analyses[36].

Adipokine analysis

Adiponectin and leptin were assessed in blood specimens stored at -80°C collected from CHAMACOS participants at birth (cord blood), and repeatedly at 5, 9, and 14 years of age. We selected samples for analysis based on individuals with plasma available at a given CHAMACOS assessment. The subset of CHAMACOS participants used in this analysis was not significantly different from the main cohort in most demographic characteristics (child sex, baseline poverty, education, gestational age, parity, child overweight status). However, mothers in this sample tended to be slightly older at delivery and had higher average BMI than those not included in the analyses. Plasma adipokines were measured using enzyme-linked immunoassay (ELISA) RayBiotech Human Adiponectin and Human Leptin kits (RayBiotech Inc., Norcross, GA, USA). The protocol developed by the manufacturer was used, with some previously described modifications[37] to increase precision for assessment in children: (a) the standard curve for adiponectin was narrowed in order to obtain better resolution at smaller readings, (b) the standard curve for leptin was broadened, and (c) plasma samples in the leptin analysis were more diluted. Final dilutions for the RayBio Human Leptin ELISA and the Human Acrp30 ELISA were 1:70 and 1:30,000, respectively. Absorbance values were obtained at a wavelength of 450 nm, with an upper absorbance cutoff of 4.0 optical density units. The minimum detectable concentrations for adiponectin and leptin were 10 and 6 pg/mL, respectively. Internal lab controls were included on each plate to ensure reproducibility between the experimental runs, with average intra- and inter-plate CVs less than 5 and 14%, respectively. All plasma samples were measured in duplicate.

Statistical analysis

We $\log_{10}$ transformed leptin concentrations in CHAMACOS study participants for use in regression and correlation analyses since they were right-skewed. Adiponectin concentrations did not require this adjustment. The interrelationships between adiponectin and log-transformed leptin levels measured at birth and ages 5, 9, and 14 were assessed by Pearson correlation coefficients. Differences in mean adipokine levels by sex were determined by t-tests, and longitudinal trends of adipokines across childhood were characterized by generalized estimating equations (GEE) with an exchangeable correlation structure[38]. Since leptin demonstrated a difference by sex, we adjusted for sex in the GEE models with leptin as the outcome.

Linear regression models with robust estimates of the standard errors were used to determine the association of continuous measures of maternal scores on the CES-D, HOME scores, and total social support scores with concurrent or future child adipokine levels and BMI z-scores, which were assessed at ages 5, 9, or 14. CES-D maternal depressive symptom, Duke-UNC social support, and raw H.O.M.E. scores were assessed as continuous variables in regression models. We included additional covariates in the models that were identified in previous literature in CHAMACOS[39] and in research assessing the relationship between adversity and obesity trends[6].

Specifically, two regression models were performed to assess the relationship between each independent home environment variable (i.e. continuous measures of maternal depressive symptoms, raw HOME scores, and total social support) and outcome (adiponectin, log-transformed leptin, and BMI z-score): Model 1, the unadjusted model and Model 2, adjusting for child sex and maternal pregnancy variables. Maternal pregnancy variables in Model 2 included continuous (i.e. maternal pre-pregnancy BMI, years in the U.S., pregnancy sugar-sweetened beverage consumption per week), categorical (maternal education level, poverty status), and

binary (smoking and alcohol consumption) variables. The maternal education variable was coded as shown in Table 5.1. Maternal baseline poverty was coded as at or below poverty, greater than poverty to 200% of the poverty level, and greater than 200% of the poverty level. As a sensitivity analysis, we also ran Model 2 with an adjustment for poverty assessed concurrently with the outcome of interest (adipokines or BMI z-score). The results were similar to those of Model 2; thus, they were not presented in the tables.

In addition, regression was used to assess the relationship between longitudinal trends of measures of the home environment and the outcomes (adipokines and BMI). We used GEE-derived standard errors based on exchangeable working correlation models to provide robust inference in the presence of repeated measures. For both the maternal depressive symptom and H.O.M.E. scores, we generated a new variable by using the values for each that were measured at the same time or prior to the assessment of adipokines or BMI. Specifically, maternal depressive symptom measurements at child age 3 corresponded to obesity measurements at age 5, and depressive symptoms at age 9 corresponded to adipokine and BMI measurements at ages 9 and 14. We used the same time points for the H.O.M.E. score variable, with the exception of the correspondence of the 10.5 year H.O.M.E. score with the 14 year outcome assessment. We used social support values from the 5 year child assessment because it was the closest value for all time points of the outcomes. Henceforth, we will designate these variables as time varying measures of the home environment to distinguish them from the original variables. We used GEE models to assess the relationship between the time varying variables for maternal depressive symptoms, social support, or home environment and repeated measurements of adipokines and BMI from ages 5, 9 and 14. In the models, we adjusted for age and child sex, since they have been previously shown to influence adipokine and BMI trends and would be the most likely confounders in the current analysis. The following model was used:

$Y_{ij} = b_0 + b_1X_{1ij} + b_2X_{2ij} + b_3X_{3i} + e_{ij}$

where Y_{ij} is either the BMI z-score or adipokine level for the i-th subject at the j-th anthropometric or adipokine assessment in childhood (j=5, 9, 14). X_{1ij} is the time varying home environment value (i.e. H.O.M.E., social support, or maternal depressive symptom score) for the i-th subject and the j-th assessment, where j is time point of the home environment measurement that is either the same time as the adipokine or BMI assessment or the measurement prior. X_2, X_3 and b_2, b_3 represent the covariates and their respective slopes for the age and child sex variables. e_{ij} is the residual error term.

As a sensitivity analysis, we ran additional models, including one with all the time varying exposure variables (maternal depressive symptoms, H.O.M.E. score, and social support) and another model with poverty at outcome assessment. We also examined the potential of social support to moderate the relationship between home environment factors and obesity. We generated interaction terms by multiplying the maternal depressive or H.O.M.E. time varying scores by the social support variable, and included the new variable in analyses. We also ran mixed effect regression models to confirm GEE findings. Given the sample size, more complicated longitudinal models did not appear to have sufficient power to detect changes in measures of obesity related to previous or concurrent home environment parameters.

We controlled for the false discovery rate (FDR) in the analysis of individual assessments of measures of the home environment with child adipokines and BMI. All statistical analysis was performed in STATA (version 12.1, STATA Corp., College Station TX). Statistical significance was set at $p<0.05$.

5.4 Results

Study sample characteristics

Demographic and anthropometric data for CHAMACOS mothers and children (n=326) with adipokines assessed at least once during childhood (delivery or ages 5, 9, or 14) are shown in Table 5.1. At pregnancy, the majority of mothers were young, had lived in the United States for ten or fewer years, did not obtain a high school diploma, and had overweight or obesity. In our study, 48% of participants were boys and 52% were girls. More than half of the children at each time point had overweight or obesity: 53% of 5 year olds, 55% of 9 year olds, and 54% of 14 year olds.

