ABSTRACT

PARK, SARAH S. Environmental Exposures in Early Life and Developmental Health Outcomes in a Prospective Pre-birth Cohort. (Under the direction of Drs. Cathrine Hoyo and James Bonner).

The past few decades have seen a rise in pediatric obesity both globally and in the US. Concomitantly, there has been an increase in the prevalence of co-morbid conditions in children including hypertension, insulin resistance, and non-alcoholic fatty liver disease. While established obesity risk factors include genetic variation and energy imbalance estimated as the difference between increased caloric input in the form of diet, and reduced caloric output in the form of physical activity, these factors alone do not account for the rapid rise in obesity prevalence. Environmental determinants which include both chemical (e.g., BPA, metals) and non-chemical (e.g., sociocultural, stress) exposures, during sensitive or susceptible windows of development, e.g., *in utero*, are hypothesized to increase the risk or accelerate the onset of chronic conditions such as obesity and its sequelae including cardiovascular disease, Type 2 diabetes, and some cancers. Furthermore, there are racial disparities in the prevalence of both exposure and morbidity related to these chronic conditions. It is therefore important to address and clarify the role of environmental exposures during pregnancy and offspring health outcomes.

Data on environmental exposure mediated health effects are largely available for single chemicals. Meanwhile, there are fewer studies examining the effects from exposure to chemical mixtures. As we are exposed to multiple pollutants simultaneously, evaluating chemical mixtures is a key area of toxicological research due to its environmental health relevance. This project focuses on mixture identification of metals due to their environmental ubiquity in perpetuity, as well as developmental toxicity. The main objectives of this PhD project were to: 1) identify metal combinations that are present in maternal blood and determine whether some of these mixtures might associate with increased risk of childhood obesity or elevated blood pressure; 2) evaluate the potential role of prenatal metal mixture exposure on neurodevelopment as it pertains to satiety and appetite regulation; and 3) identify DNA methylation differences between African American and White individuals at birth that may provide insights into racial disparities in chronic diseases. To address these objectives, we utilized data and specimens from mothers and offspring in the Newborn Epigenetics Study (NEST), a prospective pre-birth cohort. Overall, components of this PhD project examined data from the first trimester of pregnancy up to middle childhood. From our analyses, we found sex-specific associations between trace metal mixtures and offspring weight and blood pressure at age 4-6 years (Chapter Three). We also present data showing relationships between obesity associated metal mixtures and altered satiety response suggesting that altered neuro-circuitry governing food intake may be dysregulated (Chapter Four). In efforts to better understand the racial disparities of common chronic diseases such as obesity or hypertension, we analyzed epigenome wide data from umbilical cord blood DNA. From these analyses, we identified differentially methylated CpG sites by race in genes associated with inflammation and cardiovascular disease. We also present preliminary analyses on maternal nutrition and deciduous tooth phenotypes from the NEST cohort. The overarching goal of this project was to elucidate environmental risk factors and markers in childhood obesity and its correlates with the hope that our project might contribute to early detection, prevention, and treatment strategies.

Environmental Exposures in Early Life and Developmental Health Outcomes in a Prospective Pre-birth Cohort

by
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DEDICATION

I dedicate this to my parents and siblings; love and thanks from your megablock.

BIOGRAPHY

Born and raised in Washington, Sarah graduated from the University of Washington Seattle with a Bachelor of Science in Environmental Health and a minor in Public Health in 2007. Taking environmental and public health courses concurrently gave Sarah the opportunity to appreciate both the science behind environmental health concerns such as air and water pollution, and the public health impact these issues had on different communities.

This undergraduate education was followed by eight years of experience in two research laboratories. There, Sarah contributed towards researching the neurodevelopmental effects of organophosphate pesticide exposures, and later, the molecular pathways and genetics involved in craniofacial development. Sarah was given the opportunity to work on a project investigating maternal ethanol exposure on craniofacial development in mice and soon after, she enrolled in the Toxicology Program at North Carolina State University.

Outside of research, Sarah spent a few years volunteering in a needle exchange clinic in Seattle. Her experiences interacting with diverse populations equipped her with a broader perspective towards assessing behavioral and social contributors to chemical exposure-mediated health outcomes.

Sarah's undergraduate education, past research experience, and her graduate training guided her desire to pursue toxicology research.

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I thank the past and present members of the Hoyo lab for challenging, motivating, and supporting me both professionally and personally. Heartfelt thanks to David Skaar and Rachel Maguire for their guidance and wisdom.

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I am also grateful for the technical guidance from students and members in the Departments of Statistics and Bioinformatics. I acknowledge Yaxu Wang for his important contributions to Chapter Five.

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CHAPTER ONE

General Introduction

Childhood obesity and its co-morbid conditions

While historically considered adult diseases, co-morbid conditions clustering with obesity, including Type 2 diabetes, non-alcoholic fatty liver disease, and cardiovascular diseases and conditions, are increasingly being observed in children (1, 2). Around the world, 340 million children between the ages of 5-18, and 40 million children between 0-5 years of age, are overweight or obese (3). Established obesity risk factors include genetics and energy imbalance, that is, excess caloric input in the form of dietary intake, and lower output in the form of physical activity. However, these factors alone do not fully account the rise in prevalence of obesity and its co-morbid conditions (which will henceforth be designated as cardio-metabolic), as well as its onset earlier in life. A growing body of evidence suggests that environmental chemicals and non-chemical stressors including socio-cultural factors that also appear to vary by race/ethnicity also contribute to an increased risk of developing obesity and obesity-related conditions.

The Developmental Origins of Health and Disease (DOHaD) hypothesis posits that environmental factors during important windows of early life development can modulate the trajectory and risk of non-communicable conditions and diseases in later life. Nutritional imbalance, stress and trauma, infections, as well as exposures to select drugs, environmental chemicals during pregnancy have been shown to alter the risk of developing chronic diseases in offspring. These chronic conditions include obesity, metabolic syndrome, and cardiovascular disease, as well as dysregulated immune and reproductive function (4, 5). Additionally, racial disparities in childhood obesity (6) in those exposed highlight the need to elucidate the roles of these environmental factors, and their interactions in order to contribute towards an improved understanding of these disparities, and effective and timely identification of risk factors and markers of obesity. Given their presence and persistence in the environment, one area of growing interest in developmental health outcomes is in early life metal exposure.

Metals and human health

Metals can be essential and harmful to human health depending on oxidation state, exposure source, and dose. Essential metals such as calcium and magnesium are nutritive and important for regulating and maintaining cellular homeostasis. Metal micronutrients are trace elements essential for normal physiologic function in the body. Micronutrients include cobalt, chromium, iron, manganese, and zinc. These metals are tightly regulated and both deficient and excessive levels can lead to acute and chronic health effects (7). Some metals have no known biologic function in humans, and are referred to as toxic metals.

The most well-known toxic metals/metalloids are arsenic (As), cadmium (Cd), mercury (Hg), and lead (Pb). These metals are present in the water, soil, and air where they are often ingested or inhaled. These toxic metals can dysregulate cell differentiation, proliferation, and apoptosis, and generate reactive oxygen species (8). Metals can affect cellular and physiological processes via ionic or molecular mimicry. Enzymes, transporters, channels, structural proteins, and pumps utilize metals such as calcium, iron, and zinc for proper cellular function. However, other metals that ionically resemble these cations can bind to designated sites and reduce or inhibit activity, and utilize channels and transporters for intracellular uptake. For example, cadmium, a divalent cation can utilize calcium channels and divalent metal transporters (DMT) to gain entry into cells and also act as an estrogen mimic and bind to estrogen receptors. Elevated lead levels have been identified in those with iron deficiency while mercury has an affinity for thiol containing molecules including the antioxidant glutathione (9, 10).

As, Cd, Hg, and Pb are among the top ten chemicals on the ATSDR 2019 Substance Priority List (11). Because these pollutants have been shown to co-occur in the environment, humans are exposed to multiple chemicals simultaneously. Studies from our group and others have identified co-occurrence of toxic metals in blood and urine, as well as presence in the environment. Our review on the presence of As, Cd, Cr, Hg, and Pb in >1,000 EPA Superfund sites across the US, found that ~50% of the sites contained at least three of these metals. A recent NHANES analysis of the US population found ~50% had at least three of four metals (As, Cd, Hg, and Pb) in blood or urine, and that minority individuals were more likely to be exposed to mixtures (12, 13). This highlights the need to evaluate the health effects of multipollutant exposures. Research into assessing the public health impact of chemical mixtures have been increasing in the last several years (14-18).

Metal exposure can affect multiple organ systems in the body. The neurotoxic (19), osteotoxic, and nephrotoxic effects (20, 21) are well-known. These documented effects are largely based on higher levels of exposure to toxic metals often due to occupational sources (22). Meanwhile, the health impact of the much lower chronic and non-occupational exposures, especially during pregnancy and early childhood, are less studied. Metals such as cadmium are poorly excreted and can bio-accumulate in tissues including the placenta and remain in the body for up to 30 years (23). Thus, even at low non-occupational exposure levels, bio-accumulated metals can remain in organs and tissues which then become internal reservoirs of continuous exposure through circulation.

Fetal development during pregnancy is one of several developmental windows that has been established as a critical and susceptible period to environmental influences, and exposure to metals during pregnancy has been associated with developmental neurotoxicity as well as fetal growth. Multiple studies from our lab and others have reported associations between maternal exposure to toxic metals such as lead or cadmium, and lower birth weight (24-26), which is a non-specific indicator of dysregulated growth *in utero*. A lower birth weight is often followed by accelerated growth in early childhood—a consistent risk factor for cardio-metabolic impairment, including obesity, in later life (27, 28). The health effects of exposure to metal mixtures on developmental outcomes, are less examined.

In adults, exposure to toxic metals has been implicated in cardio-metabolic dysfunction including metabolic syndrome, hypertension, atherosclerosis, excess adiposity, or altered adipogenesis. These include arsenic (29-31), cadmium (32-34), lead (35, 36), as well as nickel (37, 38). Conversely, reduced levels of some metals have also been associated with increased cardio-metabolic risk including vanadium (39-41) and zinc (42, 43). Exposure to metals can have deleterious effects on multiple organs and systems, and dysregulation of crosstalk between organs may contribute.

Given the neurotoxic effects of metal exposure and the role of the brain in maintaining energy homeostasis (44), our research evaluated whether metals might affect the hypothalamic regulation of appetite and satiety. We introduce this mechanism in the context of cadmium and lead in Chapter Two, and in Chapter Four, we explore the potential role that exposure to metal mixtures play in modulating hunger and satiety response, subsequently leading to an increased risk of obesity in childhood.

In the context of cardio-metabolic health outcomes in children, fewer studies have examined associations between metal combinations and human health (15, 45, 46). As it is more than likely that humans are being exposed to multiple metals simultaneously, methods in analyzing and identifying environmental mixtures are continuing to develop. This subsequent section introduces a broad overview of select methods in mixtures research (47).

Current and developing methods in analyzing environmental mixtures emphasizes the importance of establishing a hypothesis-driven research question in order to select the most appropriate methodological approach. Some core categories in formulating a mixtures modeling question include the following objectives: 1) identifying toxic agents; 2) estimating overall effects; 3) pattern recognition of exposures; 4) interactions; and 5) *A priori* defined groups. Selected methods that are suitable for these questions include 1) Bayesian Kernel Machine Regression (BKMR), Weighted Quantile Sum Regression (WQS), Elastic Net, and Lasso; 2) BKMR and WQS; 3) Factor Analysis (FA) as a dimension reduction tool; and Hierarchical Clustering and K-means clustering; 4) BKMR; and 5) Hierarchical BKMR and Group Lasso. While these methods exist, there is no consensus on an overarching sole method that can be applied to all mixtures analysis questions. The recognition of exposure patterns and toxic chemical identification will be discussed in further detail as these two objectives are in line with the overarching research questions of this dissertation.

In exposure pattern recognition, the chemical data can potentially provide information on exposure sources and characteristics. The methods for pattern recognition are generally unsupervised, with no outcome that is guiding (supervising) patterns from a dataset. Factor Analysis (FA) used extensively in Chapter Three, is an unsupervised dimension reduction technique that attempts to identify a smaller number of latent (unobserved) factors from within the larger group of chemicals by detecting common sources of variation. Outcome supervised methods such as Elastic Net regression can select the major “bad actors” from within a chemical mixture (47-48).

In Chapter Three, FA, Elastic Net, as well as Partial Least Squares a.k.a Projection to Latent Structures, an outcome supervised method, are applied to address the research questions of what metals co-occur as factors in maternal blood during pregnancy, and do any of these factors emerge as potential contributors to elevated blood pressure or obesity risk in young children.

Environmental health disparities and epigenetics

In addition to assessing the role of early life chemical (metal) exposures in developmental health outcomes, another important area in environment health research is in understanding racial disparities in exposure and outcome. Minority populations are often at higher risk of air pollution and pesticide exposure, and experiencing stress along with the mental and physical health effects associated with these exposures (49, 50). Minority populations have a higher likelihood to be exposed to multiple toxic metals when compared to Whites (13). Health outcomes in terms of morbidity and mortality also differ by race/ethnicity. In early life, infant mortality, pre-term births, and low birth weight are higher in African Americans, and childhood prevalence of diabetes is higher in Hispanics. In adulthood, premature death from cancer, stroke, and cardiovascular disease is higher in African Americans (51). Overall, there is increased mortality in African Americans due to cancer. Rates of triple negative breast, colorectal, and lung cancer are higher in African Americans and mortality rates from breast, cervical, lung, and prostate cancers are higher in African Americans. Cervical cancer rates are higher in Hispanics and American Indians/Alaska Natives, and liver and kidney cancer rates are higher in American Indians/Alaska Natives (52).

These ethnic/racial disparities arise in response to a multitude of factors including access to healthcare, lifestyle behaviors including smoking and diet, mental and physical stress, and socioeconomic status. Early prevention and detection are some key strategies in mitigating these disparate risks. One approach that is highlighted in this document is to characterize potential cellular phenotypes that may differ by race in early life. This might provide insights into the differences with disease trajectory and mortality. Questions to address include determining whether expression of inflammatory markers at birth differ by race, or if there are differences in expression of genes regulating or involved in cell differentiation, proliferation, and apoptosis. Identifying potential epigenetic differences/alterations by race in early life is one appropriate and feasible method.

Epigenetics is the study of heritable changes (phenotype) in the genome that do not arise from changes to the genomic sequence (genotype). DNA methylation (DNAm) of CpG dinucleotides is one of the most widely studied mechanisms in epigenetics research in humans. Genome/epigenome scale analysis approaches for DNAm include the Illumina 450K or the newer MethylationEPIC array, reduced representation bisulfite sequencing, as well as whole genome bisulfite sequencing (WGBS). Logistically, DNAm analysis approaches can be cost-effective and accessible. The stability of DNA and well optimized DNA isolation techniques from a wide range of tissues and cells allows for analysis on archived samples such as placental tissue, leukocytes, and saliva.

DNAm involves the covalent addition of a methyl group to the 5th carbon of the cytosine ring via DNA methyltransferases (DNMTs) and is involved in the regulation of gene expression and chromatin structure through the recruitment of DNA binding proteins and facilitation of transcription factor binding. While DNAm can occur at any cytosine, in mammals, the majority of cytosine sites are followed by guanine in somatic cells and are known as CpG sites, while in stem cells and neuronal cells, DNAm can occur at non-CpG sites (53-55). Altered CpG methylation due to environmental factors in early life has been associated with susceptibility to chronic diseases in adulthood (56). Assessing potential CpG methylation differences by race at birth may provide insights into differences in disease course and mortality, and in doing so, possibly inform early prevention approaches. This serves as the basis of Chapter Five, where we used agnostic epigenome-scale approaches to measure and compare CpG methylation in umbilical cord blood between African American and White infants. Racial disparities in environmental health are due to multifactorial influences. These include but are not limited to disproportionate exposure to social and chemical stressors, socioeconomic status, genetics and epigenetics, and lifestyle behaviors. As such, DNAm profiles at birth may provide the opportunity to examine and define potential differences and perhaps clarify the interactions among these factors.

The Newborn Epigenetics Study (NEST)

The research conducted and reported herein was made possible with data and specimens collected in the Newborn Epigenetics Study developed and led by Dr. Cathrine Hoyo and her team. A prospective pre-birth cohort, NEST enrolled ~2,000 mother-offspring dyads between 2005-2011. One of the primary objectives of NEST was to determine how the maternal environment (both chemical and non-chemical) might influence epigenetic response/signatures in early life. Furthermore, it sought to investigate how these epigenetic signatures at birth might serve as biomarkers or prediction "metrics" for chronic disease risk, such as obesity, in childhood. Pregnant women were enrolled at median age 12 weeks gestation. Maternal data collected at enrollment included, but were not limited to anthropometric and demographic data (e.g., BMI, smoking, education, income, and race/ethnicity). From peripheral blood obtained at enrollment, circulating levels of nutrients (e.g., vitamins), and metals were also measured. At birth, umbilical cord blood and infant measurements were taken. The offspring were followed yearly from 0-6 years of age. These resources gave us the opportunity to develop and conduct research on more complex and relevant environmental health questions including metal mixture identification, (epi)gene-environment interactions, environmental exposures and neurodevelopment, and health disparities.