Age and sex adipokine trends

Adiponectin levels were highest at birth (mean ± SD, 101.2 ± 32.9 μg/mL) and were lower as children got older (age 5: 36.4 ± 16.2 μg/mL; age 9: 40.7 ± 18.1 μg/mL), and were lowest in adolescence (age 14: 23.7 ± 11.0 μg/mL) (Table 5.2, Figure 5.2). Leptin levels at birth were high (18.6 ± 18.7 ng/mL), which could indicate an influence of maternal levels of leptin. Leptin levels were much lower at age 5 (3.6 ± 3.3 ng/mL) and were higher as the children aged (age 9: 15.0 ± 16.7 ng/mL; age 14: 22.2 ± 17.2 ng/mL). Adiponectin levels did not vary by sex, with the exception of a borderline significant (p-value=0.049) difference at age 14 with higher levels in girls compared to boys (Table 5.2). Log leptin levels from delivery through age 14 were consistently higher in girls. Adiponectin levels were moderately and positively correlated across different time points, becoming stronger as children aged (Table 5.3). Adiponectin levels at a given time point were most correlated with measurements at adjacent time points (Table 5.3). Similar trends were observed in the leptin measurements. At each assessment, adiponectin levels were significantly and inversely associated with leptin levels, with the exception of delivery levels, when the negative trend was not significant (p-value=0.70) (Figure 5.3). Age was a strong predictor ($p<0.001$) of leptin and adiponectin levels in the GEE model. For adiponectin, β value was close to -5.0 (GEE β=-4.975, robust SE =0.153), and for leptin, β value was 0.013 (0.003), respectively.

Maternal depressive symptoms and home environment protective factors

Among the mothers, 50%, 43%, 26%, and 26% were at risk of clinical depression with CES-D scores ≥16 when children were assessed at 12 months, 3.5 yrs, 7 yrs, and 9 yrs, respectively (Table 5.4). Scores reported at 12 months could also reflect symptoms of postpartum depression. Median maternal social support scores were high (1y: 4.0; 5y: 4.3). Median H.O.M.E. scores varied across the collections, reflective of the use of the Infant-Toddler H.O.M.E. inventory for earlier visits (ages 6m – 2y) and the short-form of the H.O.M.E. inventory for the later time points (3.5y – 10.5y). Specifically, median scores ranged from 26 to 36 and 14 to 18 for the earlier and later visits, respectively.

Individual assessments of measures of the home environment with child adipokines and BMI

We assessed the relationship of measures of the home environment across childhood at individual time points with obesity, as measured by BMI z-score, and adipokines. Maternal depressive symptoms at child ages 3.5 and 9 were positively associated with BMI z-score at ages 9 and 14 (Table 5.5 and Supplemental Table 5.2). A one-unit increase in CES-D score when children were 3.5 and 9 corresponded to a 0.01-point increase in BMI z-score at age 9 (95% C.I.;

3.5yr: 0.003, 0.02; 9yr: 0.002, 0.02) and age 14 (95% C.I.; 3.5yr: 0.003, 0.02; 9yr: 0.006, 0.03). Social support and H.O.M.E. scores were not significantly associated with 9 yr BMI z-scores. However, a unit increase in social support at age 5 corresponded to a significant 0.2-point decrease in BMI z-score at age 14 (95% C.I.: -0.3, -0.05). Additionally, a one-unit increase in H.O.M.E. score at age 9 was associated with a 0.05-point decrease in BMI z-score at age 14 (95% C.I.: -0.09, -0.005). The associations of maternal depressive symptoms at age 9 and social support at age 5 with BMI z-score at age 14 remained significant after FDR adjustment (all $p<0.01$).

In models with adiponectin as the outcome, we observed a consistent trend across models of higher H.O.M.E. score, indicating a more enriched home environment, with increased adiponectin at age 9. Positive associations with similar magnitudes of effect sizes were with H.O.M.E. scores at ages 3.5, 7, and 9, but only age 3.5 was significant in crude models, indicating that a one-unit increase in H.O.M.E. score at age 3.5 corresponded to a 0.9-point increase in adiponectin at age 9 (95% C.I.: 0.03, 1.8). Similarly, H.O.M.E scores were positively associated with adiponectin at age 14, with larger effect sizes observed in crude models with the 7, 9, and 10.5 yr H.O.M.E. scores. Specifically, a one-unit increase in the total H.O.M.E. score at ages 7, 9, and 10.5 corresponded respectively to a 0.6-point increase (95% C.I.: 0.1, 1.2), 0.6-point increase (95% C.I.: 0.001, 1.2), and a 0.5-point increase (95% C.I.: 0.02, 1.0) in adiponectin at age 14.

We identified inverse associations of maternal depressive symptoms and 9yr adiponectin levels; however, results did not reach statistical significance. In the 14 yr adiponectin models, a one-unit increase in CES-D score when children were 7 and 9 corresponded to a 0.2-point decrease (95% C.I.; 7yr: -0.3, -0.03; 9yr: -0.3, -0.07) in adiponectin.

Social support at age 5 had a positive relationship with adiponectin at age 9 and 14. Specifically, a one-unit increase in social support at age 5 corresponded to a 1.6-point increase in 9yr adiponectin levels (95% C.I.: -0.9, 4.0), which was not significant but was similar in magnitude to a borderline significant 1.3-point increase in 14 yr adiponectin (95% C.I.: 0.003, 2.7). For the adiponectin models, only the relationship between maternal depressive symptoms and adiponectin at age 14 remained significant after FDR adjustment ($p=0.003$).

We also observed significant associations between symptoms of maternal depression, H.O.M.E. scores, and social support, predominantly in early childhood, and leptin levels in CHAMACOS children. In crude models, a one-point increase of maternal depressive symptoms at child age 3.5 corresponded to a 0.007-point increase in log leptin at age 9 (95% C.I.: 0.001, 0.013), while a unit increase in scores at age 7 significantly related to a 0.007 increase in 14 yr log leptin levels (95% C.I.: 0.001, 0.013). A unit increase in H.O.M.E. score at age 2 corresponded to a 0.03-point increase (95% C.I.: 0.003, 0.055) in log leptin levels at age 9. In the 9 and 14 yr leptin models, the direction of association between social support and leptin was consistently negative.

We also examined the relationship between positive and negative aspects of the home environment and adipokines at age 5 (Supplemental Table 5.1). Results were similar in magnitude to the 9 and 14 year adipokine and BMI models, but did not reach statistical significance, suggesting that obesity status and biomarkers of obesity in later childhood may be more sensitive to the effects of maternal depressive symptoms or protective home environment factors.