Overview of research aims

The overarching goal of this project was to evaluate how environmental exposures during pregnancy modulate offspring developmental health outcomes. Our primary environmental factors of interest were prenatal metal mixtures and the role of race/ethnicity in epigenetic signatures at birth. Our primary outcomes of interest were offspring body mass index standardized for age/sex (BMIz) levels (as a proxy for childhood obesity) and blood pressure. Key mechanisms we assessed were neurobehavioral response (biomarker and questionnaire data), and epigenetic (DNAm) signatures. We also discuss early findings on analyses looking at nutrition and race with offspring tooth phenotype.

Chapter Two is comprised of a published literature review titled "Epigenetics, obesity and early-life cadmium or lead exposure" which provides an overview of two toxic metals, cadmium and lead, and their link to childhood obesity risk. We discuss the role of epigenetic response in the separate contexts of exposure (cadmium or lead) and (childhood obesity), as well as how epigenetic mediators may clarify this exposure-outcome relationship. We also discuss the potential roles of cadmium or lead developmental neurotoxicity and dysregulation of appetite and satiety function on pediatric obesity outcomes. Chapter Two focuses on two well-studied metals with known health effects. This contributed to the discussion on the importance of metal mixtures research in Chapter Three.

Chapter Three titled "Identification of metal combinations in pregnant women and associations with offspring weight and blood pressure in childhood" is a manuscript in preparation for journal submission. The two main objectives for this chapter were to: 1) identify from a suite of 24 metals, which metals group together in maternal blood during the first trimester of pregnancy; and 2) determine whether any of these metal combinations were associated with BMIz or blood pressure outcomes in offspring between 4-6 years of age. We utilized both unsupervised and supervised methods in dimension reduction and variable selection to address these aims.

Chapter Four titled "Environmental metal mixtures, satiety function and regulation, and childhood obesity" is a first-draft manuscript in preparation for journal submission. The main objective for this chapter was to assess how exposure to metal mixtures may alter eating behavior response and markers of satiety and appetite function, thus increasing the risk of pediatric obesity. In this chapter, we were able to conduct a longitudinal analysis examining prenatal metal exposure, markers related to satiety regulation at birth, and responses to the Childhood Eating Behavior Questionnaire in early childhood, on BMIz scores in children.

Chapter Five titled "Analysis of Umbilical Cord Blood Differential DNA Methylation in African Americans and Whites" is a manuscript in preparation for journal submission. The main objective of this chapter was to compare CpG methylation profiles between African American and White infants at birth to determine whether these signatures provide insights into disparities in chronic disease outcomes and mortality in later life. We analyzed epigenome wide methylation data generated from the Illumina 450K Methylation array and employed pathway analysis tools to address this aim.

We conclude the document with future directions and perspectives, and discuss preliminary findings into how offspring deciduous tooth phenotypes are affected by prenatal nutrition and other environmental factors during pregnancy, and potential implications for later life health outcomes.

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CHAPTER TWO

Epigenetics, obesity, and early-life cadmium or lead exposure

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Epigenomics

Epigenetics, obesity and early-life cadmium or lead exposure

Obesity is a complex and multifactorial disease, which likely comprises multiple subtypes. Emerging data have linked chemical exposures to obesity. As organismal response to environmental exposures includes altered gene expression, identifying the regulatory epigenetic changes involved would be key to understanding the path from exposure to phenotype, and provide new tools for exposure detection and risk assessment. In this report, we summarize published data linking early-life exposure to the heavy metals, cadmium and lead, to obesity. We also discuss potential mechanisms, as well as the need for complete coverage in epigenetic screening to fully identify alterations. The keys to understanding how metal exposure contributes to obesity are improved assessment of exposure and comprehensive establishment of epigenetic profiles that may serve as markers for exposures.

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Approximately 17% of US children and 35% of adults are obese [1], and annual expenditures attributable to obesity and related care exceed US$190 billion [2]. Obese children are more likely to be obese as adults, and its comorbid conditions include Type 2 diabetes, hypertension and cardiovascular disease [3]. Established risk factors are genetic predisposition and energy imbalance, defined as higher caloric intake compared with output. These factors alone, however, do not fully account for the magnitude and rapid increase in the incidence of obesity, especially in early life. A compelling hypothesis receiving consideration posits that increased exposure to epigenetically disruptive chemicals during key developmental stages causes stable epigenetic alterations that may promote obesity. Due to their endocrine-disrupting properties, environmental pollutants including the heavy metals, cadmium and lead, are being investigated as risk factors for obesity. Assessing whether exposure to these chemicals increases obesity risk remains a challenge. Low-level exposure to heavy metals often elicits no immediate symptoms and there is often a long latent period between exposure and obesity outcomes. These exposures may occur as early as the prenatal period while obesity in children may not become evident until middle childhood.

Common heavy metals such as cadmium and lead are ubiquitous environmental pollutants. They frequently co-occur in the environment, and are ranked in the top ten environmental chemicals of concern by environmental health agencies [4]. This concern is driven by well-documented effects of exposure to these heavy metals on neurodevelopmental outcomes. Cadmium or lead exposure increases the risk for both neurodevelopmental disorders [5–8] and lower birth weight [9–12]. Lower birth weight, followed by rapid weight gain is a consistent risk factor for cardiometabolic impairment later in life, such as cardiovascular disease, Type 2 diabetes,

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hypertension and dyslipidemia [13–15]. Disentangling these relationships has been complicated by several methodological shortcomings, including short-term follow-up in contemporary cohorts and the inability to account for competing risk factors for cardiometabolic and neurodevelopmental disorders in older cohorts, complicating causal inference. Given the substantial cost to patients and the healthcare system associated with obesity and its sequelae, including cardiometabolic diseases, it is imperative that biomarkers are found that identify individuals at risk of obesity early in development so it can be more effectively prevented.

Because the etiology of obesity is multifactorial, a potential way of addressing this challenge is to identify epigenetic alterations that occur in response to risk factors such as heavy metal exposure, and to delineate those patterns associated with obesity. Since altering epigenetic gene regulation is a way in which organisms normally respond to environmental change, the identification of these epigenetic modifications has the potential to clarify the etiology of obesity. These epigenetic alterations can also contribute to defining obesity subtypes [16,17] or endotypes that are likely to be responsive to different interventions, if such endotypes exist. To accomplish this, however, requires the gathering of data that demonstrate relationships between epigenetic marks and both obesity and exposure. Alterations in DNA methylation – the most studied epigenetic modification in humans – are proposed to be useful in providing mechanistic insights and identifying stable exposure biomarkers [18–20]. In this report, we discuss the current research examining the epigenetic alterations associated with childhood obesity, developmental exposure to cadmium or lead, potential mechanisms at play and the potential role that cadmium- or lead-induced epigenetic dysregulation has on obesity and cardiometabolic outcomes.

Childhood obesity & epigenetics

The evidence is mounting that DNA methylation alterations at regulatory regions contribute to early onset obesity [21–24]. DNA methylation in the promoter region of genes has been studied extensively because increased methylation of regions leads to transcriptional silencing [25,26]. We conducted a literature search in PubMed using the keywords 'child, obesity, and epigenetics and/or methylation'. The search generated 168 results primarily of reviews and earlier reports on Prader–Willi syndrome, a genetic disorder associated with hyperphagia and obesity, and 24, for the purposes of our review, were relevant original research articles. Summaries of these articles are included in reverse chronological order in Table 1.

In targeted analyses using bisulfite-sequenced DNA, promoter regions of genes known to be involved in obesity or its correlates, such as dyslipidemia or hyperglycemia [29,33,36–39,41,46] and regulatory regions of imprinted genes [40,42,45,51] were among the first epigenomic regions to be interrogated. Regulatory regions of genomically imprinted genes are characterized by parent-of-origin methylation that controls gene expression. Using DNA methylation measurements, imprint control regions associated with obesity include *ZAC1* (*PLAGL1/HYMA1*), a putative nodal regulator of a large network of growth effector genes [52], and *IGF2/H19*: these genes are involved in growth regulation, lipid distribution and early obesity [42,45,53,54]. Data from targeted analyses also support that obesity in children is associated with differential DNA methylation in the regulatory regions of multiple genes, some not imprinted. These include *POMC*, *FAIM2*, *BDNF*, *HIF3A* and the *IGF2/H19* imprinted domain [29,32,33,36,37,41,42,45,46]. One study utilized a combination of *in vitro* and *in vivo* experimental approaches to evaluate the role of SOX6 in adipogenesis. The authors reported that SOX6 was an enhancer of adipogenesis through its regulation of adipogenic genes such as MEST, PPARγ, C/EBPα and FABP4. *SOX6* expression was higher in adipocytes from small for gestational age (SGA) neonates. CpGs adjacent to putative SOX6-binding sites in the *MEST* promoter were hypomethylated in SGA-differentiated adipocytes with increased expression of *MEST*. SGA has been shown to be a risk factor for obesity. In mice, SOX6 was also shown to regulate lipid metabolism where *Sox6* knockdown reduced serum and liver triglycerides and serum cholesterol levels. Loss of Sox6 in zebrafish larvae also resulted in reduced adipogenesis [55]. These data support the role of epigenetics in the genesis of obesity; however, the regions interrogated thus far remain limited.

Agnostic experimental approaches, primarily using array technology of preselected CpG dinucleotides, have also identified regions associated with obesity in children. These studies utilized the 450K methylation array [28,30–32] or 385K methylation array [35], and alternative and older methods: the 27K methylation array, GoldenGate, MassARRAY [34,47,48,50,56] and global methylation [44,49]. Consistent relationships have been found between *LINE-1* hypomethylation and obesity [44,49]. Gene-specific methylation associated with obesity that were identified using these agnostic approaches include *CORO7* [34], *FZD7*, *PRLHR*, *EXOSC4* and *EIF6* [35], as well as *TAOK3*, *PIWIL4* and *FYN* [30]. Furthermore, differential DNA methylation of miRNA-coding regions in obese compared with nonobese children were identified [28] as were differences in the distribution of differentially meth-

Table 1. Relationship between childhood obesity and its correlates and epigenetic alterations.

Author (year)	PMID	Study location	Sample size/characteristics	Measured outcomes	Tissue source and assay type	Results	Ref.
Dalgaard (2016)	26824653	Germany	n = 18 obese and n = 22 nonobese children ages 2–15 years (prepubertal)	Mice: glucose tolerance, basal metabolic rate, levels of fasting plasma hormones, fatty acids, adipokines, adipocyte histology, size, and number	Mice: perigonadal white adipose tissue. Humans: subcutaneous white adipose tissue; qRT-PCR, RNA-seq, and reduced representation bisulfite sequencing	Trim28 dependent network can trigger obesity in an on/off manner. An obesity 'on' position is associated with the reduced expression of *Nnat*, *Peg3*, *Cdkn1c*, and *Plagl1*. Humans cluster into Trim28 associated subpopulations	[27]
Mansego (2016)	26780939	Spain	n = 12 obese and n = 12 nonobese children and n = 95 in validation sample	BMI	Peripheral blood leukocytes; 450K and validation through MassARRAY EpiTYPER	16 differentially methylated CpGs identified between obese and nonobese children. Three miRNAs, *miR-1203*, *412*, and *216A* were associated with BMI. KEGG pathway analysis identified 19 obesity related biological pathways	[28]
Wang (2015)	26717317	China	n = 110 severely obese and n = 110 nonobese children ages 7–17 years, age and sex matched	Height, weight, hip and waist circumference, fasting levels of glucose, total cholesterol, triglycerides, HDL-C, LDL-C, ALT	Peripheral blood leukocytes; MassARRAY EpiTYPER on *HIF3A*	*HIF3A* methylation is associated with childhood obesity and is positively associated with ALT levels independent of BMI	[29]
Huang (2015)	26646899	Australia	n = 54 severely obese and n = 54 nonobese children (each group pooled for methylation analysis). For validation, n = 78 obese and n = 71 nonobese children with mean age: 12–13 years (which includes the discovery set)	BMI, fasting insulin and glucose, blood pressure, cholesterol, LDL, HDL, triglycerides	Pooled DNA from whole blood; 450K and pyrosequencing for validation on individual samples	129 differentially methylated CpG loci in 81 genes with >10% difference in methylation. Candidate genes validated and identified include *FYN* (hypermethylated), *PIWIL4*, *TAOK3* (hypomethylated)	[30]
Cao-Lei (2015)	26098974	Canada	n = 31 (19 male and 12 female adolescents at mean age 13.3 years)	Height, weight, waist circumference	T cells from blood; 450K	Prenatal maternal stress is associated with BMI and central adiposity and is mediated by DNA methylation of genes in Type 1 and 2 diabetes pathways with a potentially protective role	[31]

ALT: Alanine aminotransferase; ASMM; Allele-specific methylated multiplex; DMC: Differentially methylated CpG site; DMR: Differentially methylated region; DVC: Differentially variable CpG site; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; MS; Methylation specific; PAH; Polycyclic aromatic hydrocarbon; qRT; Quantitative reverse transcriptase; RTQ; Real-time quantitative.

Table 1. Relationship between childhood obesity and its correlates and epigenetic alterations (cont.).

Author (year)	PMID	Study location	Sample size/characteristics	Measured outcomes	Tissue source and assay type	Results	Ref.
Pan (2015)	26011824	Singapore	n = 991 infants (weight and subscapular and triceps skinfolds measured between birth and 24 months)	Weight, length, and subscapular and triceps skinfold	Umbilical cord tissue; 450K	Reported positive association between *HIF3A* methylation, birth weight, and adiposity	[32]
Wu (2015)	25922107	China	n = 59 obese and n = 39 nonobese children ages 8–18 years	BMI, glucose, total cholesterol, triglycerides, HDL, LDL. Questionnaire about sedentary behavior and physical activity	Peripheral blood leukocytes; MassARRAY EpiTYPER on *FAIM2* promoter	Associations between *FAIM2* promoter methylation, sedentary behavior, and physical activity in obese children compared to nonobese children	[33]
Eriksson (2015)	25887538	Greece	n = 24 obese and n = 23 nonobese pre-adolescent females; n = 11 obese and n = 11 nonobese pre-adolescent males ages 9–13 years	BMI	Peripheral whole blood; 27K	Genome wide DNA methylation reveals lower *CORO7* methylation in obese children. In mice *Coro7* is expressed in the brain in regions involved with appetite and regulation of energy homeostasis. Studies in drosophila identified increased resistance to starvation with knockdown of *pod1* (a homolog of *CORO7*) and increased expression of *pod1* when fed a protein and sugar rich diet	[34]
Ding (2015)	25871514	China	n = 32 obese and n = 32 nonobese children sex and age matched ages 3–6 years	BMI	Peripheral blood leukocytes; 385K and validation of select genes using pyrosequencing	251 promoters and 575 CpG islands demethylated in obese compared to nonobese children and 141 promoters and 277 CpG islands hypermethylated and a chromosomal imbalance of demethylated promoters and CpG islands on chromosomes 3,16,17, and 19 and more differentially methylated promoters and CpG islands on chromosome X over Y. Validated differentially methylated promoters of *FZD7*, *PRLHR*, *EXOSC4* and *EIF6*	[35]

ALT: Alanine aminotransferase; ASMM: Allele-specific methylated multiplex; DMC: Differentially methylated CpG site; DMR: Differentially methylated region; DVC: Differentially variable CpG site; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; MS: Methylation specific; PAH: Polycyclic aromatic hydrocarbon; qRT: Quantitative reverse transcriptase; RTQ: Real-time quantitative.

Table 1. Relationship between childhood obesity and its correlates and epigenetic alterations (cont.).

Author (year)	PMID	Study location	Sample size/characteristics	Measured outcomes	Tissue source and assay type	Results	Ref.
Gardner (2015)	25779370	USA	n = 32 obese and n = 32 nonobese African–American children ages 5–6 years	BMI, percent body fat, questionnaire on food and satiety responsiveness	Saliva; DNA methylation analysis on the promoters of seven candidate obesity genes: *FTO*, *MAOA*, *SH2B1*, *LEPR*, *DNMT3B*, *BDNF* and *CCKAR*	Food and satiety responsiveness were respectively higher and lower in obese female children than nonobese females. *BDNF* promoter methylation associated with altered satiety response in females	[36]
Wu (2015)	25696115	China	n = 59 obese and n = 39 nonobese children ages 8–18 years	Weight, height, and full metabolic panel	Peripheral blood leukocytes; MassARRAY *FAIM2* promoter	Methylation of *FAIM2* promoter associated with obesity and independently with dyslipidemia	[37]
Yan (2014)	25347678		*In vivo* (mouse). Prenatal PAH exposure	Weight, body composition, and adipose cell size	Inguinal white adipose tissue and interscapular brown adipose tissue for RNA expression (qRT-PCR) of adipose related genes *C/ebp*α, *Ppar*γ, *Cox2*, *Fas*, and adiponectin and DNA methylation of *Ppar*γ (pyrosequencing)	Increased exposure to PAH led to increases in weight, fat mass, and adipose gene expression in offspring and also grandoffspring. Higher expression of *C/ebp*α, *Ppar*γ, *Cox2*, *Fas*, and adiponectin along with lower methylation of *Ppar*γ	[38]
Garcia-Cardona (2014)	24549138	Mexico	n = 106 (66 male and 40 female adolescents ages 10–16 years)	BMI, fasting glucose, cholesterol, triglycerides, leptin, total adiponectin	Peripheral blood leukocytes; *LEP* and *ADIPOQ* promoter MS-PCR	*LEP* and *ADIPOQ* promoter methylation associated with BMI, dyslipidemia, and insulin resistance in obese adolescents	[39]
Azzi (2014)	24316753	France	n = 254 mother–infant pairs	Biparietal diameter, head and abdominal circumferences, femur length, weight, height, and C-peptide levels	Umbilical cord blood; ASMM-RTQ-PCR of the *ZAC1 (PLAGL1)* DMR	Positive association between *ZAC1* (*PLAGL1*) DMR methylation and fetal, birth, and infant weight and BMI. Maternal alcohol and vitamins B2 and B12 intake positively associated with *ZAC1* DMR methylation	[40]
Yoo (2014)	24222450	South Korea	n = 90 mother–infant pairs and follow-up at ages 7–9 years	Height, weight, waist circumference, glucose, triglycerides, cholesterol, HDL cholesterol	Umbilical cord blood from infants and blood from the median cubital vein in children after overnight fasting; pyrosequencing of *POMC*	Hypermethylation of *POMC* associated with lower birth weight and higher triglyceride and insulin levels in children	[41]

ALT: Alanine aminotransferase; ASMM: Allele-specific methylated multiplex; DMC: Differentially methylated CpG site; DMR: Differentially methylated region; DVC: Differentially variable CpG site; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; MS: Methylation specific; PAH: Polycyclic aromatic hydrocarbon; qRT: Quantitative reverse transcriptase; RTQ: Real-time quantitative.