Longitudinal assessment of measures of the home environment with child adipokines and BMI

Longitudinal models of adipokines and BMI z-score showed significant relationships with the most recent measures of maternal depressive symptoms and social support (Table 5.6). Maternal depressive symptoms were significantly associated with increased BMI z-score (β: 0.007; 95% C.I.: 0.0004, 0.014) and decreased adiponectin (β: -0.248; 95% C.I.: -0.376, -0.119). Social support at age 5, which was the closest assessment to all childhood adipokine measurements (age 5, 9 and 14) was negatively associated with BMI z-score (β: -0.162; 95% C.I.: -0.273, -0.050) and leptin (β: -0.056; 95% C.I.: -0.096, -0.016). Cumulative H.O.M.E. score was not significantly associated with longitudinal levels of adiponectin across childhood (β: -0.285; 95% C.I.: -0.694, 0.124; p=0.17), contrary to the results observed in the individual models. In the sensitivity analysis, we found no significant differences in the model including all the exposure variables (maternal depressive symptoms, H.O.M.E. score, and social support) and another model with poverty at outcome assessment. The results of the analyses of a potential social support moderation of the relationships of H.O.M.E. score and maternal depressive symptoms with obesity indicated a lack of interaction. Mixed effect models produced similar results to the GEE models.

5.5 Discussion

In this longitudinal study, we examined adipokine levels and BMI throughout childhood and assessed their relationship with early life measures of the home environment in a Mexican-American birth cohort. Adiponectin levels in CHAMACOS children were high at birth and, on average, lower as the children aged. Leptin levels were high at birth and during adolescence, but lower during early childhood (age 5). Previous mixture modeling analyses that reported trajectory clusters for adiponectin and leptin in a subset of CHAMACOS children up to age 9 observed similar patterns in adipokine levels, including the large difference in adiponectin levels at birth and early childhood [24]. We found consistent positive associations between H.O.M.E. score in late childhood and adiponectin at ages 9 and 14 indicating that a protective home environment is associated with beneficial profiles of adiponectin in children. Significant associations were observed between maternal depressive symptoms and H.O.M.E. score in early childhood and leptin at ages 9 and 14, but effect sizes were relatively small. We also report positive associations between maternal depressive symptoms at child age 3 and 9 with BMI z-scores at ages 9 and 14, suggesting that increased maternal depressive symptoms are related to higher BMI z-scores in offspring. This data indicate that aspects of the home environment of CHAMACOS children could lead to altered obesity status and the expression of biomarkers of obesity. Clinical implications of these findings could include the direct impact of psychosocial factors on child health status and indirect effects, such as increased medical costs due to obesity comorbidities.

In addition to previous adipokine trajectories research in the CHAMACOS cohort[24], there have been only a few studies that have assessed trends of repeated measurements of adipokines throughout childhood. Mantzoros et al.[40] examined the relationship between cord blood and 3 year adipokine levels in 588 children in the Project Viva cohort. The authors found that while there was not a significant relationship between adiponectin levels at birth and age 3, leptin levels at the assessments were positively associated (crude model p=0.0003). Gruszfeld et al.[41] studied trends in adipokines in 459 children from infancy to age 8, observing a decrease in adiponectin and leptin levels from infancy, with an increase around school age for adiponectin

only. This finding is similar to our observations in the CHAMACOS cohort over a longer period from birth to 14 years of age. Another study in 519 Japanese children age 9 to 10 years old observed positive associations ($p<0.001$) between adipokine levels at baseline and values measured at the three-year follow-up[42]. In the current study, we also observed positive associations between adipokine assessments throughout childhood, with the strongest correlation observed for consecutive measurements. At each time point, we found leptin values were higher in girls compared to boys, which is consistent with previous reports[42, 43]. The sex difference observed for leptin, but not for adiponectin, could be a result of differences in body composition, as well as the potential of serum androgen levels to reduce the amount of leptin in boys[44].

In addition to assessment of trends in adipokine levels across childhood and by child sex, we examined the relationship of BMI, adiponectin, and leptin with maternal depressive symptoms and protective factors of the home environment, including social support and H.O.M.E. scores. Previous research has identified relationships of early life aspects of the home environment[26-28], maternal depression[23, 29], and offspring social support[30] with obesity risk in offspring. In the CHAMACOS cohort, we identified consistent and positive associations between maternal depressive symptoms at child age 3 and 9 with BMI in adolescence. The positive relationship identified in the current study is similar to a previous study conducted by Audelo et al.[23] in 332 7-year old CHAMACOS children, where youth whose mothers demonstrated depressive symptoms at the 1, 3.5 and 7 year child assessments had 2.4 times the adjusted odds of overweight/obesity relative to those whose mothers never experienced symptoms of depression. A similar study[29], including 1,090 children with anthropometric assessments at 3 time points during early childhood, reported that children with mothers exhibiting depressive symptoms, assessed by CES-D when the child was 1 month, as well as 2 and 3 years old, were 1.7 times more prone to be overweight than their counterparts whose mothers did not report depressive symptoms. Taken together, these studies suggest a possible role of maternal depression in offspring obesity status and a potential avenue for obesity intervention.

Most of the research regarding the relationship between adversity and adipokine measurements has been conducted in cross-sectional assessments of adult populations. Positive associations were found between abuse, family instability, and depressive symptoms, in early childhood or adulthood, and adult leptin levels[9, 10, 21, 45], whereas negative relationships were observed between home and family measures and adiponectin in adulthood[9, 11, 15-17, 46]. In our prospective analysis in Mexican-American children, we also saw positive associations between maternal depressive symptoms and leptin levels throughout the child's youth, and negative associations with adiponectin, particularly in older children. In addition, the positive associations we observed between H.O.M.E score and adiponectin at 9 and 14 years concurs with trends seen in the literature; in this case, as H.O.M.E. scores decrease, indicating poor home learning environment, adiponectin levels decline. Since adiponectin has many beneficial immunological and metabolic properties, a profile of lower levels is less desirable. The association between H.O.M.E. scores and adiponectin in the longitudinal models did not reach statistical significance ($p=0.17$); and thus, was unable to completely corroborate the individual time point findings.

Although the current study relies on maternal assessment of adversity as children age into adolescence, it is plausible that maternal depressive symptoms, social support, and home environment could impact mental well-being, and biomarkers of obesity, of their children through several pathways. Numerous studies have demonstrated associations between stress and depressive symptoms in mothers and altered risk of obesity in their children, potentially mediated by parenting behaviors (i.e. through unhealthy dietary practices or sedentary lifestyles)

or parent-child interactions[47-50]. Maternal stress can induce a subsequent stress response in their children, as a result of augmented levels of cortisol[51]. In addition, pervasive stress during childhood has been shown to activate the hypothalamic-pituitary-adrenal (HPA) axis, resulting in the release of hormones such as glucocorticoids that have downstream effects on obesity risk[52].

The biological pathways whereby family instability, stress, and depression in an individual can affect their adipokine profiles are well-documented in humans. As an anti-inflammatory adipokine, adiponectin can inhibit the function of macrophages and tumor necrosis factor-α, and increase the secretion of anti-inflammatory cytokines, including interleukin-10 and interleukin-1 receptor antagonist[53-55]. However, during symptoms of depression, pro-inflammatory cytokines, such as interleukin-6, can alter HPA activity, releasing glucocorticoids that can prevent adiponectin from exerting its anti-inflammatory effects[56]. Conversely, glucocorticoid secretion as a response to stressors enhances the production of leptin in humans[57, 58].

The main strengths of the current study include the use of data collected longitudinally to assess the relationship between maternal depressive symptoms, social support, and the home environment and adipokine trends in a well-characterized Mexican-American birth cohort. Understanding the relationship between the home environment and biomarkers of obesity in this population is particularly relevant due to the high prevalence of both parental pre-pregnancy and childhood obesity. However, we recognize certain limitations, since we did not examine more data on child behavior, adverse experiences, and emotional well-being, which could mediate the relationship between maternal psychosocial factors and child obesity risk. In addition, other home environment factors that were not examined in the current study, such as single parent household and crowding, could influence adipokine levels in children. Given the projected growth in the Hispanic population in the United States[59] and the high prevalence of obesity in Mexican-American children[3], which we have observed in the CHAMACOS cohort, this research can have health implications in the general population and in minority populations at high risk of obesity. This study provides novel support for the relationship between early life exposure to maternal depressive symptoms and protective factors, such as social support and an enriching home environment, and their association with adipokines. This research can inform potential public health strategies to address early childhood factors that contribute to the obesity epidemic and comorbidities of obesity.

5.6 Acknowledgements

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5.7 Publication

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5.8 Tables

Table 5.1 Demographic characteristics of CHAMACOS mothers and children with adipokine data at delivery or age 5, 9, or 14 years (n=326)

Characteristic	N (%)
Maternal age at pregnancy	
18-24	134 (41)
25-29	107 (33)
30-34	54 (17)
35-45	31 (10)
Number of years mother lived in US at pregnancy	
Less than 1 year	57 (17)
1-10 years	174 (53)
11 or more years	95 (29)
Parity	
0	105 (32)
≥ 1	221 (68)
Education	
≤ 6th grade	144 (44)
7-12th grade	115 (35)
≥ High school graduate	67 (21)
Maternal pre-pregnancy BMI[a]	
Underweight (< 18.5 kg/m^2)	2 (1)
Normal (18.5-24.9 kg/m^2)	112 (34)
Overweight (25-29.9 kg/m^2)	127 (39)
Obese (≥ 30 kg/m^2)	84 (26)
Child sex	
Boy	155 (48)
Girl	171 (52)
Gestational age	
34-36 weeks	23 (7)
≥ 37 weeks	303 (93)
Child birthweight	
Low birthweight (< 2500 g)	12 (4)
Normal birthweight (≥ 2500 g)	314 (96)
Child 5 year BMI (CDC categories)[a]	
Normal (<85 percentile)	139 (47)
Overweight (≥85 percentile, < 95 percentile)	61 (20)

Characteristic	N (%)
Obese (≤ 95 percentile)	98 (33)
Child 9 year BMI (CDC categories)[a]	
Normal (<85 percentile)	134 (45)
Overweight (≥85 percentile, < 95 percentile)	50 (17)
Obese (≤ 95 percentile)	114 (38)
Child 14 year BMI (CDC categories)[a]	
Normal (<85 percentile)	136 (46)
Overweight (≥85 percentile, < 95 percentile)	56 (19)
Obese (≤ 95 percentile)	103 (35)

[a]Total number of observations for BMI varies due to missing data

Table 5.2 Plasma adipokine levels at delivery and ages 5, 9, and 14

	N	Mean	SD	Min	Max	P-value
Adiponectin (µg/mL)						
Delivery						
Boys	103	97.7	30.3	2.5	163.0	0.14
Girls	114	104.3	34.9	7.4	186.9	
All	217	101.2	32.9	2.5	186.9	
Age 5						
Boys	105	35.5	15.9	0.1	86.1	0.46
Girls	122	37.1	16.5	3.9	97.4	
All	227	36.4	16.2	0.1	97.4	
Age 9						
Boys	119	40.6	19.2	8.2	93.3	0.92
Girls	131	40.8	17.2	2.8	92.4	
All	250	40.7	18.1	2.8	93.3	
Age 14						
Boys	107	22.2	11.9	5.5	57.7	0.049
Girls	126	25.0	10.2	4.4	47.6	
All	233	23.7	11.0	4.4	57.7	
Leptin (ng/mL)						
Delivery						
Boys	98	13.7	15.0	1.9	84.6	<0.001
Girls	113	22.8	20.5	1.4	97.1	
All	211	18.6	18.7	1.4	97.1	
Age 5						
Boys	82	3.0	2.3	0.2	16.4	0.073
Girls	119	4.1	3.7	0.3	22.2	
All	201	3.6	3.3	0.2	22.2	
Age 9						
Boys	113	11.4	12.2	0.9	66.1	0.017
Girls	131	18.1	19.3	0.7	93.3	
All	244	15.0	16.7	0.7	93.3	
Age 14						
Boys	102	14.2	13.7	0.4	55.4	<0.001
Girls	126	28.6	17.1	2.8	73.8	
All	228	22.2	17.2	0.4	73.8	

P-values obtained from t-tests comparing adipokine levels in boys and girls at different assessments. SD=standard deviation, Min=minimum, Max=maximum

Table 5.3 Pearson correlation matrix of adipokines assessed at delivery and ages 5, 9, and 14

	A_0	A_5	A_9
A_5	**0.26**	-	-
A_9	**0.26**	**0.49**	-
A_{14}	**0.21**	**0.35**	**0.52**

	L_0	L_5	L_9
L_5	**0.19**	-	-
L_9	**0.37**	**0.51**	-
L_{14}	**0.28**	**0.35**	**0.56**

Bolded values are significant at $p<0.05$

A_0= adiponectin at delivery, A_5= adiponectin at age 5, A_9= adiponectin at age 9, A_{14}= adiponectin at age 14, L_0= log-transformed leptin at delivery, L_5= log-transformed leptin at age 5, L_9= log-transformed leptin at age 9, L_{14}= log-transformed leptin at age 14

Table 5.4 Distribution of home environment parameters in CHAMACOS participants

Adversity Parameter	Child Age	N	Mean	SD	Min	Max
Maternal Depressive Symptoms (CES-D)	1 yr	301	16.7	11.0	0.0	50.0
	3.5 yr	291	15.0	10.9	0.0	49.0
	7 yr	308	10.8	9.5	0.0	43.0
	9 yr	278	10.5	10.4	0.0	46.0
Duke Social Support	1 yr	303	3.8	1.0	1.0	5.0
	5 yr	299	4.0	1.0	1.1	5.0
Total H.O.M.E Score	6 mo	325	31.8	4.3	16.0	43.5
	1 yr	325	35.6	3.3	19.3	43.5
	2 yr	325	25.8	2.7	13.3	31.0
	3.5 yr	325	17.2	2.4	11.1	23.9
	7 yr	319	17.7	2.8	9.0	24.0
	9 yr	307	14.3	2.8	5.0	20.0
	10.5 yr	298	14.5	2.8	6.0	20.0