Table 1. Relationship between childhood obesity and its correlates and epigenetic alterations (cont.).

Author (year)	PMID	Study location	Sample size/characteristics	Measured outcomes	Tissue source and assay type	Results	Ref.
Deodati (2013)	23774180	Italy	n = 85 obese children age ~11 years	Oral glucose tolerance, blood levels of C-peptide, insulin, and glucose, blood pressure, body composition (DXA scan), height, weight, birth weight, triglycerides, total cholesterol, HDL, LDL, adiponectin and leptin	Blood lymphocytes; Methyl-Profiler DNA Methylation qPCR Assay for *IGF2* methylation	Association between the degree of *IGF2* methylation and lipid profile in obese children	[42]
Xu (2013)	23644594	USA	n = 48 obese (24 females, 24 males) and n = 48 (sex and age-matched) nonobese African–American youth ages 14–20 years	BMI	Peripheral blood leukocytes; 450K	Both DMCs and DVCs can predict obesity status	[43]
Perng (2013)	23638120	Colombia	n = 553 children ages 5–12 years	BMI-for-age Z-score, waist circumference Z-score, skinfold thickness ratio (subscapular to triceps) Z-score, height-for-age Z-score	Peripheral blood leukocytes; pyrosequencing *LINE-1*	Lower *LINE-1* methylation associated with adiposity development in male children (BMI and skinfold thickness)	[44]
St-Pierre (2012)	22907587	Canada	n = 50 mother–infant pairs	Birth and placenta weight, height, head and thorax circumferences	Maternal and umbiliical cord blood and placental tissue biopsy (maternal and fetal sides) intervillous tissue and chorionic villi and fetal villous tissue; pyrosequencing of *IGF2*-DMR and *H19*-DMR	Placental DNA methylation changes of *IGF2/H19* locus associated with fetal developmental and birth weight	[45]
Kuehnen (2012)	22438814	Germany	n = 91 females and n = 80 males obese average age 11 years and n = 55 females and n = 35 males nonobese average age 17.9 years and n = 21 from longitudinal birth cohort study with peripheral blood DNA at ages 5 or 13 years (normal weight) and at 13 or 20 years (obese), and newborn screening cards (peripheral blood DNA from Guthrie spots)	BMI	Peripheral blood; bisulfite sequencing of *POMC*	DNA hypermethylation variant at intron 2-exon 3 boundary in *POMC* associated with obesity. *POMC* exon 3 hypermethylation shown to interfere with the binding of P300, a transcription enhancer leading to a reduction in *POMC* transcript expression	[46]

ALT: Alanine aminotransferase; ASMM: Allele-specific methylated multiplex; DMC: Differentially methylated CpG site; DMR: Differentially methylated region; DVC: Differentially variable CpG site; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; MS: Methylation specific; PAH: Polycyclic aromatic hydrocarbon; qRT: Quantitative reverse transcriptase; RTQ: Real-time quantitative.

Table 1. Relationship between childhood obesity and its correlates and epigenetic alterations (cont.).

Author (year)	PMID	Study location	Sample size/characteristics	Measured outcomes	Tissue source and assay type	Results	Ref.
Relton (2012)	22431966	UK	Two birth cohorts. n = 24 (11–13 years) for gene expression analysis and n = 178 (~9 years) for DNA methylation analysis	BMI, birth weight, and body composition–fat and lean mass (DXA scan)	Peripheral blood and umbilical cord blood; sodium bisulfite pyrosequencing and GoldenGate assay	DNA methylation in umbilical cord blood has some association with altered gene expression, body size and composition in childhood	[47]
Almen (2012)	22234326	Greece	n = 23 obese and n = 24 nonobese pre-adolescent females ages ~10–12 years	Height and weight	Peripheral whole blood; 27K	Methylation level differences in five sites (six genes) between homozygous carriers of normal allele and obesity risk allele of *FTO*. The authors also identified 20 differentially methylated genes in obese pre-adolescent females	[48]
Michels (2011)	21980406	USA	n = 319 mother– infant pairs	Birth weight, gestational age, birth weight/placenta weight ratio, height	Umbilical cord blood; *LINE-1* pyrosequencing	Lower *LINE-1* methylation levels in infants born with low or high birth weight or born prematurely	[49]
Godfrey (2011)	21471513	UK	Two cohorts. n = 78 infants then as 9 year olds and n = 239 infants then as 6 year olds	Adiposity (measured by DXA scan), birth weight	Umbilical cord tissue; MassARRAY EpiTYPER of five candidate genes *RXRA*, *eNOS*, *SOD1*, *IL8*, and *PI3KCD*	Higher methylation of *RXRA* chr9:136355885+ and *eNOS* chr7:150315553+ associated with childhood fat mass and % fat mass in the first cohort In the second cohort, no association between *eNOS* + methylation but associations between *RXRA* + methylation, fat mass and % fat mass	[50]

ALT: Alanine aminotransferase; ASMM: Allele-specific methylated multiplex; DMC: Differentially methylated CpG site; DMR: Differentially methylated region; DVC: Differentially variable CpG site; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; MS: Methylation specific; PAH: Polycyclic aromatic hydrocarbon; qRT: Quantitative reverse transcriptase; RTQ: Real-time quantitative.

ylated regions between chromosomes where specific chromosomes were over-represented for demethylation of promoters and CpG islands in obese versus nonobese children [35]. A combination of methylation array and genome-wide genetic variant analysis showed an enrichment for obesity-related genes [43]. The strong relationships between DNA methylation and RNA expression supports the functional significance of many differentially methylated regions identified [57].

While the number of studies is growing, replicating the multiple CpGs identified has remained a challenge. First, these earlier human studies were conducted in DNA obtained from accessible specimens, such as saliva and peripheral blood leukocytes, which may not have direct relevance to obesity, as methylation marks are cell specific. Second, these studies are often underpowered with the majority of reviewed studies interrogating epigenetic marks in <200 individuals. Third, the scope of CpGs investigated thus far using existing array technology is also relatively small compared with the >28 million present in the human genome [58]. Coverage is based on annotated genes, promoters and CpG islands, which excludes most of the genome, including most intergenic regions and large portions of intragenic regions. Also, some imprint control regions are not covered partly due to their distance from genes as well as their low CpG content. Furthermore, comparisons of available data are also complicated by differences in the obesity indicators to which the CpGs are evaluated in different studies, with varying use of overall weight with or without adjusting for height, age or sex, waist circumference or skinfold thickness, and other indicators of early truncal fat accrual. Thus, it is still unclear which CpG dinucleotides are associated with patterns of childhood obesity. Although the identification of epigenomic regions related to childhood obesity has provided clues about the potential pathways leading to obesity in children, a comprehensive analysis tool that captures the entirety of the epigenome, and relating these to specific obesity outcomes, is needed.

As the cost of treating obesity and its comorbidities increases with age, it is critical to identify epigenetic perturbations that occur during early development and use these data to better focus early intervention efforts for obesity endotypes based on epigenetic biomarkers. Identification of such biomarkers will require comprehensive and unbiased screening, with tools such as whole-genome bisulfite sequencing at sufficient depth to measure DNA methylation at most cytosines, including atypical non-CpG sites, to identify obesity related regions. For clinical utility, it will also be important to demonstrate that biomarkers identified in surrogate cell types, accessible without invasive sampling from otherwise healthy humans, are relevant to cell types targeted by the exposure. Only then can methylation marks identified from agnostic approaches be useful in identifying the endotypes of obesity. Recently, this endotyping approach was employed to identify epigenetically labile regions in the peripheral blood of obese asthmatic children [59]. Overlapping genes and pathways identified in these obesity studies could potentially provide patterns of epigenetically dysregulated genes that characterize the obesity endotypes.

Exposure to cadmium or lead & obesity

An example of the application of epigenetic endotyping is in addressing the emerging question of whether epigenetic mechanisms mediate, at least in part, observed associations between early exposure to heavy metals and obesity risk in children. Cadmium or lead exposure during the prenatal period has long been associated with lower birth weight and SGA [9–12,60]. Low birth weight, which is often followed by rapid adiposity gain is a consistent risk factor for cardiovascular and metabolic impairment later in life [13]. Some but not all [61–63] human observational studies demonstrate a positive association between lead or cadmium exposure and obesity [64,65] as well as cardiovascular disease or metabolic syndrome [66,67]. In support of these human observations, animal studies of perinatal lead exposure show increased fat mass, body weight or food intake in adulthood [68–71]. Early-life cadmium exposure has also been shown to increase fat mass in male mice. This study utilized the transplantation of fecal microbiota from cadmium-exposed male mice to recipient controls and they exhibited increased fat mass and body fat percentage compared with recipient controls from unexposed control donors [72]. Cadmium exposure has also been associated with altered adipocyte differentiation [73].

Thus far, there are limited data available to demonstrate associations between prenatal or early postnatal exposure to these compounds and subclinical markers of cardiometabolic impairment during childhood. Given the evidence linking cadmium and lead exposure to low birth weight and data linking low birth weight to rapid weight gain and obesity in childhood, it is important to determine if these heavy metals alter the epigenome early in development. If informative epigenetic marks are identified, these marks could serve as a predictive tool for identifying children at risk for obesity or cardiovascular diseases in later life.

A PubMed literature search using keywords 'child, epigenetics or methylation, and lead exposure or cadmium' generated 35 results of which five were primary research articles. This was further augmented with a

Table 2. Relationships between cadmium or lead exposure and obesity and its correlates.

Author (year)	PMID	Study location	Sample size/ characteristics	Exposure	Measured outcomes	Results	Ref.
Ba (2016)	27634282		*In vivo* (mice)	Early-life cadmium exposure	Adiposity (body fat, lean mass, and total mass), plasma TC, LDL, VLDL, HDL, plasma and liver TG, plasma free fatty acids, plasma leptin, gut microbiota, and hepatic gene expression	In male mice, LDC exposure led to fat accumulation and increased levels of plasma TC, TG, and free fatty acids, and liver TG, alterations in gut microbiota, and hepatic gene expression related to fatty-acid and lipid metabolism was enhanced. Transplant of fecal microbiota from LDC exposed male mice into unexposed male controls led to increased mass and percent body fat in these recipients	[72]
Wu (2016)	26962054		*In vivo* (mice)	Early-life lead exposure	Gut microbiota composition and body weight	Increased adult body weight in male mice. Decrease of aerobes and increase of anaerobes in lead exposed mice. Changes in gut microbiota and body weight in male mice	[70]
Cassidy-Bushrow (2016)	26358768	USA	n = 299 children (ages 2–3 years)	Early-life lead exposure	BMI	Having detectable blood lead levels associated with smaller body size at 2–3 years of age	[61]
Faulk (2014)	25105421		*In vivo* (mice)	Early-life lead exposure	Energy expenditure, spontaneous activity, food intake, body weight and composition and glucose tolerance	Increases in food intake at differing ages for females and males. Increased body fat, body weight, and insulin response in males	[68]
Delvaux (2014)	24742724	Belgium	n = 114 children ages 7–9 years (n = 57 females and 57 males)	Prenatal cadmium exposure	BMI, abdominal fat (waist circumference) and subcutaenous fat (skinfolds)	Inverse association between prenatal cadmium exposure and body weight, BMI, abdominal fat and subcutaenous fat in females	[62]
Scinicariello (2013)	24099784	USA	NHANES data 1999–2006 children and adolescents ages 3–19 years	Lead exposure	BMI	Inverse association between blood lead levels and BMI	[63]
Tian (2009)	19404590	China	n = 106 infants measured again at ~4.5 years	Prenatal cadmium exposure	Birth weight and height, weight and height at ~4.5 years, WPPSI-R	Higher levels of cord blood cadmium associated with lower birth weight and length and at ~4.5 years, lower height and WPPSI-R-IQ full scores	[11]
Leasure (2008)	18335103		*In vivo* (mice)	Early-life lead exposure	Body weight, motor activity, dopamine levels	Late onset obesity in 1-year-old male mice and motor abnormalities in male mice	[69]

HDL: High-density lipoprotein; LDC: Low dose cadmium; LDL: Low-density lipoprotein; SGA: Small for gestational age; TC: Total cholesterol; TG: Triglycerides; TLBW: Term low birth weight; TMBW: Term mean birth weight; VLDL: Very low-density lipoprotein.

Table 2. Relationships between cadmium or lead exposure and obesity and its correlates (cont.).

Author (year)	PMID	Study location	Sample size/ characteristics	Exposure	Measured outcomes	Results	Ref.
Berkowitz (2006)	16376613	USA	n = 169,878 birth certificate data for five communities in proximity to the Bunker Hill Superfund site	Prenatal lead exposure (due to lead smelter fire). Air emissions of high concentrations of lead	Preterm birth, SGA, TLBW and TMBW among term infants	Maternal lead exposure associated with increased risk of TLBW and SGA, and reduced TMBW	[9]
Sanin (2001)	11331680	Mexico	n = 329 mother–infant pairs	Early-life lead exposure	Weight at age 1 month and weight gain from birth to 1 month	Maternal lead burden inversely associated with infant weight at one month of age and weight gain between birth and one month of age	[60]
Gonzalez-Cossio (1997)	9346987	Mexico	n = 272 mother–infant pairs	Early-life lead exposure	Birth weight	Maternal bone-lead burden inversely associated with birth weight	[10]
Kim (1995)	8529592	USA	n = 236 at age ~7 years (1975–1978) and follow-up 13 years later n = 58 at age ~20 years (1989–1990)	Lead exposure	Weight and height	Dentin lead levels were positively associated with BMI in 1975–1978 and increase in BMI between 1975–1978 and 1989–1990	[64]

HDL: High-density lipoprotein; LDC: Low dose cadmium; LDL: Low-density lipoprotein; SGA: Small for gestational age; TC: Total cholesterol; TG: Triglycerides; TLBW: Term low birth weight; TMBW: Term mean birth weight; VLDL: Very low-density lipoprotein.

Table 3. Relationships between cadmium or lead exposure and epigenetic alterations.

Author (year)	PMID	Study location	Sample size/characteristics	Exposure	Measured outcomes	Tissue source and assay type	Results	Ref.
Nye (2016)	NA	USA	n = 321 mother–infant pairs	Prenatal lead exposure	Birth weight, changes in WHZ between birth to 1 year, 1–2 years, and 2–3 years of age, and DNA methylation	Peripheral blood leukocytes (umbilical cord); pyrosequencing of *H19*, *MEG3*, *PEG3*,and *PLAGL1* DMRs	Prenatal lead exposure inversely associated with birth weight, positively associated with WHZ change by 2–3 years, and hypermethylation at the *MEG3* DMR regulatory region	[74]
Sen (2015)	26417717	USA	n = 35 mother–infant pairs	Prenatal lead exposure	DNA methylation	Dried blood spots: MNBS, CNBS, CCBS; 450K	564 loci with altered DNA methylation in the CNBS of children whose mothers had high neonatal blood lead levels	[75]
Vidal (2015)	26173596	USA	n = 319 mother–infant pairs	Prenatal cadmium exposure	Birth weight and DNA methylation	Peripheral blood leukocytes (umbilical cord); pyrosequencing of *IGF2/H19, MEG3, MEST, NNAT, PEG3, SGCE/PEG10,*and *PLAGL1*	Higher maternal cadmium levels associated with lower birth weight and lower DNA methylation at the *PEG3* DMR in female infants	[12]
Li (2016)	26115033	USA	n = 64 females and n = 41 males ages 25–30 years (Blood lead concentration data available for these individuals at ages birth to 78 months)	Early-life lead exposure	DNA methylation of 22 imprinted genes	Peripheral blood leukocytes; MassARRAY EpiTYPER	Early-life lead exposure associated with sex-dependent DNA methylation differences in the imprinted gene DMRs of *PEG3, IGF2/H19,* and *PLAGL1/HYMA1*	[76]
Sen (2015)	26077427	USA	n = 25 males and n = 18 females from ages 3 months to 5 years	Early-life lead exposure	DNA methylation	Dried blood spots; 450K	Early-life lead exposure leads to 5-mC clustering into three subtypes: sex-specific and conserved. In the conserved subtype, increased DNA methylation around the transcription start site of *LEP* was identified. *HIF3A* is among the genes differentially methylated and associated with lead exposure in females	[77]

CNBS: Child neonatal blood spots; CCBS: Child's current blood spot; Dnmts: DNA methyl-transferases; hESCs: Human embryonic stem cells; MIRA: Methylated CpG island recovery assay; MNBS: Maternal neonatal blood spots; NA: Not available; WHZ: Weight-for-height Z score.