SD=standard deviation, Min=minimum, Max=maximum, CES-D=Center for Epidemiological Studies Depression Scale, HOME=Home Observation for the Measurement of the Environment

Table 5.5 Relationship of aspects of the home environment during childhood with adipokines or body mass index Z-score at age 14

	β[1] (95% Confidence Interval)						
	Depression Scale				Duke SS		
	1Y	3Y	7Y	9Y	1Y	5Y	
BMI Z-score							
Model 1	0.010 (-0.001, 0.021)	**0.014 (0.003, 0.024)**	0.009 (-0.002, 0.020)	**0.016 (0.006, 0.026)***	-0.022 (-0.140, 0.097)	**-0.164 (-0.278, -0.050)***	
Model 2	0.006 (-0.005, 0.017)	0.009 (-0.002, 0.019)	0.009 (-0.002, 0.020)	**0.012 (0.001, 0.023)**	-0.009 (-0.127, 0.110)	**-0.157 (-0.268, -0.046)***	
Adiponectin							
Model 1	-0.078 (-0.204, 0.048)	-0.102 (-0.235, 0.031)	**-0.169 (-0.311, -0.027)**	**-0.203 (-0.336, -0.070)***	-0.391 (-1.854, 1.073)	**1.342 (0.003, 2.682)**	
Model 2	-0.028 (-0.160, 0.103)	-0.059 (-0.202, 0.084)	**-0.177 (-0.326, -0.028)**	**-0.172 (-0.318, -0.026)**	-0.496 (-2.026, 1.033)	1.236 (-0.187, 2.660)	
Leptin							
Model 1	0.004 (-0.001, 0.009)	**0.006 (0.001, 0.011)**	**0.007 (0.001, 0.013)**	0.004 (-0.002, 0.010)	-0.047 (-0.102, 0.009)	-0.053 (-0.106, 0.001)	
Model 2	0.002 (-0.004, 0.007)	0.002 (-0.003, 0.007)	**0.006 (0.0004, 0.012)**	0.003 (-0.002, 0.009)	-0.032 (-0.087, 0.023)	-0.054 (-0.110, 0.002)	
	H.O.M.E. Score						
	6M	1Y	2Y	3Y	7Y	9Y	10.5Y
BMI Z-score							
Model 1	-0.007 (-0.034, 0.020)	-0.002 (-0.040, 0.036)	0.033 (-0.016, 0.082)	0.005 (-0.042, 0.052)	-0.033 (-0.073, 0.007)	**-0.046 (-0.086, -0.005)**	-0.016 (-0.057, 0.026)
Model 2	-0.013 (-0.041, 0.016)	-0.00001 (-0.037, 0.037)	0.030 (-0.019, 0.079)	0.004 (-0.041, 0.048)	-0.016 (-0.054, 0.023)	-0.029 (-0.069, 0.011)	0.0002 (-0.04, 0.04)
Adiponectin							
Model 1	0.172 (-0.152, 0.495)	0.323 (-0.141, 0.786)	0.241 (-0.351, 0.834)	0.100 (-0.423, 0.623)	**0.641 (0.104, 1.178)**	**0.588 (0.001, 1.176)**	**0.523 (0.018, 1.028)**
Model 2	0.120 (-0.243, 0.483)	0.211 (-0.293, 0.716)	0.089 (-0.583, 0.760)	0.064 (-0.484, 0.612)	0.493 (-0.09, 1.075)	0.354 (-0.28, 0.988)	0.325 (-0.229, 0.878)
Leptin							
Model 1	0.003 (-0.011, 0.016)	0.002 (-0.017, 0.021)	0.019 (-0.004, 0.041)	-0.002 (-0.030, 0.025)	-0.018 (-0.040, 0.004)	-0.009 (-0.030, 0.012)	0.004 (-0.018, 0.025)
Model 2	-0.004 (-0.019, 0.010)	0.009 (-0.006, 0.025)	0.014 (-0.007, 0.036)	-0.0002 (-0.024, 0.024)	-0.008 (-0.029, 0.013)	-0.005 (-0.027, 0.016)	0.003 (-0.018, 0.024)

[1]Linear regression coefficient
Model 1: crude
Model 2: crude + sex + smoking + alcohol + education + pre-preg bmi + preg ssb + baseline poverty + years US
Bolded values are significant at $p<0.05$; *significant after FDR adjustment

Table 5.6 Results of GEE analyses of the relationship of longitudinal trends in aspects of the home environment with adipokines or body mass index z-score

Exposure	Outcome	β[1] (95% Confidence Interval)
Depressive Symptoms	BMI z-score	**0.007 (0.0004, 0.014)**
	Adiponectin	**-0.248 (-0.376, -0.119)**
	Log Leptin	-0.0004 (-0.004, 0.003)
Social Support	BMI z-score	**-0.162 (-0.273, -0.050)**
	Adiponectin	1.191 (-0.236, 2.618)
	Log Leptin	**-0.056 (-0.096, -0.016)**
H.O.M.E. score	BMI z-score	-0.002 (-0.022, 0.018)
	Adiponectin	-0.285 (-0.694, 0.124)
	Log Leptin	-0.009 (-0.021, 0.003)

[1]Coefficient from GEE models adjusting for age and sex
Bolded values are significant at $p<0.05$

5.9 Figures

Figure 5.1 Schematic of analysis.

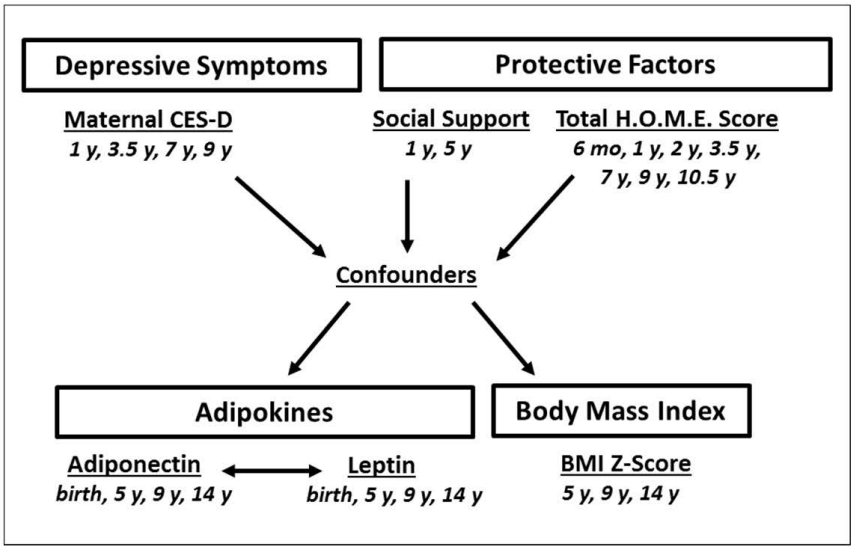


Figure 5.2 Box plots of adipokine trends in CHAMACOS newborns and children at ages 5, 9, and 14 years old. The figure presents distributions of adipokines in all subjects with data available at each time point. In addition, GEE models were used to model longitudinal changes in adipokines by age. Age was a significant predictor of adipokine levels ($p<0.001$ for all models) for leptin and adiponectin.

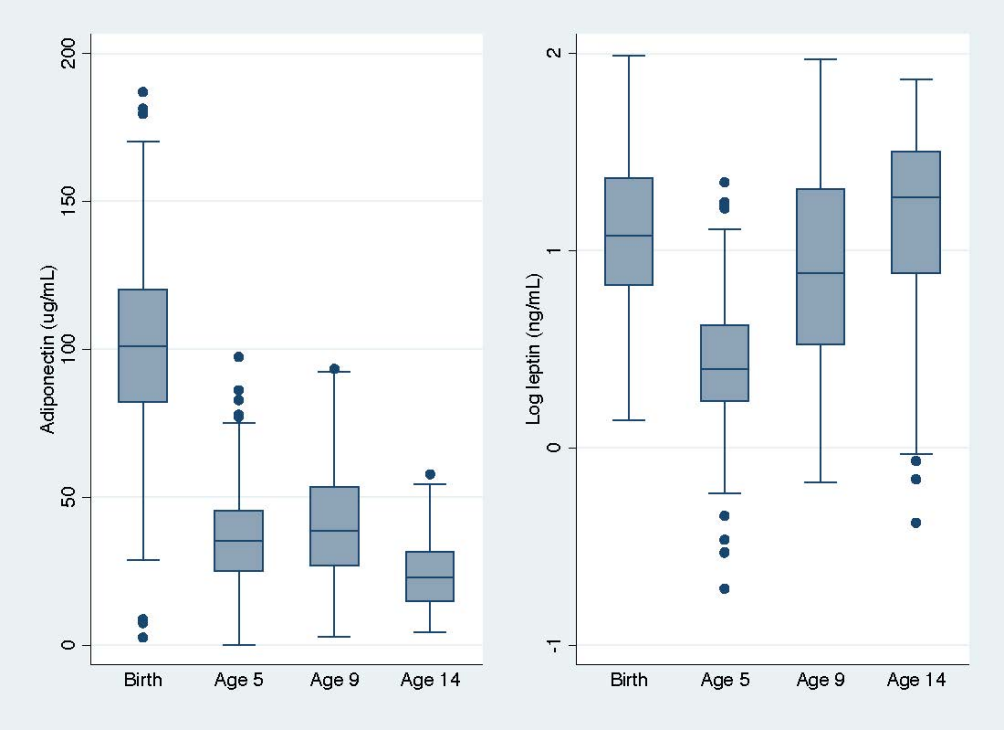
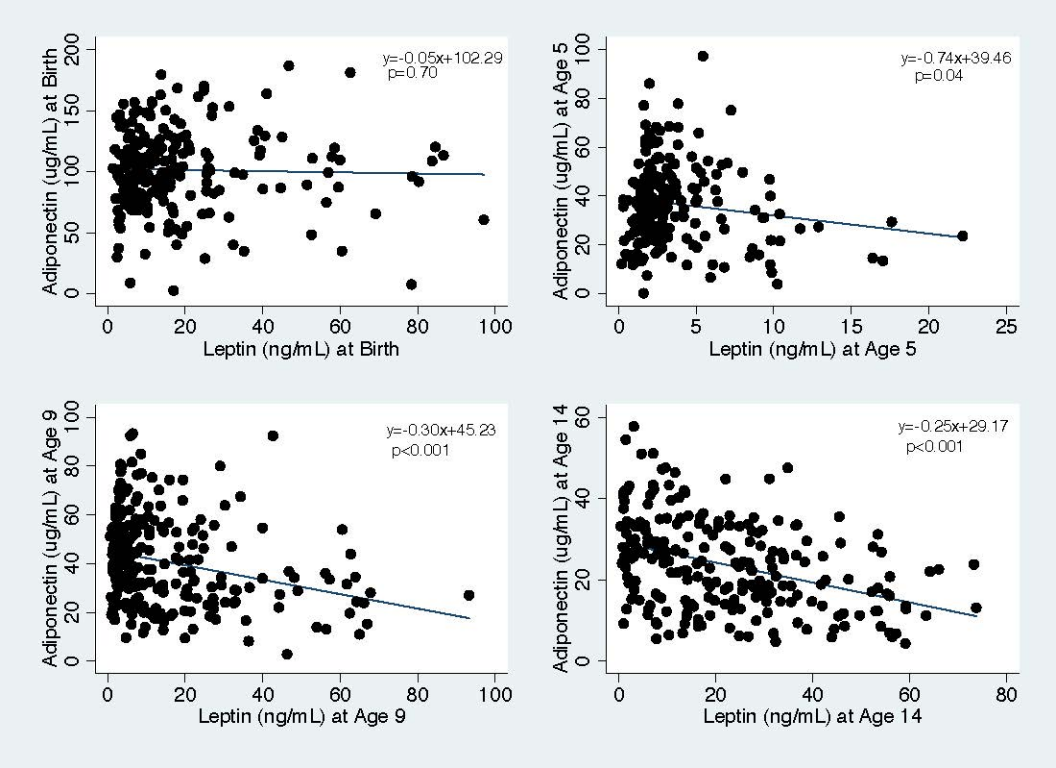


Figure 5.3 The relationships between leptin and adiponectin in CHAMACOS children at birth and age 5, 9, and 14. The figure shows the relationship between adiponectin and leptin measurements at a given assessment. At all time points, with the exception of birth, leptin and adiponectin are significantly and inversely associated.



5.10 Supplementary Materials

Supplementary Table 5.1 Relationship of aspects of the home environment during childhood with adipokines or body mass index z-score at age 5