Table 3. Relationships between cadmium or lead exposure and epigenetic alterations (cont.).

Author (year)	PMID	Study location	Sample size/characteristics	Exposure	Measured outcomes	Tissue source and assay type	Results	Ref.
Sen (2015)	26046694	Mexico	n = 24 female and n = 24 male infants and *in vitro* (hESCs)	Prenatal lead exposure	DNA methylation	Umbilical cord blood; 450K and MeDIP-450K (modified 450K)	Lead exposure associated 5-mC and 5-hmC clusters identified. These can be divided into sex-independent and sex-dependent categories with possible roles as early biomarkers of lead exposure	[78]
Senut (2014)	24519525		*In vitro* (hESCs)	Lead exposure	Neuronal differentiation and DNA methylation	hESCs; 450K	Lead exposure affects neuronal differentiation of hESCs altering number and morphology of generated neurons. Lead exposure also alters DNA methylation of genes involved in neuro-developmental pathways	[112]
Sanders (2014)	24169490	USA	n = 17 mother–infant pairs	Prenatal cadmium exposure	DNA methylation	Maternal venous blood and umbilical cord blood; MIRA	Cadmium exposure associated patterns of DNA methylation in maternal and newborn DNA	[80]
Faulk (2013)	24059796		*In vivo* (viable yellow agouti [A^{vy}] mice)	Early-life lead exposure	Body weight and DNA methylation	Tail DNA; coat color classification and pyrosequencing of imprinted *Igf2* and *Igf2r*, and metastable epiallele loci $Cabp^{IAP}$ and A^{vy}	Dose and sex-specific effects were identified. Increase in wean body weight in males developmentally exposed to lead. Male specific effects at A^{vy} locus. Altered coat color in A^{vy}/a offspring	[71]
Kippler (2013)	23644563	Bangladesh	n = 127 mother–infant pairs and n = 56 children age 4.5 years	Prenatal cadmium exposure	Birth weight and DNA methylation	Umbilical cord blood and blood mononuclear cells from the 4.5-year-old children; 450K	Maternal cadmium exposure associated with sex-specific changes to DNA methylation. CpG sites associated with cadmium exposure identified in both newborns and 4.5 year old children and cadmium-associated changes in methylation related to lower birth weight	[81]

CNBS: Child neonatal blood spots; CCBS: Child's current blood spot; Dnmts: DNA methyl-transferases; hESCs: Human embryonic stem cells; MIRA: Methylated CpG island recovery assay; MNBS: Maternal neonatal blood spots; NA: Not available; WHZ: Weight-for-height Z score.

Table 3. Relationships between cadmium or lead exposure and epigenetic alterations (cont.).

Author (year)	PMID	Study location	Sample size/characteristics	Exposure	Measured outcomes	Tissue source and assay type	Results	Ref.
Schneider (2013)	23246732		*In vivo* (rats)	Early-life lead exposure	Protein expression of Dnmts and methyl cytosine-binding proteins	Hippocampus; Western blot of DNA methyltransferases (Dnmt1, Dnmt3a) and methyl cytosine-binding protein (MeCP2, Mbd1)	Lead exposure affects Dnmt1 and Dnmt3a expression in the rat hippocampus. Expression of MeCP2 is affected by lead exposure in females	[82]

CNBS: Child neonatal blood spots; CCBS: Child's current blood spot; Dnmts: DNA methyl-transferases; hESCs: Human embryonic stem cells; MIRA: Methylated CpG island recovery assay; MNBS: Maternal neonatal blood spots; NA: Not available; WHZ: Weight-for-height Z score.

search for articles in the reference sections of relevant papers pertaining to epigenetics, birth weight or adiposity generating 18 additional articles, for a total of 23 articles included in Tables 2 & 3.

Studies of targeted methylation analysis of DNA from human embryonic kidney cells exposed in culture to lead, and tissues exposed *in vivo* to lead report epigenetic perturbations at the regulatory regions of imprinted genes and altered expression of DNA methyltransferases [79,82,83]. Targeted DNA methylation analysis identified differential methylation at the imprinted loci of *PEG3, PEG1/MEST, IGF2/H19* and *DLK1/MEG3* that is attributable to prenatal cadmium or lead exposure, and associated with dysregulated growth outcomes in both mice and humans [12,71,73,74].

In humans, agnostic approaches using global methylation screening of *Alu* and *LINE-1* elements demonstrated hypomethylation of *LINE-1* related to increased patellar lead levels [84,85]. Sex-specific effects resulting from cadmium [81] and lead [77,78] exposure as well as multigenerational effects from lead exposure [75] have also been reported.

For future work to be comprehensive, tools such as whole-genome bisulfite sequencing are needed to identify DNA methylation patterns and genes that are dysregulated by exposure to cadmium or lead in cell types relevant to the genesis of obesity. It will also be important to identify the overlap in epigenetic profiles associated with cadmium or lead exposure and those associated with obesity.

Potential mechanisms by which cadmium & lead may alter obesity risk

Cadmium and lead have well-established roles as neurotoxins impacting neurodevelopment [86–88]. The relationship between obesity and brain function is also established [89,90]. The role of neurodegeneration on obesity mediated by neurotoxic heavy metals was reviewed [91]. One mechanism by which heavy metal exposure might lead to obesity may involve the effects of metal neurotoxicity on brain function and signaling related to appetite and satiety. Since brain development is affected by both lead and cadmium, a disruption in energy balance could result from dysregulated appetite and satiety response, with consequent increased caloric intake. For example, both cadmium and lead exposures have been shown to reduce the levels of BDNF [8,92,93], an obesity related gene that regulates energy balance [94]. Meanwhile, lower methylation of *BDNF* promoter in the salivary DNA of obese adolescents has also been reported [36], while increased adiposity is related to decreased levels of circulating BDNF [95]. Likewise, prenatal lead exposure results in decreased sponta-

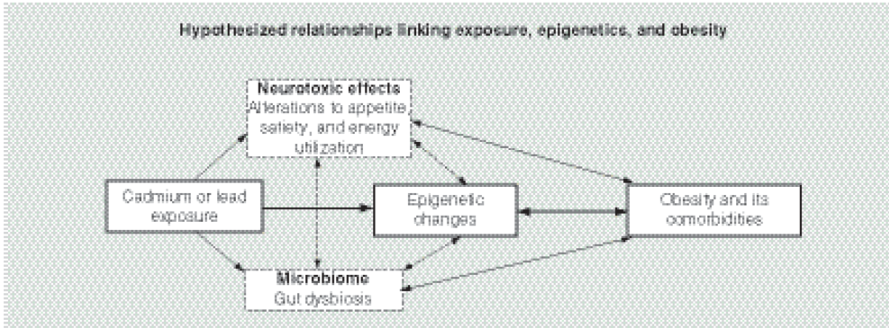

Figure 1. Hypothesized relationships linking exposure, epigenetics and obesity. This schema summarizes the hypothesized relationships between an exposure such as to heavy metals and increased risk of obesity and its comorbidities including cardiovascular disease, Type 2 diabetes and dyslipidemia. Epigenetic alterations may provide a means by which metal exposure alters obesity risk, but the known neurodevelopmental effects and remodeling of gut microbiota by metal exposures may also contribute, influencing behavior and metabolism. Bidirectional interactions between neurodevelopmental effects, the microbiome and the epigenome could together alter each of these factors to individually or synergistically contribute to obesity. The suggested complexity of interactions highlights the need for comprehensive ascertainment of exposure and their effects.

neous motor activity, altered dopamine levels and obesity in adult male mice [69]. In humans, early-life cadmium or lead exposure is also associated with higher risk of attention-deficit/hyperactivity disorder [96,97], a neurodevelopmental condition that is linked with obesity [98]. In addition, *PLAGL1*, found to be involved in neocortical development [99], had a positive association between its methylation and lead exposure [76], while its reduced expression is associated with obesity [27]. Inflammation and oxidative stress may play a mechanistic role. Increased oxidative stress and inflammation are associated with childhood obesity [100–102], as is early-life exposure to cadmium [103,104], and the brain is a primary target of cadmium-mediated oxidative stress [105]. However, determining cause-and-effect remains a challenge. If regions of the epigenome targeted by these heavy metals are determined, epigenetics may play a key role in clarifying the relationships and pathways connecting neurotoxicity and obesity.

Another area warranting further study is the link between the diversity of specific gut microbial species with environmental exposures [106] and obesity. In male mice, prenatal lead and early-life cadmium exposure have been shown to alter gut microbiota and lead to increases in adult body weight [70,] and fat mass [72]. Pathways epigenetically perturbed by cadmium and lead, especially the pathways altered in obesity, will be important for understanding its pathogenesis potentially via the microbiota–gut–brain axis [107–109] in childhood. We summarize these putative relationships in Figure 1.

Conclusion

Epigenetics can be a powerful tool in understanding the etiology of complex diseases. In the context of obesity, a multifactorial and chronic disease, epigenetic patterns may contribute to delineating the pathways that contribute to comorbidities and severity. Furthermore, connecting exposure and effect is often challenging and epigenetics has the potential to elucidate relationships between the two. In this report, we review and discuss the utility and application of comprehensive DNA methylation analysis as an epigenetic screening tool in childhood obesity, however, methodological shortcomings remain.

Future perspective

This report provides a summary of how patterns of epigenetic response could be used to characterize early exposure to ubiquitous environmental toxicants of concern such as cadmium and lead. This approach could be useful in endotyping obesity, improving exposure assessment, identifying epigenetic profiles to serve as indicators for specific heavy metal exposures and also for clarifying the role of the microbiota–gut–brain axis. Furthermore, studying populations with known exposures to heavy metals [9,110] and a higher incidence of obesity [111] may help expand and clarify these links. This can only be accomplished by increasing the capability of next-generation sequencing to produce whole-genome methylation maps from humans and animal models for multiple exposures known to be risk factors for common chronic diseases including obesity. We anticipate that these methylation maps will have the ability to:

subdivide obesity phenotypes; and provide gene targets for expression studies and therapeutic intervention. We also anticipate that animal models will soon determine the extent of epigenetic alterations due to chronic low-level exposure to heavy metals, and alterations in the gut–brain axis. It is in this context that human studies can identify, with specificity, heavy metal-induced epigenetic changes that occur during early development that contribute to obesity risk. Overall, the identification of epigenetic alterations in response to environmental exposures such as cadmium and lead exposure will elucidate mechanisms that may be involved in the genesis of obesity and cardiometabolic disease, allow for exposure detection, and provide a new means for reducing obesity incidence and its severity.

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Executive summary

- Epigenetic alterations as a consequence of early exposure to ubiquitous environmental pollutants, such as cadmium and lead, may contribute to our understanding of the development of obesity, and effects on lifetime risk.
- A mechanism by which early cadmium or lead exposure could initiate obesity is through its neurotoxic role as the brain is a target for both metals. Altered brain function could lead to subsequently dysregulated appetite, impulsivity and lack of satiety, thereby resulting in increased caloric intake and altered energy expenditure.
- The role of the gut microbiome on obesity in the context of cadmium or lead exposure, is another area that warrants further investigation.
- Changes in the epigenome may provide insight into the genesis of heavy metal-induced obesity, and serve as a reliable method for predicting its development.
- The keys to understanding how metal exposure affects obesity are to improve direct exposure assessment, and establish epigenetic profiles that serve as markers for specific exposures.
- This report summarizes studies identifying DNA methylation profiles associated with childhood obesity, and the extent to which they can be used to link early cadmium and lead exposure to obesity, potentially providing novel endotypes of obesity in children.

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CHAPTER THREE

Identification of metal combinations in pregnant women and associations with offspring weight and blood pressure in childhood

This manuscript is currently in preparation for journal submission

Abstract

Background: Toxic metals including arsenic, cadmium, and lead are chemical pollutants that often co-occur in the environment. Prenatal exposure to these metals individually can affect proper growth and development *in utero*, which is a risk factor for obesity and other co-morbid conditions in later childhood. Our objective is to identify trace metal combinations in the first trimester that are associated with an increased risk for offspring obesity and elevated blood pressure in children aged 4-6 years.

Methods: We measured blood concentrations of 24 trace metals/metalloids in n=310 women during the first trimester of pregnancy using ICP-MS, and applied both unsupervised and supervised statistical methods to identify metal mixtures associated with body mass index z (BMIz) score and systolic and diastolic blood pressure in offspring at 4-6 years of age. We also performed semi-speciation analysis of chromium.

Results: After adjusting for pre-pregnancy BMI, smoking, ethnicity and socioeconomic status, we observed significant associations between the metal mixture comprising barium, calcium, cobalt, copper, and strontium and higher BMIz ($\hat{\beta}$: 0.26, se: 0.12, p=0.03): this association may be stronger in females ($\hat{\beta}$: 0.31, se: 0.15, p=0.05) than males ($\hat{\beta}$: 0.19, se: 0.18, p=0.28). In contrast, the mixture comprising arsenic, cadmium and scandium was near-significantly associated with higher systolic blood pressure in females ($\hat{\beta}$: 3.28, se: 1.70 p=0.06). We also observed significant associations between individual metals including chromium, and obesity and blood pressure outcomes.

Conclusion: These data suggest that first trimester exposure to multiple trace metals, including calcium, barium, copper, arsenic, cadmium, scandium, chromium, and arsenic is associated with higher risk of obesity and higher blood pressure in young children. If confirmed in larger studies,

these data would support the hypothesis that prenatal exposure to multiple metals may contribute to cardio-metabolic conditions in children.

Introduction

Trace metals are widely used in medicine, electronics, and industrial processes, which results in the possibility of environmental release and subsequent human exposure. Arsenic (As), mercury (Hg), lead (Pb), and cadmium (Cd), are among the top ten chemicals of concern on the Agency for Toxic Substances and Disease Registry (ATSDR) 2019 Substance Priority List, due to their frequency, toxicity, and exposure potential in humans at National Priorities List sites (1). These pollutants can co-occur in the environment (2), and data from a representative sample of Americans support that humans are often exposed to multiple chemicals simultaneously (3). In blood samples of pregnant women consecutively enrolled from prenatal clinics that serve the Duke Health System in Durham, NC, a sizable proportion had elevated levels of both Cd and Pb, or both As and Pb (4). Previous studies have suggested that exposure to metal mixtures can have greater than additive human health effects, however, few reports have explored these effects on early life development.

While the health effects of occupational exposure to high concentrations of individual toxic trace metals are well-documented (5), data on the impact of low-level chronic exposure (characteristic of the general population) are limited and conflicting, especially in children. In adults, associations between the toxic metals As, Cd, and Pb and cardiovascular disease have been reported (6). Thus, evaluating the impact of metal mixtures on common childhood conditions is important.

A recent comprehensive review reported consistent associations between Cd, Pb or Hg and metabolic syndrome, a condition that includes obesity, dyslipidemia, insulin resistance, and hypertension, among *in vivo* and *in vitro* model systems. However, findings from epidemiologic studies were inconsistent (7). These discrepancies may complicate public health advocacy and policy decisions regarding follow-up public health action.

While reasons for the inconsistent findings are still unclear, data from animal models suggest that health effects may vary by dose and sex (8, 9). Analysis on model systems collected by the ATSDR suggests that the joint toxic effects of As, Cd, Cr, and Pb (primarily for binary combinations) are greater than additive (10), even though individually, concentrations may be below threshold levels for public health action. Inconsistent findings could also stem from differences in oxidation state of measured metals, which contributes to their toxicity. Finally, the age at which the health effects of the individuals are evaluated may contribute to reported inconsistencies in findings. Data accumulated in the last decade and a half support that the first 1,000 days of life are a period of heightened susceptibility in development where exposure can produce subsequent chronic health outcomes later in life (11, 12) yet the majority of data are cross-sectional, among adults (7).

In this study, we explore associations between exposure to trace metal mixtures during the early prenatal period and risks of subclinical indicators of metabolic dysfunction in male and female children aged 4-6 years. We report associations between metal combinations and obesity or blood pressure.

Methods

Participant recruitment and covariates

Previously reported in (4) and briefly summarized herein, data and specimens of 310 mother-infant dyads enrolled as part of the Newborn Epigenetics STudy (NEST) cohort were analyzed for this study. Between April 2009 and October 2011, English or Spanish speaking pregnant women ≥18-years of age attending a prenatal clinic serving Duke University and

Durham Regional obstetric facilities were recruited for study participation. Of the 2,548 eligible individuals approached, 1,700 (66.7%) consented. These analyses are limited to the first 310 enrolled women in whom metals were measured. The 310 mothers did not differ significantly from the remainder of the cohort, with respect to maternal smoking, race/ethnicity, and offspring BMIz/BP measurements ($p>0.05$); however, pregnant women included in these analyses were less likely to be obese before pregnancy and be of lower socioeconomic status ($p<0.05$) than those not included.

Metals measurement

Trace metals were measured in whole blood. Detailed methods have been previously described (4, 13). Briefly, frozen whole blood samples were calibrated and processed for inductively coupled plasma mass spectrometry (ICP-MS) analyses using the Perkin Elmer Dynamic Reaction Cell II Axial Field ICP-MS at GeoMed Analytical. The following 24 metals were measured in 200 µl of maternal whole blood drawn at median gestational age 12 weeks, via inductively coupled plasma mass spectrometry (ICP-MS): aluminum (Al), arsenic (As), barium (Ba), calcium (Ca), cadmium (Cd), chromium (Cr), cobalt (Co), copper (Cu), iron (Fe), lead (Pb), magnesium (Mg), manganese (Mn), mercury (Hg), molybdenum (Mo), nickel (Ni), rubidium (Rb), scandium (Sc), selenium (Se), silicon (Si), strontium (Sr), tin (Sn), titanium (Ti), vanadium (V), and zinc (Zn). Metal concentrations were re-scaled using log[x+1] transformation. Instrumentation and quality control analysis were performed as previously reported in (4, 14).