	β[1] (95% Confidence Interval)			
	Depression Scale		Duke SS	
	1Y	3Y	1Y	5Y
BMI z-score				
Model 1	0.006 (-0.006, 0.018)	0.008 (-0.002, 0.018)	0.017 (-0.113, 0.147)	-0.102 (-0.233, 0.030)
Model 2	0.004 (-0.009, 0.016)	0.004 (-0.007, 0.015)	0.024 (-0.109, 0.157)	-0.098 (-0.239, 0.043)
Adiponectin				
Model 1	-0.063 (-0.250, 0.125)	-0.155 (-0.351, 0.040)	0.014 (-2.315, 2.342)	0.055 (-1.950, 2.060)
Model 2	-0.016 (-0.222, 0.189)	-0.107 (-0.326, 0.112)	0.29 (-2.341, 2.922)	0.161 (-2.003, 2.325)
Leptin				
Model 1	0.001 (-0.004, 0.005)	-0.002 (-0.006, 0.002)	-0.002 (-0.050, 0.046)	-0.040 (-0.089, 0.009)
Model 2	-0.0004 (-0.006, 0.005)	-0.003 (-0.008, 0.002)	0.005 (-0.046, 0.055)	-0.038 (-0.093, 0.016)
	H.O.M.E. Score			
	6M	1Y	2Y	3Y
BMI z-score				
Model 1	-0.004 (-0.030, 0.022)	-0.012 (-0.052, 0.027)	0.008 (-0.044, 0.061)	-0.018 (-0.067, 0.030)
Model 2	-0.006 (-0.038, 0.026)	-0.003 (-0.045, 0.040)	0.016 (-0.041, 0.074)	-0.023 (-0.073, 0.026)
Adiponectin				
Model 1	0.137 (-0.280, 0.553)	0.099 (-0.492, 0.691)	-0.062 (-0.870, 0.747)	0.378 (-0.575, 1.331)
Model 2	0.183 (-0.309, 0.675)	0.227 (-0.437, 0.892)	0.398 (-0.465, 1.261)	0.553 (-0.414, 1.520)
Leptin				
Model 1	-0.003 (-0.012, 0.006)	-0.001 (-0.014, 0.013)	-0.007 (-0.027, 0.012)	0.012 (-0.007, 0.031)
Model 2	-0.003 (-0.014, 0.008)	0.004 (-0.011, 0.019)	-0.005 (-0.028, 0.017)	0.013 (-0.006, 0.031)

[1]Linear regression coefficient

Model 1: crude

Model 2: crude + sex + smoking + alcohol + education + pre-preg bmi + preg ssb + baseline poverty + years US

Bolded values are significant at $p<0.05$

Supplementary Table 5.2 Relationship of aspects of the home environment during childhood with adipokines or body mass index z-score at age 9

	β[1] (95% Confidence Interval)					
	Depression Scale				Duke SS	
	1Y	3Y	7Y	9Y	1Y	5Y
BMI z-score						
Model 1	0.008 (-0.003, 0.019)	**0.013 (0.003, 0.024)**	-0.0001 (-0.012, 0.012)	**0.012 (0.002, 0.023)**	0.021 (-0.108, 0.149)	-0.068 (-0.198, 0.063)
Model 2	0.005 (-0.006, 0.015)	**0.011 (0.0001, 0.022)**	0.0001 (-0.012, 0.012)	0.010 (-0.002, 0.021)	0.024 (-0.101, 0.149)	-0.060 (-0.190, 0.069)
Adiponectin						
Model 1	-0.158 (-0.365, 0.050)	-0.005 (-0.209, 0.199)	0.059 (-0.191, 0.310)	-0.208 (-0.434, 0.017)	0.587 (-1.777, 2.951)	1.577 (-0.867, 4.020)
Model 2	-0.151 (-0.370, 0.067)	0.061 (-0.158, 0.281)	0.061 (-0.194, 0.315)	-0.174 (-0.414, 0.067)	0.437 (-2.004, 2.879)	1.547 (-0.859, 3.954)
Leptin						
Model 1	0.004 (-0.001, 0.010)	**0.007 (0.001, 0.013)**	-0.0001 (-0.007, 0.007)	0.004 (-0.002, 0.010)	-0.043 (-0.108, 0.021)	-0.056 (-0.121, 0.009)
Model 2	0.004 (-0.001, 0.009)	0.005 (-0.001, 0.010)	0.001 (-0.005, 0.007)	0.005 (-0.001, 0.010)	-0.044 (-0.104, 0.015)	-0.052 (-0.116, 0.011)
	H.O.M.E. Score					
	6M	1Y	2Y	3Y	7Y	9Y
BMI z-score						
Model 1	0.0002 (-0.026, 0.026)	-0.001 (-0.041, 0.040)	0.026 (-0.025, 0.077)	0.014 (-0.032, 0.059)	-0.006 (-0.048, 0.036)	-0.030 (-0.073, 0.012)
Model 2	-0.008 (-0.036, 0.019)	-0.012 (-0.049, 0.026)	0.008 (-0.042, 0.059)	-0.001 (-0.044, 0.042)	0.001 (-0.039, 0.041)	-0.020 (-0.066, 0.025)
Adiponectin						
Model 1	0.118 (-0.404, 0.639)	0.130 (-0.548, 0.809)	-0.202 (-1.039, 0.636)	**0.933 (0.031, 1.835)**	0.726 (-0.016, 1.469)	0.768 (-0.031, 1.568)
Model 2	0.374 (-0.220, 0.968)	0.333 (-0.421, 1.087)	0.143 (-0.828, 1.115)	**1.165 (0.202, 2.129)**	**0.842 (0.067, 1.617)**	0.677 (-0.196, 1.550)
Leptin						
Model 1	0.003 (-0.011, 0.016)	0.006 (-0.015, 0.028)	**0.029 (0.003, 0.055)**	0.011 (-0.012, 0.034)	-0.003 (-0.025, 0.018)	-0.002 (-0.025, 0.022)
Model 2	-0.004 (-0.017, 0.009)	0.005 (-0.015, 0.024)	0.017 (-0.009, 0.043)	0.002 (-0.020, 0.023)	-0.003 (-0.023, 0.018)	-0.005 (-0.028, 0.018)

[1]Linear regression coefficient
Model 1: crude
Model 2: crude + sex + smoking + alcohol + education + pre-preg bmi + preg ssb + baseline poverty + years US
Bolded values are significant at $p<0.05$

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CHAPTER 6. CONCLUSIONS

The epidemic of obesity has a high economic and health burden on society. Identifying additional risk factors for pediatric obesity beyond unhealthy diet and lack of exercise is especially critical considering the numerous co-morbidities and increased risk of obesity in adulthood. Understanding how environmental exposures can impact human health and epigenetic mechanisms is essential. DNA methylation is of particular interest since this epigenetic mechanism is involved in numerous biological processes, including imprinted gene control, and altered DNA methylation has been associated with numerous diseases. One of the main goals of this dissertation was to explore whether exposures during pregnancy, specifically to phthalates and lipid metabolites, are related to DNA methylation levels in newborns. The use of metabolomic data expanded our ability to characterize exposure to numerous metabolites in biological samples, which is a large improvement compared to traditional exposure methods and is more aligned with the goals of exposomics research. We were particularly interested in lipid and phthalate metabolites for their potential associations with obesity. Another objective was to determine if the home environment during childhood is associated with obesity risk, as measured by child BMI status and adipokine levels.

We examined these relationships in the CHAMACOS cohort, which includes mothers and children followed for almost 20 years with extensive information on health and exposures, over 350,000 banked biological samples, and a high prevalence of obesity. Using multiple omics platforms including epigenomics, metabolomics, and molecular markers of obesity and oxidative stress, we addressed key knowledge gaps in the associations between environmental exposure, DNA methylation, and obesity risk, which are described below.