Chromium oxidation state estimation

Because chromium toxicity varies significantly depending on the oxidation state of the metal, we investigated the oxidation state of chromium among 28 individuals with residential addresses in the geographic area in Durham, NC in which a Pb and Cd geographic cluster was previously described (4). Total chromium was successfully measured in 23 of 28 individuals in whom red blood cells were available using ICP-MS. Direct speciation of chromium in blood presents analytical challenges related to the short hold time of hexavalent chromium and extraction of the metal and sample cleanup while preserving the oxidation state of the metal. Thus, we approximated hexavalent chromium based on selective uptake of hexavalent chromium in red blood cells (15). Using this approach, isolation of RBCs from whole blood and subsequent analysis of chromium content can provide a useful diagnostic for exposure to hexavalent chromium.

The speciation analysis was performed by digestion of RBCs and matching whole blood samples. Up to 300 uL of whole blood and 50 mg of red blood cells (RBCs) were transferred gravimetrically to digestion vessels and 500 uL of high purity nitric acid was added to each vessel, and all were heated in a graphite digestion block at 95 °C for 30 minutes. After allowing to cool, 250 uL of 30% hydrogen peroxide was added to each vessel and all vessels were returned to the graphite heating block to heat at 95 °C for another 30 minutes. Samples were allowed to cool and spiked with Pr as an internal standard at a final concentration of 5 ng/mL. Whole blood and RBC samples were analyzed for total Cr content on an X-series II ICP-MS (Thermo Fisher) with He gas as a collision cell gas to exclude polyatomic interferences. Method blanks were analyzed to allow background subtraction of any analyte signal resulting from the digestion reagents or process and method blank spikes were analyzed to assess the impact of the preparation method on analyte recovery. Spike recoveries were an average of 91% with an RSD of 9.8%.

BMIz and blood pressure measurements in children ages 4-6

Weight, height and arterial blood pressure were measured during study visits or in clinic as part of well-visits. Whether measured by study or clinic staff, weight and height were measured using a stadiometer and a Tanita scale and from the oldest available measurements between age 4-6 years, BMIz scores were calculated for each child using the Centers for Disease Control SAS macro (16). Arterial blood pressure was measured using a standard sphygmomanometer. Systolic and diastolic blood pressure (SBP and DBP) measurements were considered as separate outcomes. Mean systolic and diastolic blood pressure readings for each individual at the oldest age available between 4-6 years were utilized.

Statistical analysis

Identification of metal mixtures associated with BMIz and BP

To generate metal combinations of likely functional significance, Factor Analysis (FA) was the primary analytic strategy used, and multiple linear regression models were employed in sex-specific models. FA was run with the Principal Axis factoring method and varimax rotation using the cut-off value, loading $\geq|0.30|$. We selected the top eight factors, with eigenvalues ≥ 1.0, which explained 52% of the metal variation. Ordinary least squares regression was employed to evaluate these eight factors in relation each outcome variable (i.e., BMIz or blood pressure).

In addition to FA, which forms metal groups in an unsupervised fashion, we also used outcome-supervised selection methods, i.e., projection to latent structures (PLS), a.k.a. partial least squares approach, to explore metal mixture patterns. We applied the non-linear iterative partial least squares (NIPALS) and focused on the top 4-5 components that retained the most variations between predictors (metals) and outcomes (BMIz or BP). The variable importance plots (VIP) shown in Figure 1 were also utilized to determine metals of significance to the outcomes of BMI and BP. Specifically, metals were considered as important when their VIP score was ≥ 1.0 or coefficient score $\geq |0.30|$. PLS and FA findings were also compared to the complementary variable selection methods using Adaptive Elastic Net (EN) regression, which accounts for the collinearity among the co-occurring metals (e.g., Spearman's ρ of pairwise correlations ranged from -0.47-0.77 $p<0.001$). The Akaike information criterion (AIC) with correction (AICc) validation method as well as an $\alpha = 0.50$ was applied to the models. Non-zero regression coefficients were considered as metals of potential importance. Analysis was conducted in JMP Pro 14 and R.

Covariate data

The following covariates were considered as potential confounders in the statistical models and were included in FA regression, PLS, and EN regression: maternal BMI at time of last menstrual period used as a continuous variable, maternal smoking during pregnancy (Yes/No), self-reported race/ethnicity (African American, White, Hispanic, or other), latest age available for BMI and BP measurements, and socioeconomic status (SES). The SES variable was generated using maternal education and household income, where maternal educational attainment greater than high school graduate or GED equivalent and household income greater than $49,999 was considered "higher SES = 0" otherwise "lower SES = 1".

Results

Study participant information

Maternal and offspring sociodemographic characteristics and anthropometric measurements are presented in Table 1. The female to male ratio was 1:1, and mean BMIz and diastolic blood pressure measurements were comparable by sex ($p>0.05$) but females had slightly lower (~3 mmHg) systolic blood pressure than males ($p<0.01$).

Table 1. Participant characteristics for the n = 310 mother-offspring pairs

	Altogether	Females	Males
	Mean ± SD (N) or N (%)		
Maternal data			
Maternal BMI (kg/m^2)			
	27.2 ± 7 (307)	27.6 ± 7.01 (154)	26.8 ± 6.97 (153)
Smoking during pregnancy			
No	254 (84.7)	125 (82.7)	129 (86.5)
Yes	46 (15.3)	26 (17.2)	20 (13.4)
Race/ethnicity			
Black	110 (35.5)	61 (39.6)	49 (31.4)
Hispanic	98 (31.6)	50 (32.4)	48 (30.7)
Other	12 (3.9)	5 (3.2)	7 (4.4)
White	90 (29)	38 (24.6)	52 (33.3)
Socioeconomic status (SES)			
Lower SES	105 (41)	54 (42.1)	51 (39.8)
Higher SES	151 (58.9)	74 (57.8)	77 (60.1)
Offspring data			
Sex			
Female	154 (49.7)		
Male	156 (50.3)		
BMIz 4-6yrs			
	0.478 ± 1.14 (166)	0.482 ± 1.11 (89)	0.474 ± 1.19 (77)
Blood pressure (mmHg)			
Systolic	98.4 ± 8.14 (169)	97 ± 7.89 (88)	100 ± 8.18 (81)
Diastolic	61 ± 6.76 (169)	60.6 ± 6.9 (88)	61.5 ± 6.62 (81)

Co-occurring metals in the NEST cohort

The variables in FA comprise both positive and negative loadings; within a factor, metals that are grouped together may contribute in opposing directions. Table 2 shows the loadings of the top 8 factors used in the subsequent regression of BMIz and BP outcomes, and together, they account for 52% of the variance, ranging from 13.4% for Factor 1 to 2.4% for Factor 8. Together, these factors demonstrate that pregnant women are exposed to multiple trace metals simultaneously, and that known toxic metals such as Cd, Pb, As and Hg co-occur with other trace elements such as Ba and Sn, as well as Cr.

Table 2. Factor Loadings for the 24 metals measured in 1st trimester maternal blood

	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5	Factor 6	Factor 7	Factor 8
Fe	**0.885**	0.223	0.108	-0.059	0.033	0.089	-0.026	0.094
Si	**0.871**	-0.044	0.133	0.128	-0.270	-0.005	0.011	0.157
Cr	**0.699**	0.129	**0.311**	0.176	-0.194	-0.075	-0.033	0.048
V	**0.560**	0.270	0.042	0.013	0.043	0.183	-0.058	-0.108
Mg	**0.500**	**0.470**	0.095	0.173	0.124	0.178	-0.208	-0.051
Zn	0.274	**0.786**	0.060	-0.024	-0.054	-0.066	0.067	-0.033
Rb	-0.027	**0.685**	0.088	0.063	0.226	-0.051	-0.078	0.271
Ni	0.145	**0.569**	-0.018	0.026	-0.013	0.064	0.046	-0.188
Mn	0.265	0.171	**0.644**	0.039	0.259	0.125	-0.120	-0.125
Ti	**0.446**	-0.159	**0.616**	0.108	0.213	-0.151	-0.133	-0.121
Se	0.086	0.128	**0.576**	0.269	-0.016	-0.070	-0.048	-0.097
Co	-0.012	0.017	**0.535**	**0.302**	0.028	0.053	-0.011	0.022
Mo	0.048	-0.008	**0.418**	-0.050	0.048	0.051	-0.019	0.042
Sn	-0.029	0.027	0.028	0.027	0.027	-0.050	-0.042	**-0.358**
Ca	0.155	0.000	0.085	**0.712**	0.003	0.229	-0.056	-0.008
Cu	-0.031	0.163	0.217	**0.541**	0.142	-0.192	0.139	-0.180
Sr	0.135	-0.083	0.168	**0.412**	**0.361**	0.094	0.050	-0.029
Cd	-0.155	0.042	0.222	0.013	**0.621**	0.238	**0.301**	-0.184
Pb	-0.200	0.255	0.145	0.203	**0.535**	**-0.323**	-0.008	0.005
Al	0.121	-0.013	0.047	0.079	0.007	**0.634**	0.057	0.065
Ba	-0.118	0.166	0.121	**0.312**	**0.312**	**0.378**	0.009	0.234
Sc	-0.037	0.121	-0.031	0.124	0.195	0.262	**0.653**	0.044
As	-0.052	-0.070	-0.124	-0.037	-0.008	-0.088	**0.592**	0.038
Hg	**0.329**	0.020	-0.065	-0.174	**-0.313**	0.014	-0.037	**0.355**

Variance Explained by Each Factor

Factor	Variance	Percent	Cum Percent
Factor 1	3.2189	13.412	13.412
Factor 2	1.9922	8.301	21.713
Factor 3	1.9259	8.024	29.737
Factor 4	1.4279	5.950	35.687
Factor 5	1.3549	5.645	41.332
Factor 6	1.0213	4.256	45.588
Factor 7	0.9951	4.146	49.734
Factor 8	0.5854	2.439	52.173

The contribution (loading) of individual metals for each factor (left). Loadings of ≥|0.30| are in bold. Values <0 represent negative loadings. The amount of variance attributed to each factor along with its contribution to the total variance of 52% are also displayed (right).

Association between trace metals mixtures and children's BMIz scores and blood pressure

In sex-specific models evaluating the associations between each Factor (Factors 1-8) and BMIz (Table 3a), we found that exposure to trace metals in Factor 4 comprising Ba, Ca, Co, Cu, and Sr, was positively associated with obesity in female children at age 4-6 years ($\hat{\beta}$: 0.31, se: 0.15, p=0.048). Although associations in a similar direction were also found in males ($\hat{\beta}$: 0.19, se: 0.18, p=0.28), these associations were not statistically significant. These associations were adjusted for established risk factors for childhood obesity, including pre-pregnancy BMI, race/ethnicity, SES, and smoking. Repeating these analyses among all subjects to increase power (n = 135), and further adjusting for sex, we identified Factors 4 and 8 to be significantly associated with BMIz ($\hat{\beta}$: 0.26, se: 0.12, p=0.026 and $\hat{\beta}$: 0.37, se: 0.14, p=0.011, respectively).

Models with the eight factors were also built to evaluate associations with mean systolic and diastolic blood pressure measured at ages 4-6 years (Table 3b & 3c). In models combining males and females, no factors were significantly associated with either systolic or diastolic blood pressure. However, in sex-stratified analyses, the directions of associations appeared sex-specific. For example, Factor 7 (comprising As, Cd, and Sc) was associated with increased SBP in females ($\hat{\beta}$: 3.28, se: 1.7, p=0.06) whereas in males, an inverse association was identified ($\hat{\beta}$: -2.04, se :1.22, p=0.10). We also observed an inverse association between Factor 3 (comprising Cr, Mn, Ti, Se, Co and Mo) and SBP in females (p=0.12).

Because FA identifies the latent metal factors purely based on the correlation patterns among metals, we complemented the analysis with outcome-supervised PLS (Table 4 and Figure 1), which identifies metals that capture the high amount of relationship between metal mixtures and outcomes, as well as Adaptive Elastic Net (Table 5), which identifies important individual metals under a multivariate regression setting while allowing collinearity among metals. PLS analyses support that key elements in Factor 4 (i.e., Ba, Ca, Co, and Sr) were associated with higher BMIz in females. For blood pressure, both PLS and EN analyses identified Factor 7 (i.e., As) to be associated with higher SBP in females. Additionally, both PLS and EN analyses also identified elements that were not identified in the FA regression (i.e., Cr) to be positively associated with both females and males, and Rb and Zn to be respectively positively and inversely associated with BMIz in males, and Se to be inversely associated with SBP in females.

Qualitative speciation of chromium

Chromium (Cr) was associated with increased BMIz in females and males using PLS and EN approaches, and with SBP in both males and females using PLS. Because Cr co-occurs with known toxic metals previously associated with obesity or hypertension including Cd, As and Pb

(7, 17-20), semi-speciation analysis of chromium was undertaken, by comparing Cr concentrations in whole blood and red blood cells. Chromium concentrations ranged from 0.126-4.20 ng/g in whole blood and 0.137-9.21 ng/g in red blood cells; these concentrations in whole blood are comparable to those of a representative sample of the US population (0.29-6.24 ug/L) (21). The detection of chromium in red blood cells suggests the presence of hexavalent chromium in these samples.

Discussion

During pregnancy, the developing fetus is exposed to numerous toxic metals simultaneously, some (e.g., Cd) accumulating in the placenta potentially decreasing placental sufficiency (22), while others (e.g., Pb) may accumulate in the fetal compartment (23). As the body burden of many of these chemicals are individually below reportable/actionable levels, identifying the combinations associated with adverse health outcomes in children is of importance. We undertook an analysis to explore combinations of trace metal exposures (measured in samples obtained at median 12 weeks gestation) that are associated with indicators of metabolic syndrome in children aged 4-6 years, adjusting for established prenatal and postnatal factors. We identified co-occurring metals in maternal blood including combinations containing toxic metals (e.g., Cd, Ba, and Pb, as well as As, Sc, and Cd) and potentially nutritive metals (e.g., Mo, Mn, Se). Furthermore, Factor 4 (the mixture comprising Ba, Co, Ca, Cu, Sr) and Factor 8 (Hg, Sn) were associated with higher BMIz at age 4-6 years, while Factor 7 (Cd, As, Sc) was associated with higher blood pressure. In both outcomes, associations may have been stronger in female offspring. Similar findings were apparent when PLS, elastic net, and ordinary least squares regression (not shown) models were used. Finally, while sample size limits inference, our data from a sub-study suggest the presence of hexavalent chromium in these blood samples.

Our data demonstrate the importance of assessing metals in combination. For example, Factor Analysis of the 24 metals revealed metal combinations that could suggest intake of a nutritive supplement, such as a multivitamin (some members of Factors 1-3), or potential exposure to industrial applications (Factor 5) such as heat stabilizers for PVC plastic polymers (49), or manufacturing (recycling) waste products of previously widely used, but now obsolescent, cathode ray tubes (50, 51), though the likelihood of non-occupational exposure is low. Further characterization and clarification of these observations are needed, including ascertainment of potential exposure sources (e.g., dietary intake, occupation).

Given the relatively high cost of multiplex measurement of trace metals, which has only recently begun to come down, few studies had examined the co-occurrence of multiple metals, especially beyond the core toxic metals (As, Cd, Cr, Hg, and Pb) (24-28). Some of the co-occurring metals that we identified including Cd/Pb in one factor and Cd/As in another have been previously reported in both humans and the environment, perhaps owing to their historical applications (3, 4, 29). In a report on Superfund sites in North Carolina (2), 29 out of 158 contaminants were detected in ~25% of Superfund sites, a list which included As, Cd, Cr, Pb, Ba, and Hg. While all of these elements are naturally occurring, their presence at Superfund sites is generally a result of anthropogenic release in the environment, often as a result of industrial use. As such, these co-occurrences may be a result of specific industrial processes. It will be important to develop a compendium of what metals co-occur in specific populations and geographic regions in future studies of chemical mixtures to predict future disease risk.

Our finding that prenatal exposure to metal mixture comprising Ba, Ca, Co, Cu, and Sr (Factor 4) increases the risk of obesity in children is consistent with studies that have identified individual components of this factor to be associated with obesity. For example, while essential metals such as Ca are widely considered to be beneficial for weight loss and prevention of obesity (30), positive associations with Ca and elevated triglyceride levels, metabolic syndrome (T2DM), as well as BMI (31, 32) have also been reported in cross-sectional studies. One potential explanation of the observed maternal levels of calcium and increasing BMI could be that other metals (for example, cadmium) may be displacing its proper utilization in cells and thus leading to altered calcium homeostasis. Similarly, chromium supplementation has largely been shown to mitigate the effects of obesity and diabetes (33, 34). However, others have reported positive associations between chromium levels and metabolic measures (35, 36). Both urinary and blood levels of barium in children and adults have been associated with increased BMI and waist circumference (37-39). One study identified multiple metals including chromium and barium to be associated with increased markers of oxidative stress (40). Oxidative stress is an established risk factor as well as outcome for inflammatory diseases such as obesity and higher blood pressure.

Barium as a single metal has established associations with cardio-metabolic indicators strengthening our finding. Barium was also included in multiple factors, highlighting its importance as a potential chemical of interest. We also identified membership of barium, cadmium, and lead in Factor 5. While cadmium and lead were not in the metal mixture significantly associated with BMIz in females, several reports indicate that prenatal cadmium or lead exposure can be related to childhood cardio-metabolic health outcomes.

We have previously reported associations between Cd and obesity at age 5 years, adjusting for Pb and As exposure. In attempts to isolate this exposure, the relationship between Cd and lipid accumulation was recapitulated in zebrafish (8). Our present analysis did not find associations between factors where Cd is a member and BMIz. However, in EN analysis, we found Cd to be associated with BMIz when analyzed altogether. We did not find associations between a factor that included barium, cadmium, and lead (Factor 5) and BMIz. Studies analyzing NHANES data identified positive associations with barium and obesity but inverse associations with cadmium and lead (38, 39). Further clarification and examination of the role of identifying a single metal associated with an outcome and its correlation structure with other metals are required.

Our findings are consistent with previous reports of positive associations between arsenic and increased blood pressure in females. In adults, arsenic has been implicated in increasing blood pressure and cardiovascular outcomes (26, 41-44). Arsenic was also found to increase SBP in adolescents (42). The identification of metals both positively and negatively associated with blood pressure highlights the need to analyze them in the context of their beneficial (as potential micronutrients) or deleterious contributions as well as their likely sources.

We found As and Cr (DBP) to be associated with higher blood pressure while metals including Se were associated with lower blood pressure in females. Other studies that measured metals in serum were consistent with our findings: Cr and Se were identified in the same directions (with our study) in the serum of pregnant pre-eclamptic women (45, 46). The metalloid selenium is a micronutrient essential for human health. Selenium deficiency has been associated with an increased risk of hypertension (47) however, a review on the relationship between selenium and blood pressure found conflicting reports (48). This highlights the importance of considering the role of multiple metals, chemical species, exposure source and timing, and dose. Our semi-speciation data for chromium suggest the presence of hexavalent chromium. However, given our limited sample size, these findings require replication in larger studies.

The strengths of this study include the life course framework, with exposure assessment made in the first trimester, to foster causal inference. For example, many of the metals attach to red blood cells which have a lifespan of ~120 days. Thus, specimens obtained at 12 weeks gestation increase the likelihood that these reflect metals present at conception. Additional strengths include using sex specific analysis in identifying metal mixtures associated with outcomes in children, measurements of metals beyond the commonly assayed toxic heavy metals As, Cd, Cr, Hg, and Pb, and the utilization of multiple complementary approaches.

The main limitation of this study was that we may be underpowered for some of the regression models, which could result in undetected significant metal mixtures and the subset associated with poorer outcomes in children. Secondly, the follow-up time between the first trimester of pregnancy and early childhood is relatively short. As knowledge of placental sequestration of some metals is unclear and therefore unaccounted for, elucidation into the exposure levels in the offspring is needed: longer term *in vivo* studies and postnatal measurements in offspring biological specimens such as cord blood, hair, fingernails, urine, and deciduous teeth may help clarify the stability of these associations. To better understand the physicochemical properties of these metals, ascertaining both metal species and source of exposure, will also be important. Larger studies that can directly measure hexavalent chromium and oxidation states of other metals that may be more toxic to human health, are needed. Furthermore, in this study, associations appear to vary by race/ethnicity and SES, thus necessitating stratified analysis. However, the limited sample size precludes such an evaluation. Structural confounding by race is a subject warranting further analysis.

Despite these limitations, we have provided novel data on potential correlations between exposures to metal mixtures early in life and long-term metabolic outcomes. Our results contribute to elucidating correlations between trace metals and metabolic and vascular dysfunction in early life. These findings also highlight the importance of considering not only the combination of toxic metals but the co-occurrence of nutritive and toxic metals, and when possible, characterizing properties such as source and oxidation state.

Table 3. Regression analysis of BMIz, SBP, and DBP on individual factors

3a. BMIz

BMIz (β̂, se, p-value)																											
All (n = 135)	**Females (n = 75)**	**Males (n = 60)**	**Factor**	**Fe**	**Si**	**Cr**	**V**	**Mg**	**Zn**	**Rb**	**Ni**	**Mn**	**Ti**	**Se**	**Co**	**Mo**	**Sn**	**Ca**	**Cu**	**Sr**	**Cd**	**Pb**	**Al**	**Ba**	**Sc**	**As**	**Hg**
-0.04, 0.12, 0.73	-0.08, 0.18, 0.65	0.003, 0.167, 0.98	**1**	+	+	+	+	+					+														+
-0.01, 0.11, 0.95	-0.002, 0.16, 0.99	-0.07, 0.157, 0.67	**2**					+	+	+	+																
0.08, 0.13, 0.56	0.18, 0.18, 0.32	-0.12, 0.206, 0.57	**3**			+						+	+	+	+	+											
0.26, 0.12, 0.026	**0.31, 0.15, 0.048**	0.19, 0.18, 0.28	**4**												+			+	+	+				+			
-0.02, 0.14, 0.88	-0.005, 0.22, 0.98	0.019, 0.21, 0.93	**5**																	+	+	+		+			-
0.0004, 0.12, 0.99	0.088, 0.16, 0.58	-0.102, 0.20, 0.61	**6**																			-	+	+			
-0.02, 0.14, 0.88	0.16, 0.22, 0.48	-0.001, 0.18, 0.99	**7**																		+				+	+	
0.37, 0.14, 0.011	0.32, 0.19, 0.1	0.42, 0.23, 0.07	**8**														-										+

3b. Systolic blood pressure

SBP (β̂, se, p-value)																											
All (n = 132)	**Females (n = 72)**	**Males (n = 60)**	**Factor**	**Fe**	**Si**	**Cr**	**V**	**Mg**	**Zn**	**Rb**	**Ni**	**Mn**	**Ti**	**Se**	**Co**	**Mo**	**Sn**	**Ca**	**Cu**	**Sr**	**Cd**	**Pb**	**Al**	**Ba**	**Sc**	**As**	**Hg**
-0.86, 0.85, 0.31	0.47, 1.36, 0.73	-1.75, 1.1, 0.12	**1**	+	+	+	+	+					+														+
0.54, 0.78, 0.49	1.41, 1.23, 0.26	-0.22, 1.05, 0.83	**2**					+	+	+	+																
-1.1, 0.89, 0.22	-2.05, 1.29, 0.12	-0.03, 1.32, 0.98	**3**			+						+	+	+	+	+											
-0.38, 0.81, 0.64	-1.27, 1.18, 0.29	0.43, 1.1, 0.70	**4**												+			+	+	+				+			
0.61, 0.96, 0.53	1.71, 1.61, 0.29	-0.09, 1.28, 0.94	**5**																	+	+	+		+			-
0.60, 0.81, 0.46	0.72, 1.16, 0.54	0.41, 1.27, 0.75	**6**																			-	+	+			
-0.17, 0.97, 0.86	**3.28, 1.7, 0.06**	-2.04, 1.22, 0.10	**7**																		+				+	+	
-0.55, 0.96, 0.57	-1.48, 1.41, 0.30	0.59, 1.43, 0.68	**8**														-										+

3c. Diastolic blood pressure

DBP ($\hat{\beta}$, se, p-value)																											
All (n = 132)	**Females (n = 72)**	**Males (n = 60)**	**Factor**	**Fe**	**Si**	**Cr**	**V**	**Mg**	**Zn**	**Rb**	**Ni**	**Mn**	**Ti**	**Se**	**Co**	**Mo**	**Sn**	**Ca**	**Cu**	**Sr**	**Cd**	**Pb**	**Al**	**Ba**	**Sc**	**As**	**Hg**
0.73, 0.75, 0.33	1.16, 1.16, 0.32	0.46, 1.05, 0.66	**1**	+	+	+	+	+					+														+
-0.29, 0.69, 0.68	0.84, 1.06, 0.43	-1.07, 0.97, 0.27	**2**					+	+	+	+																
0.12, 0.79, 0.88	-1.3, 1.12, 0.24	2.1, 1.2, 0.08	**3**			+						+	+	+	+	+											
-0.73, 0.72, 0.31	-1.31, 1.01, 0.20	-0.12, 1.06, 0.91	**4**												+			+	+	+				+			
-0.11, 0.85, 0.90	-0.37, 1.4, 0.79	-0.01, 1.2, 0.99	**5**																	+	+	+		+			-
0.74, 0.72, 0.31	-0.12, 1.0, 0.91	2.29, 1.14, 0.05	**6**																			-	+	+			
0.27, 0.87, 0.76	1.36, 1.5, 0.37	-0.87, 1.2, 0.46	**7**																		+				+	+	
-1.03, 0.85, 0.23	-0.41, 1.23, 0.74	-1.7, 1.3, 0.21	**8**														-										+

Offspring outcomes of BMIz (a), SBP (b), and DBP (c) were each regressed on individual factors and covariates (maternal BMI, SES, race, smoking, age of child at BMIz or SBP/DBP measurement, and sex [when analyzed altogether]). The estimate of the regression coefficient ($\hat{\beta}$), standard error (se), and p-values are presented for analyses run altogether and by sex. Metals/metalloids with loadings ≥|0.30| are indicated in a simplified diagram of Table 2 where "+" represents positive loadings and "-" represents negative loadings for each respective factor.

Table 4. Partial Least Squares (PLS) regression analysis of BMIz, SBP, and DBP

4a. Residualized BMIz

% var explained altogether (BMI)	Component	Fe	Si	Cr	V	Mg	Zn	Rb	Ni	Mn	Ti	Se	Co	Mo	Sn	Ca	Cu	Sr	Cd	Pb	Al	Ba	Sc	As	Hg
11.9	1												+			+						+			
5.4	2	+			+	+				+	+														
4.5	3	+	+	+														-							
2	4	-	-												+				+						-

% var explained females (BMI)	Component	Fe	Si	Cr	V	Mg	Zn	Rb	Ni	Mn	Ti	Se	Co	Mo	Sn	Ca	Cu	Sr	Cd	Pb	Al	Ba	Sc	As	Hg
19.5	1												+			+		+				+			
6.2	2	+			+	+				+		+													
7	3	+	+	+																					
3.3	4		-						+										+	+				+	-
4.9	5						+	+				-								+	-				

% var explained males (BMI)	Component	Fe	Si	Cr	V	Mg	Zn	Rb	Ni	Mn	Ti	Se	Co	Mo	Sn	Ca	Cu	Sr	Cd	Pb	Al	Ba	Sc	As	Hg
19.6	1				+			+				+								+		+			
12.1	2					+			+	+	+														
11.6	3			+							+								+			-			
4.7	4						+	+	+		-							-							

4b. Residualized systolic blood pressure

% var explained altogether (SBP)	Component	Fe	Si	Cr	V	Mg	Zn	Rb	Ni	Mn	Ti	Se	Co	Mo	Sn	Ca	Cu	Sr	Cd	Pb	Al	Ba	Sc	As	Hg
5.1	1	+	+	+							+	+													
3.4	2				+	+			+	+															
2.2	3						+	+	+													+			
0.8	4																+			+	-				

% var explained females (SBP)	Component	Fe	Si	Cr	V	Mg	Zn	Rb	Ni	Mn	Ti	Se	Co	Mo	Sn	Ca	Cu	Sr	Cd	Pb	Al	Ba	Sc	As	Hg
13.2	1		-	-							-	-											+	+	
5.9	2	+			+	+																			
3.7	3						+	+	+													+			
2.6	4																+	+		+				+	

% var explained males (SBP)	Component	Fe	Si	Cr	V	Mg	Zn	Rb	Ni	Mn	Ti	Se	Co	Mo	Sn	Ca	Cu	Sr	Cd	Pb	Al	Ba	Sc	As	Hg
14.6	1	+	+	+	+																				
8.0	2					+		+		+		+												-	
7.2	3							+									+								
3.0	4										+								+	+					-

4c. Residualized diastolic blood pressure

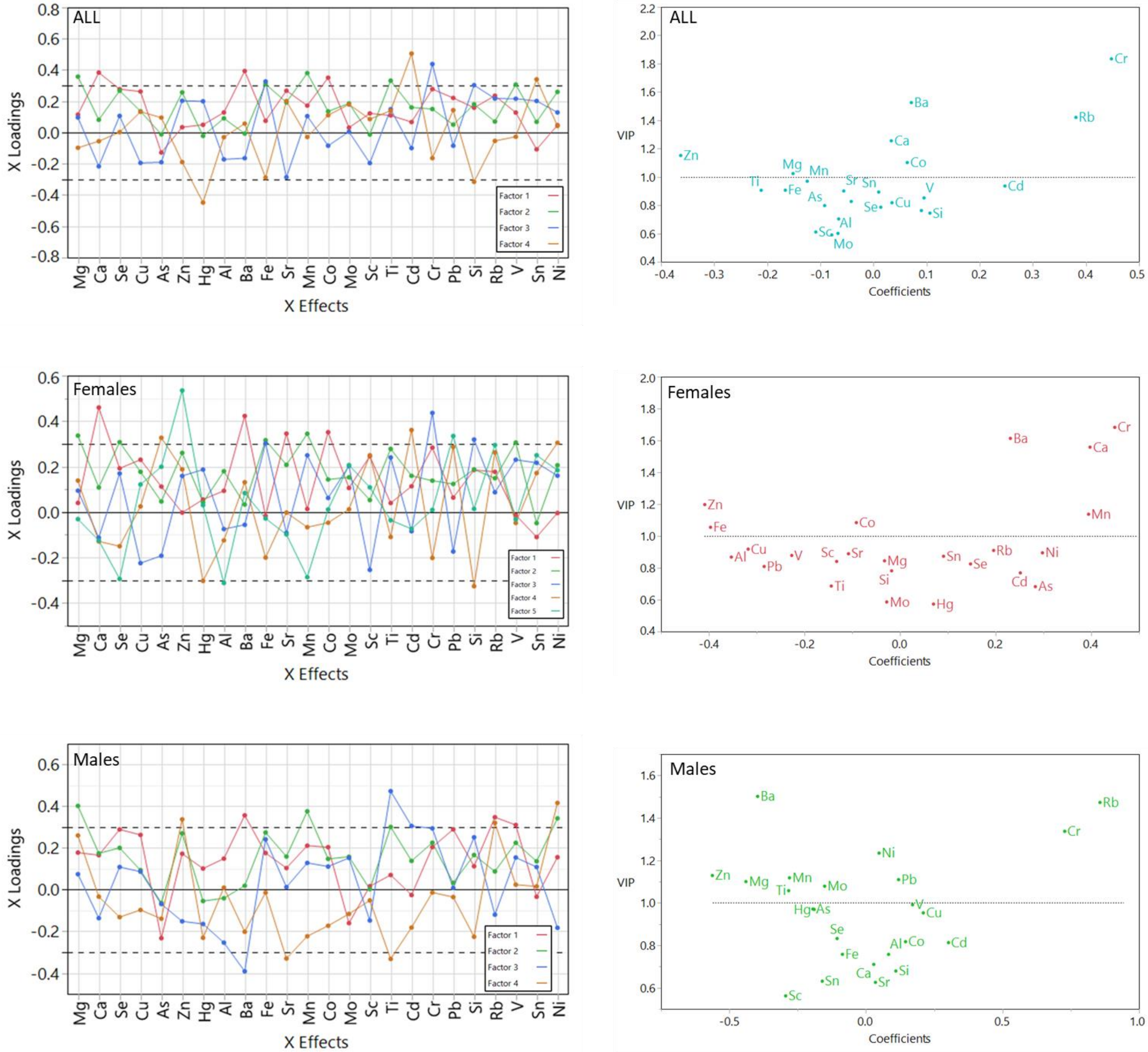
% var explained altogether (DBP)	Component	Fe	Si	Cr	V	Mg	Zn	Rb	Ni	Mn	Ti	Se	Co	Mo	Sn	Ca	Cu	Sr	Cd	Pb	Al	Ba	Sc	As	Hg
7.3	1	+	+												+		-			-					
2.5	2	+	+	+	+							+													
2.6	3									+						+									
2.2	4														+			+	+						-

% var explained females (DBP)	Component	Fe	Si	Cr	V	Mg	Zn	Rb	Ni	Mn	Ti	Se	Co	Mo	Sn	Ca	Cu	Sr	Cd	Pb	Al	Ba	Sc	As	Hg
18.4	1										-	-	-												
4.9	2	+	+	+		+																			
7.1	3				+	+	+		+																
2.6	4														-							+		+	

% var explained males (DBP)	Component	Fe	Si	Cr	V	Mg	Zn	Rb	Ni	Mn	Ti	Se	Co	Mo	Sn	Ca	Cu	Sr	Cd	Pb	Al	Ba	Sc	As	Hg
17.8	1									+	+					+			+						
7.7	2					+											+	+		+					
4.5	3				+		+	+	+									-							
3.9	4	-	-	-		-																			