- Research in humans on the relationship between phthalate exposure *in utero* and DNA methylation of imprinted genes, many of which are involved in early growth, has been limited to two imprinted genes (*H19* and *IGF2*) and has only been examined in placenta samples[1, 2]. We assessed the relationship between prenatal phthalate exposure and methylation of nine imprinted gene differentially methylated regions in 296 CHAMACOS newborns. Average pregnancy concentrations of metabolites of di-2-ethylhexyl phthalate (DEHP) were significantly and positively associated with methylation at the *MEG3* DMR. This is the first study to demonstrate that maternal urinary metabolites of DEHP, which is a chemical commonly added to plastics to increase flexibility, was linked to methylation profiles of children at birth in *MEG3*, a gene associated with early growth, tumorigenesis, and diabetes. Additionally, it is the only study that has examined this relationship in cord blood, which is more reflective of the fetal methylome. This research adds to the growing body of evidence of the health effects of phthalates and demonstrates a potential mechanism, imprinted gene methylation, whereby exposure can contribute to altered birth outcomes and future disease development. **CHAPTER 3**
- Animal models have identified relationships between maternal dietary supplementation with fatty acids and offspring methylation status[3, 4]. A single study in a small, primarily Caucasian cohort from Michigan reported associations between prenatal exposure to lipid metabolites and DNA methylation profiles in newborns[5]. Using targeted metabolomics, we significantly expanded this line of discovery and identified a significant relationship between a prenatal fatty acid metabolite and newborn methylation. Although most of the

existing research has focused on fatty acids, we were able to uncover novel associations between maternal phospholipid and lysolipid levels and DNA methylation at delivery. In general, higher lipid metabolites during pregnancy were related to lower methylation levels at CpG sites mapped to genes involved in fetal development. The study is one of the first to show that numerous classes of maternal lipid metabolites, which can cross the placenta and act as biomarkers of exposure, are related to infant methylation in humans. Since the prenatal period is a particularly sensitive period for epigenetic structuring of the fetus, exposure to maternal lipid levels could shape expression of genes necessary for development via alteration of DNA methylation. **CHAPTER 4**

- Previous research has identified relationships of early life home environment[6-8], maternal depression[9], and social support[10] with infant or child adiposity. Additionally, increasing evidence suggests that family, home, and individual mental stability may impact the levels of biologically-active markers of obesity secreted by adipose tissue, including the adipokines: leptin and adiponectin[11-20]. However, research was generally limited to populations of African-American and White adults, was cross-sectional in nature, and focuses on the role of potentially detrimental psychosocial parameters, instead of protective factors such as social support and home learning environment. In our longitudinal study of Mexican-American children, we assessed the contribution of the home environment, perceived social support, and maternal depressive symptoms on childhood obesity risk. We observed that increased maternal depressive symptoms in early childhood were associated with higher BMI Z-score and leptin and lower adiponectin in adolescents. On the contrary, greater home enrichment was related to lower 14 year BMI Z-scores and higher adiponectin. Results were similar comparing early life home environment parameters with obesity status at age 9. One of the strengths of this research is the use of adipokines, which are recognized biological markers of obesity and help to understand aspects of the metabolic health status of an individual that are not captured by BMI. Using a molecular epidemiology approach, we generated stronger evidence that targeting mothers exhibiting symptoms of depression and enhancing the home learning environment may help prevent childhood obesity. **CHAPTER 5**

The concept of the Developmental Origins of Health and Disease postulates that exposures during tissue and organ development can influence future disease risk. In this dissertation, we have addressed knowledge gaps of the relationships of prenatal exposure to phthalates, chemicals found ubiquitously in humans, and maternal lipid metabolites with newborn DNA methylation. Emerging and exciting omics technologies, such as metabolomics, epigenomics, and molecular biomarkers, enabled us to make new discoveries regarding the role of the environment in shaping methylation profiles at genes important in early growth, with potential implications for children's environmental health. The results highlight the importance of multi-faceted intervention strategies that account for environmental, biological, and psychosocial risk factors in obesity prevention.

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CHAPTER 7. FUTURE DIRECTIONS

Understanding the molecular modifications that result from environmental exposure, particularly during the sensitive period of gestation, is essential for disease prevention. DNA methylation, an epigenetic mechanism that has been shown to relate to both exposures and health status, may be one pathway through which environmental exposures exert their effect. In this dissertation, we have examined the interplay between chemical and psychosocial exposures, DNA methylation of newborns, and pediatric obesity. Key future directions that build upon this research include:

- **Determination of the role of postnatal phthalate exposure on DNA methylation patterns**. It will be critical to assess the relationship between childhood phthalate exposure and DNA methylation, both across the human genome and in targeted assessment of imprinted genes, many of which are involved in biological pathways important in growth. Since phthalates are known endocrine disrupting chemicals and we have observed in the CHAMACOS cohort that early life exposure is associated with persistent effects on childhood obesity risk, it is essential to understand the role of exposure to these chemicals on children as they develop through puberty, a period of hormonal shifts.
- **Assessment of the relationships between biological, parental, and environmental parameters on DNA methylation at later time points (i.e. 5, 9, and 14 yr) in the CHAMACOS children**. Much of the current research in CHAMACOS and as part of the Pregnancy and Child Epigenetics (PACE) consortium focuses on the effects of early life exposure on newborn methylation. Given the availability of methylation data at later time points in the CHAMACOS cohort, it will be important to verify if changes in methylation profiles observed at birth persist as children age, and to determine whether age and other environmental exposures can impact certain areas of the human genome that are more relevant as children transition into adolescents.
- **Characterization of the effect of maternal stress and home environment on DNA methylation of newborns**. Most of the existing research on the relationship between maternal stress and offspring DNA methylation has utilized a targeted approach. As part of the PACE consortium, we are interested in examining how maternal stressful life events can impact offspring DNA methylation. Given the observed associations described in this dissertation of maternal depression, perceived social support, and home learning environment with obesity risk in children, we would also like to make use of this wealth of data in the CHAMACOS cohort to assess the impact of maternal well-being and home environment on newborn and child methylation, and whether epigenetic mechanisms mediate the relationship between early life environment and child obesity risk.
- **Exploration of the interplay between environmental exposures and health effects using novel omics technologies**. New technology continues to develop in order to keep pace with the expanding omics disciplines, particularly epigenomics. One of the promising new areas of research includes assessment of 5-hydroxymethylcytosine, a marker of demethylation of DNA. Using existing pilot 5-hydroxymethylcytosine in CHAMACOS newborns, we plan to explore the relationship between early life exposures and demethylation of DNA. We are also interested in examining additional epigenetic

markers, such as miRNAs and histone modifications, and how they are affected by environmental exposure.

- **Validation of findings**. The results described in this dissertation would benefit from validation in animal models and in other cohorts, such as those of the PACE consortium. PACE includes researchers and study participants around the world and has a focus on early life environmental impacts on human disease using DNA methylation in newborns and children. As a member of PACE, we have explored numerous knowledge gaps of the effects of environmental exposure on child epigenetics, including the impact of maternal BMI, maternal smoking, and additional projects that will yield high impact publications.