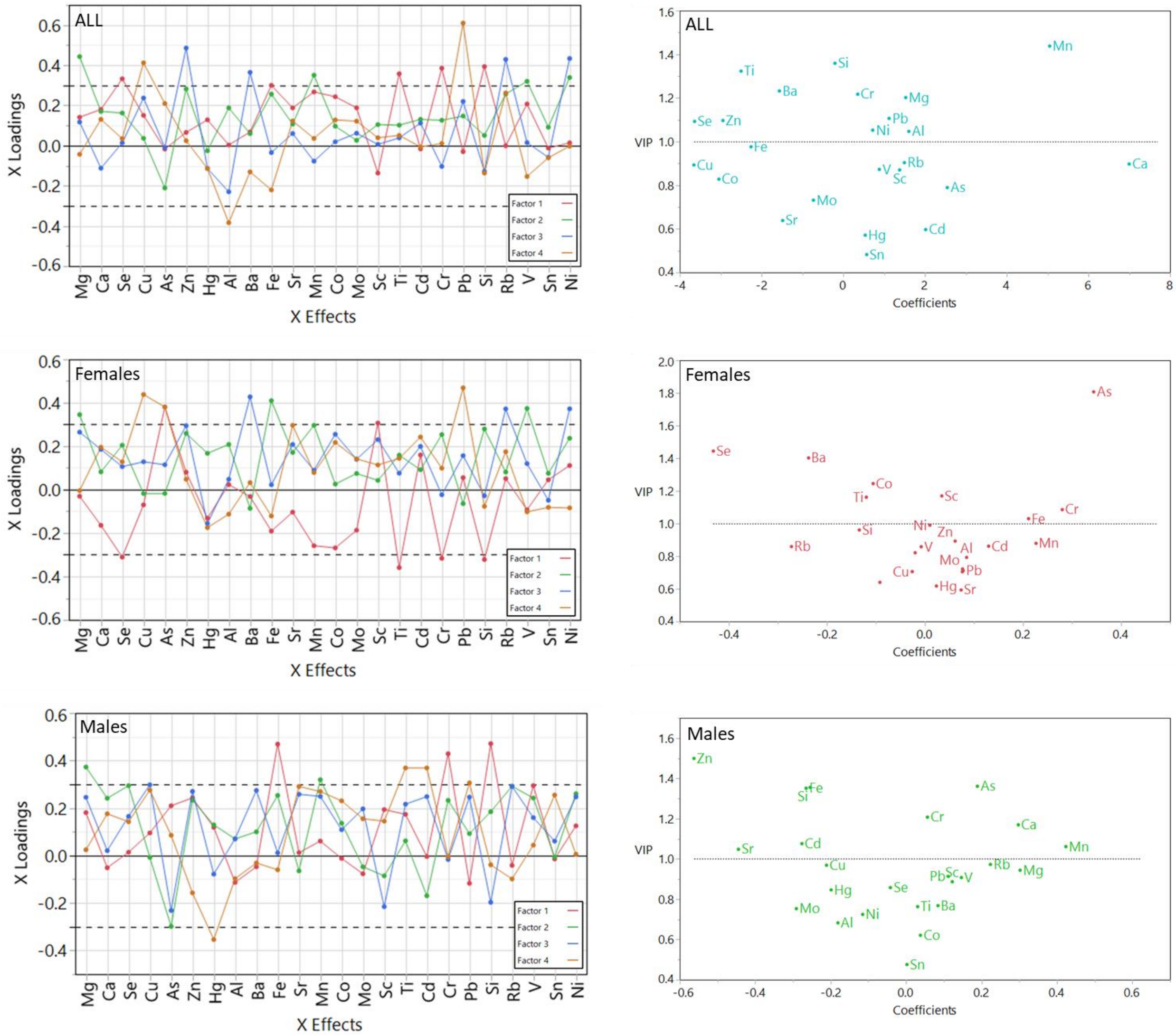
Variables of potential importance are highlighted based on the same coefficient cut-off in FA (≥|0.30|) as indicated by component loading plots (Figure 1a-c). PLS components are ranked by how much each respective individual component contributes to the variance in outcome. We present the top 4 component loadings (5 for BMIz analysis in females) which account for the majority of the total variance explained. Prior to running the PLS models, BMIz, SBP, and DBP outcomes were each residualized on covariates (maternal BMI, SES, smoking, race, age at measurement, and sex [when analyzed altogether]). SBP = systolic blood pressure and DBP = diastolic blood pressure.

1a. BMIz



ALL
Females
Males
X Loadings
X Effects
Factor 1
Factor 2
Factor 3
Factor 4
Factor 5
VIP
Coefficients

1b. Systolic blood pressure



1c. Diastolic blood pressure

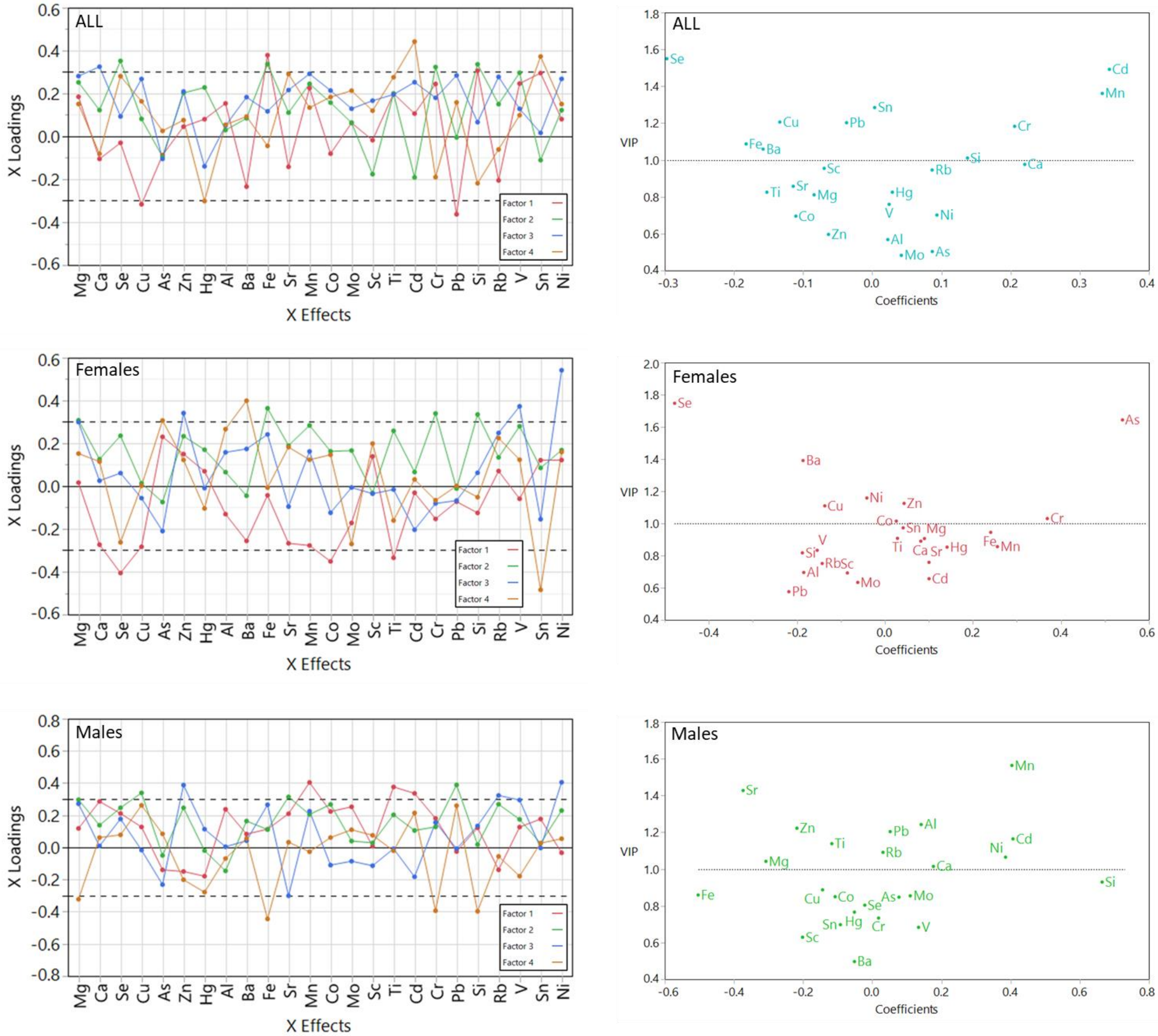


Figure 1. Loading plots
Component loading plots (left) and Variable Importance in Projection (VIP) vs Coefficient Loading plots (right) from PLS regression analysis for BMIz (a), SBP (b), and DBP (c). Variable Importance in Projection vs. Coefficient loading plots summarizes the PLS components and plots the metals accordingly. Component loadings for the first 4-5 PLS components were graphed. Metals that were members of the first 4-5 factors and with X loading scores of ≥|0.30| were tabulated in Table 3 (which also notes the percent variation explained (Component 1 has the highest % variation)

Table 5. Elastic Net regression analysis

5a. BMIz

BMI	Fe	Si	Cr	V	Mg	Zn	Rb	Ni	Mn	Ti	Se	Co	Mo	Sn	Ca	Cu	Sr	Cd	Pb	Al	Ba	Sc	As	Hg
ALL	-		+		-	-	+		-	-								+						
Females			+																					
Males			+		-	-	+		-															

5b. Systolic blood pressure

SBP	Fe	Si	Cr	V	Mg	Zn	Rb	Ni	Mn	Ti	Se	Co	Mo	Sn	Ca	Cu	Sr	Cd	Pb	Al	Ba	Sc	As	Hg
ALL																								
Females	+										-												+	
Males																								

5c. Diastolic blood pressure

DBP	Fe	Si	Cr	V	Mg	Zn	Rb	Ni	Mn	Ti	Se	Co	Mo	Sn	Ca	Cu	Sr	Cd	Pb	Al	Ba	Sc	As	Hg
ALL																								
Females	+		+								-								-		-		+	
Males																								

Identification of individual metals with non-zero regression coefficient estimates using Adaptive Elastic Net. Validation method is AICc, alpha is 0.5, covariates (maternal BMI, SES, smoking, race, age at measurement, and sex [when analyzed altogether]) are loaded but not included in the penalty.

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CHAPTER FOUR

Environmental metal mixtures, satiety function and regulation, and childhood obesity

This first-draft manuscript is currently in preparation for journal submission

Abstract

Background: Metals such as lead, manganese, and mercury are neurotoxic chemicals and developmental exposure has been associated with cognitive and motor impairment. The deficiency of metals such as zinc and manganese can also be neurotoxic. Both toxic metal exposure and nutritive metal deficiency have been linked to an increased risk of obesity. However, mechanistic insights that could guide intervention efforts, are limited.

Objective: The purpose of this study was to test the hypothesis that prenatal exposure to metal mixtures alters appetite regulation at birth and in early childhood, and increases the risk of pediatric obesity.

Methods: Regression models were run between metal mixtures (previously identified to be associated with obesity in Chapter Three), two plasma markers of appetite function (BDNF and NPY), and eating behavior responses from the Childhood Eating Behavior Questionnaire (CEBQ). Analyses were run altogether and stratified by sex and race. Models were adjusted for pre-pregnancy BMI, smoking during pregnancy, SES or education, and in overall models, sex and race.

Results: The domains of Satiety Responsiveness (SR) and Slowness in Eating (SE) were associated with BMIz at 5 years of age. The metal mixture comprised of Ba, Ca, Co, Cu, and Sr is associated with reduced SR and SE. as well as decreased BDNF levels (an appetite suppressing peptide).

Conclusion: These data provide early evidence linking prenatal exposure to metals to satiety response and increased risk of obesity in children. Larger studies are needed to confirm these findings.

Introduction

Around 40 million children (0-5 years of age) in the world are classified as overweight or obese (1). Later life health complications due to obesity are well-established and include cardiovascular and metabolic conditions (e.g., diabetes and heart disease) as well as some cancers (e.g., breast and colon) (2). Moreover, conditions that typically manifest in adulthood are increasingly being identified in overweight or obese children including non-alcoholic fatty liver disease (NAFLD) (3) and hypertension (4). The organism-wide health impact of obesity shows that the mechanisms that may contribute to increased obesity or vascular dysfunction likely involve the crosstalk between multiple organ systems.

The thrust of public health obesity prevention and treatment strategies is aimed at increasing physical activity and reducing caloric intake to impact energy balance. Environmental chemicals including ubiquitous trace metals, such as cadmium, mercury, lead and arsenic are increasingly being implicated both directly and indirectly as obesogens (5, 6). However, mechanistic insights that could contribute to actionable intervention are limited, as these trace metals are established neurotoxic chemicals, especially during development (7). To elucidate the mechanisms that may link these trace metal exposure to childhood obesity, understanding the neurodevelopmental mechanisms involved in appetite and satiety regulation is critical. One region of the brain that is affected by metals such as cadmium is the hypothalamus (8). While the hypothalamus has many different roles including regulating emotion, temperature, hormone balance, it also regulates appetite. However, the extent to which these toxic metals modify neurobehavioral parameters involved in appetite and satiety to contribute to childhood obesity research is understudied. Elucidating these potential neurodevelopmental effects would help inform public health interventions and strategies for mitigating the effects of toxic metal exposures.

This purpose of our study was to test the hypothesis that prenatal exposure to metal combinations modulates appetite and satiety related neuro-circuitry, which alters eating behavior responses leading to an increased risk for childhood obesity. In order to accomplish this, four key analyses were undertaken to examine: 1) the relationship between appetitive traits obtained from Childhood Eating Behavior Questionnaire responses between 2-5 years of age, and BMIz score at 5 years; 2) whether there are associations of identified maternal metal combinations during the first trimester of pregnancy and CEBQ response; 3) if these metal mixtures affect, at birth and measured in umbilical cord blood, levels of Brain Derived Neurotrophic Factor (BDNF) and Neuropeptide Y (NPY), key peptides involved in appetite suppression and stimulation, respectively; and 4) whether at birth, these markers can determine obesity development/risk in later childhood and serve as early-life indicators of childhood obesity risk (9-11). Developmental stages corresponding to the prenatal (environmental exposure), perinatal (intermediary biomarkers), and early childhood (eating behavior and BMI outcomes) are assessed. Here we report that the domains of satiety responsiveness and slowness in eating were associated with BMIz at 5 years, and prenatal exposure to metal mixtures and neuropeptide levels at birth may modulate this relationship.

Methods

Participant recruitment

Utilizing data from the Newborn Epigenetics Study, a longitudinal birth cohort (previously described in (12, 13)), we restricted the present analyses to children with available Childhood Eating Behavior Questionnaire (CEBQ) data (n=221), as well as the mother-infant dyads with available first trimester metals data (n =310) as previously described in Chapter Three. The CEBQ dataset of n = 221 was used for the initial evaluation of questionnaire responses and BMIz levels in children. Meanwhile, the metals dataset of n = 310 was used for analyzing the associations between metal combinations and BDNF and NPY levels. While maternal BMI and sex of the child did not differ between the two datasets, the CEBQ dataset had predominantly African American (42.5%) and White individuals (51.6%), and a higher proportion of college educated individuals (~60%). The metals dataset had roughly even distributions of African American (35.5%), Hispanic (31.6%), and White (29%) individuals. Compared to the CEBQ dataset, there was a lower percentage of college educated individuals (~33%). In order to analyze the effects of metal combinations on CEBQ response, these two datasets were merged, and n = 69 individuals had both available maternal metal levels and CEBQ data after adjusting for the covariates of maternal BMI, smoking, socioeconomic status (SES), race, and sex. In analyses utilizing CEBQ response, data on Hispanic individuals are not available. Hispanic mothers were not approached as Spanish language surveys were not available during the time of study follow-up visits (14).

Childhood Eating Behavior Questionnaire

The Childhood Eating Behavior Questionnaire (CEBQ) developed by (15) utilizes a Likert scale ranging from 1-5 (never to always) for 35 questions corresponding to eight eating domains: food responsiveness (FR); enjoyment of food (EF); emotional overeating (EOE); desire to drink (DD); satiety responsiveness (SR); slowness in eating (SE); emotional undereating (EUE); and food fussiness (FF). The CEBQ is regarded as a reliable and temporally stable measure. In NEST, the CEBQ was completed by women when their offspring were between 2-6 years of age and a subset of participants completed the CEBQ at a second timepoint, 3-5 years later. Our current study is limited to the initial questionnaire data administered to offspring between 2-5 years of age and six eating domains: food approaching FR, EF, EOE, and DD; and food avoiding SE and SR, as well as an additional variable combining satiety responsiveness and slowness in eating (SRSE). Methods for assessing questionnaire data are detailed in (14). Scores from the questions corresponding to the respective eating domains were averaged.

Covariates in analysis

The following covariates were applied to all of the following regression models: sex (when analyzed altogether), maternal BMI, maternal smoking, race (when analyzed altogether), education as a proxy for SES in the analysis between the CEBQ domains and BMIz, or socioeconomic status (as previously described in Chapter Three) for all other analyses. These covariates were selected for three key reasons: 1) maintaining consistency (to the extent possible) with the analyses from Chapter Three; 2) consideration of the established roles of maternal obesity, cigarette smoking, and ethnicity in child obesity outcomes; and 3) identification of race/ethnicity and education differences present in our datasets. All statistical analyses were run in JMP Pro 14 and GraphPad Prism 8.

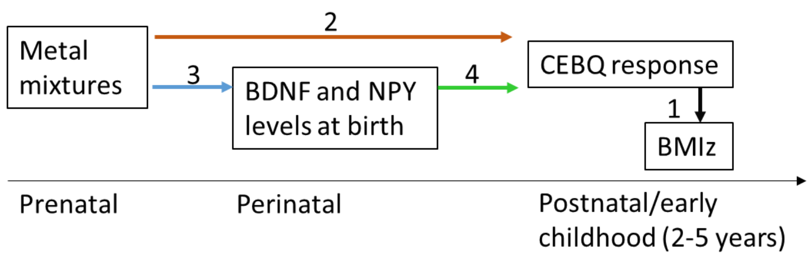


Figure 1. Analysis diagram. This figure serves as a guide for the multi-step analyses undertaken to examine intermediary relationships during the pre-, peri-, and post-natal life course. This figure will precede the tables with the respective analysis circled in red.

1. ***Analysis of eating behavior and child BMI***

In the CEBQ dataset of n = 221, BMIz at 5 years of age (n = 107; females: 55 and males: 52; African Americans: 49 and Whites: 49) was regressed on the four food approaching (DD, EF, EOE, and FR) and two food avoidant (SR, SE, and combined SRSE) domains adjusted for covariates and run altogether and also stratified by sex and race. The race/ethnicity stratified analysis was limited to African Americans (AA) and Whites (W) due to the small sample size of other race/ethnicities. Domains that associated with BMIz were then evaluated in the subsequent analyses.

2. ***Analysis of co-occurring metals and eating behaviors***

To determine whether co-occurring trace metals during pregnancy are associated with eating behaviors, we evaluated the association between metal combinations/latent structures (designated as Factor A and B corresponding to previously described Factors 4 and 8, respectively) identified and described in Chapter Three, and eating behavior domains. Factor A is comprised of the following metals: barium (Ba), calcium (Ca), cobalt (Co), copper (Cu), and strontium (Sr), and Factor B is comprised of mercury (Hg) and tin (Sn). The CEBQ domains of Satiety Responsiveness (SR), Slowness in Eating (SE), and a combined measured of SR and SE (SRSE) were regressed on Factor A and Factor B adjusting for maternal BMI, smoking, SES, race* and sex* (*when run altogether). These analyses were run altogether and also stratified by sex and race. After covariate adjustment, data were available for n= 69 individuals (females: 32 and males: 37 | AA: 22, W: 45).

3. ***Analysis of metal groupings on BDNF and NPY levels at birth***

The metal combinations comprising Factor A and B were analyzed in relation to BDNF and NPY levels adjusting for maternal BMI, smoking, SES, race/ethnicity, and sex. In exploratory analyses, these models were also stratified by sex and race/ethnicity. There were n =

177 individuals (females: 92 and males: 85 | AA: 65, Hispanic (H): 44, Other: 7, W: 61) with available BDNF and metals data, and n = 204 individuals (females: 102 and males: 102 | AA: 72, H: 50, Other: 9, and W: 73) with available NPY and metals data.

4. Analysis of BDNF/NPY and eating behavior

To examine whether BDNF and NPY levels at birth longitudinally associate with eating behavior outcomes 2-5 years later, we regressed the CEBQ domains of SR, SE, and SRSE on BDNF or NPY adjusting for covariates and run altogether and stratified by sex and race/ethnicity. In this analysis, there were n = 53 individuals for BDNF (females: 26 and males: 27 | AA: 19 and W: 33) and n = 59 individuals for NPY (females: 26 and males: 33 | AA: 20 and W: 38).

Measurement of BDNF and NPY

Archived plasma from umbilical cord blood stored at -80°C was used for the measurement of BDNF and NPY. Both markers were quantitated using ELISA kits (RayBiotech and R&D Systems, respectively) according to manufacturer's instructions. Samples for BDNF were run individually with at least 3-5 samples in duplicate per plate. Standards were assayed in duplicate for each plate. In all, for BDNF, n = 227 samples were run with 225 samples available for analysis. Samples for NPY and standards were run in duplicate and in all, n = 260 samples were run and 255 samples were available for analysis.

Results

Participant characteristics (by analysis)

Table 1a-f displays the participant characteristics for the four key analyses (Figure 1) given the different subsets and subsequent sample sizes within the larger population. Table 1a shows that in sex and race specific comparisons among the 107 participants in whom BMIz and CEBQ data were available, self-reported pre-pregnancy BMI was ~2 kg/m^2 higher in females and AA. The prevalence of prenatal cigarette smoking was higher in African Americans (22%) compared to Whites (4%). The percentage of college graduates was lower in African Americans (35%) when compared to Whites (80%). These differences were accounted for in statistical models as covariates.

While education was used as a proxy variable for SES in the first analysis between CEBQ and BMIz, the composite SES variable was used for all other analyses. There were significant differences by race with SES (with an average of ~60% of African American individuals compared to ~90% White individuals categorized as having "higher" socioeconomic status). Pre-pregnancy BMI was also ~2 kg/m^2 higher in AA than W for all analyses except for between BDNF/NPY and CEBQ response where BMI was similar. In some of our sub-analyses, African American mothers had a higher proportion of female offspring and White mothers had a higher proportion of male offspring.

While the analyses that examine associations with CEBQ scores are largely limited to African American and White mother-offspring dyads, the analyses examining metal Factors A or B with BDNF or NPY levels (Table 1c & 1d) had a sufficient number of Hispanic (H) individuals (n: 44 or 50). Hispanic individuals were significantly different in terms of SES (16-18% higher SES compared to ~60% of African Americans and ~90% of Whites). Hispanic individuals had slightly lower BMI than African Americans and higher BMI than Whites. In our analysis, Hispanic mothers were also the least likely to smoke cigarettes (~4% compared to ~30% African American and ~10% White mothers). The male to female ratio was 1:1.

Together, these participant characteristics highlight differences in maternal characteristics that necessitated examining the analyses by race/ethnicity.

Table 1. Participant characteristics by each sub-analysis

1a. CEBQ and BMIz at 5 years

	ALL (n=107)		Males (n=52)		Females (n = 55)		AA (n=49)		W (n = 49)	
	N (%)	Mean (SD)	N (%)	Mean (SD)	N (%)	Mean (SD)	N (%)	Mean (SD)	N (%)	Mean (SD)
Maternal Smoking										
Yes	14 (13.1)		7 (13.5)		7 (12.7)		11 (22.4)		2 (4.1)	
No	93 (86.9)		45 (86.5)		48 (87.3)		38 (77.6)		47 (95.9)	
Pre-pregnancy BMI										
kg/m^2		27.74 (7.14)		26.71 (7.18)		28.72 (7.03)		29.14 (7.57)		26.84 (6.80)
Education										
Less than high school	15 (14)		6 (11.5)		9 (16.4)		11 (22.4)		2 (4.1)	
High school grad/GED	14 (13.1)		8 (15.4)		6 (10.9)		9 (18.4)		3 (6.1)	
Some college	18 (16.8)		9 (17.3)		9 (16.4)		12 (24.5)		5 (10.2)	
College graduate	60 (56.1)		29 (55.8)		31 (56.4)		17 (34.7)		39 (79.6)	
Race/ethnicity										
African American	49 (45.8)		23 (44.2)		26 (47.3)					
Hispanic	0		0		0					
Other	9 (8.4)		3 (5.8)		6 (10.9)					
White	49 (45.8)		26 (50)		23 (41.8)					

1b. Metal factors and CEBQ

	ALL (n=69)		Males (n=37)		Females (n = 32)		AA (n=22)		W (n = 45)	
	N (%)	Mean (SD)	N (%)	Mean (SD)	N (%)	Mean (SD)	N (%)	Mean (SD)	N (%)	Mean (SD)
Maternal Smoking										
Yes	8 (11.6)		2 (5.4)		6 (18.8)		7 (31.8)		1 (2.2)	
No	61 (88.4)		35 (94.6)		26 (81.3)		15 (68.2)		44 (97.8)	
Pre-pregnancy BMI										
kg/m^2		25.80 (6.41)		24.81 (6.75)		26.95 (5.90)		26.11 (6.43)		25.63 (6.59)
SES										
Lower	12 (17.4)		6 (16.2)		6 (18.8)			8 (36.4)		3 (6.7)
Higher	57 (82.6)		31 (83.8)		26 (81.3)			14 (63.6)		42 (93.3)
Race/ethnicity										
African American	22 (31.9)		7 (18.9)		15 (46.9)					
Hispanic	0		0		0					
Other	2 (2.9)		0		2 (6.2)					
White	45 (65.2)		30 (81.1)		15 (46.9)					
Offspring sex										
Male							7 (31.8)		30 (66.7)	
Female							15 (68.2)		15 (33.3)	

1c. Metal factors and BDNF

	ALL (n=177)		Males (n=85)		Females (n = 92)		AA (n=65)		H (n=44)		W (n = 61)	
	N (%)	Mean (SD)	N (%)	Mean (SD)	N (%)	Mean (SD)	N (%)	Mean (SD)	N (%)	Mean (SD)	N (%)	Mean (SD)
Maternal Smoking												
Yes	28 (15.8)		12 (14.1)		16 (17.4)		19 (29.2)		2 (4.5)		7 (11.5)	
No	149 (84.2)		73 (85.9)		76 (82.6)		46 (70.8)		42 (95.5)		54 (88.5)	
Pre-pregnancy BMI												
kg/m^2		26.99 (7.07)		26.59 (6.92)		27.36 (7.22)		28.21 (8.68)		27.96 (5.42)		25.67 (6.06)
SES												
Lower	75 (42.4)		36 (42.4)		39 (42.4)		27 (41.5)		37 (84.1)		9 (14.8)	
Higher	102 (57.6)		49 (57.6)		53 (57.6)		38 (58.5)		7 (15.9)		52 (85.2)	
Race/ethnicity												
African American	65 (36.7)		23 (27.1)		42 (46.2)							
Hispanic	44 (24.9)		22 (25.9)		22 (24.2)							
Other	7 (4)		3 (3.5)		4 (4.4)							
White	61 (34.7)		37 (43.5)		23 (25.3)							
Offspring sex												
Male	85 (48)						23 (35.4)		22 (50)		37 (60.7)	
Female	92 (52)						42 (64.6)		22 (50)		24 (39.3)	

1d. Metal factors and NPY

	ALL (n=204)		Males (n=102)		Females (n = 102)		AA (n=72)		H (n=50)		W (n = 73)	
	N (%)	Mean (SD)	N (%)	Mean (SD)	N (%)	Mean (SD)	N (%)	Mean (SD)	N (%)	Mean (SD)	N (%)	Mean (SD)
Maternal Smoking												
Yes	31 (15.2)		13 (12.7)		18 (17.6)		21 (29.2)		2 (4)		7 (9.6)	
No	173 (84.8)		89 (87.3)		84 (82.4)		51 (70.8)		48 (96)		66 (90.4)	
Pre-pregnancy BMI												
kg/m^2		26.88 (7.16)		26.75 (7.32)		27.02 (7.02)		28.73 (9.17)		27.52 (5.39)		25.32 (5.58)
SES												
Lower	86 (42.2)		43 (42.2)		43 (42.2)		30 (41.7)		41 (82)		12 (16.4)	
Higher	118 (57.8)		59 (57.8)		59 (57.8)		42 (58.3)		9 (18)		61 (83.6)	
Race/ethnicity												
African American	72 (35.3)		27 (26.5)		45 (44.1)							
Hispanic	50 (24.5)		25 (24.5)		25 (24.5)							
Other	9 (4.4)		5 (4.9)		4 (3.9)							
White	73 (35.8)		45 (44.1)		28 (27.5)							
Offspring sex												
Male	102 (50)						27 (37.5)		25 (50)		45 (61.6)	
Female	102 (50)						45 (62.5)		25 (50)		28 (38.4)	

1e. BDNF and CEBQ

	ALL (n=53)		Males (n=27)		Females (n = 26)		AA (n=19)		W (n = 33)	
	N (%)	Mean (SD)	N (%)	Mean (SD)	N (%)	Mean (SD)	N (%)	Mean (SD)	N (%)	Mean (SD)
Maternal Smoking										
Yes	7 (13.2)		2 (7.4)		5 (19.2)		6 (31.6)		1 (3.0)	
No	46 (86.8)		25 (92.6)		21 (80.8)		13 (68.4)		32 (97.0)	
Pre-pregnancy BMI										
kg/m^2		25.81 (6.90)		24.88 (7.68)		26.77 (5.99)		25.69 (6.42)		25.95 (7.35)
SES										
Lower	10 (18.9)		5 (18.5)		5 (19.2)		8 (42.1)		2 (6.1)	
Higher	43 (81.1)		22 (81.5)		21 (80.8)		11 (57.9)		31 (93.9)	
Race/ethnicity										
African American	19 (35.8)		5 (18.5)		14 (53.8)					
Hispanic	0		0		0					
Other	1 (1.9)		0		1 (3.8)					
White	33 (62.3)		22 (81.5)		11 (42.3)					
Offspring sex										
Male	27 (50.9)						5 (26.3)		22 (66.7)	
Female	26 (49.1)						14 (73.7)		11 (33.3)	

1f. NPY and CEBQ

	ALL (n=59)		Males (n=33)		Females (n = 26)		AA (n=20)		W (n = 38)	
	N (%)	Mean (SD)	N (%)	Mean (SD)	N (%)	Mean (SD)	N (%)	Mean (SD)	N (%)	Mean (SD)
Maternal Smoking										
Yes	7 (11.9)		2 (6.1)		5 (19.2)		6 (30)		1 (2.6)	
No	52 (88.1)		31 (93.9)		21 (80.8)		14 (70)		37 (97.4)	
Pre-pregnancy BMI										
kg/m^2		25.51 (6.41)		25.00 (7.01)		26.16 (5.62)		25.59 (6.27)		25.52 (6.64)
SES										
Lower	11 (18.6)		6 (18.2)		5 (19.2)		8 (40)		3 (7.9)	
Higher	48 (81.4)		27 (81.8)		21 (80.8)		12 (60)		35 (92.1)	
Race/ethnicity										
African American	20 (33.9)		6 (18.2)		14 (53.8)					
Hispanic	0		0		0					
Other	1 (1.7)		0		1 (3.8)					
White	38 (64.4)		27 (81.8)		11 (42.3)					
Offspring sex										
Male	33 (55.9)						6 (30)		27 (71.1)	
Female	26 (44.1)						14 (70)		11 (28.9)	

Satiety responsiveness (SR) and slowness in eating (SE) domains are associated with BMIz at age 5 years with sex-specific differences

Of the six CEBQ domains analyzed (Figure 2), satiety responsiveness (SR) and slowness in eating (SE) were significantly inversely associated with BMIz at 5 years. As this study aimed to assess the potential role of metal exposures on neuroregulatory mechanisms of hunger and satiety in obesity, all subsequent analyses are restricted to these two food avoidant domains (SR and SE) as well as the combined variable SRSE. After adjusting for maternal BMI, smoking, education, sex, and race/ethnicity, we found (Table 2) that overall, children with higher satiety responsiveness scores had lower BMIz scores [$\hat{\beta}$: -0.608, se: 0.175, p=0.0008]. These associations may be stronger in females [β: -0.940, se: 0.286, p=0.002] than males [$\hat{\beta}$: -0.299, se: 0.223, p=0.19] and similar in African Americans [$\hat{\beta}$: -0.677, se: 0.292, p=0.025] when compared to Whites [$\hat{\beta}$: -0.622, se: 0.251, p=0.018]. These directional associations were also consistent for slowness in eating with females [$\hat{\beta}$: -0.661, se: 0.288, p=0.026] with potentially stronger associations when compared to males [$\hat{\beta}$: -0.233, se: 0.241, p=0.339], and similar between African Americans and Whites. When satiety responsiveness and slowness in eating were combined as a domain (SRSE), these significant inverse associations persisted [$\hat{\beta}$: -0.75, se: 0.206, p<0.001 overall] and [$\hat{\beta}$: -1.122, se: 0.327, p=0.001 in females]. Together, these inverse relationships suggest that children with decreased satiety responsiveness or slowness in eating (increased food intake may be required to be satiated and/or due to faster eating) are more likely to be obese.

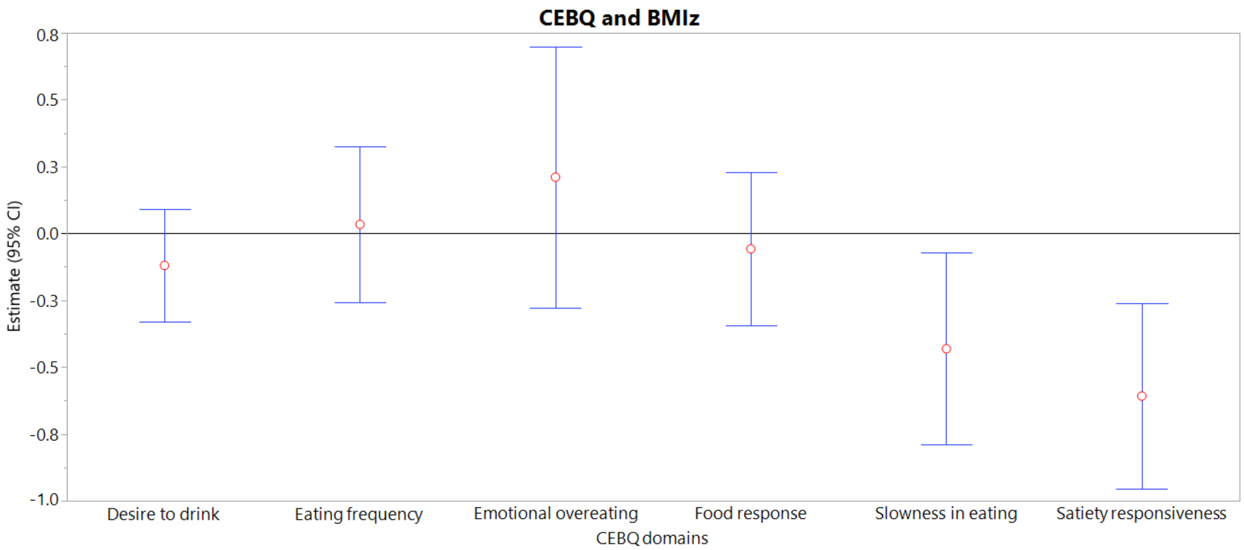
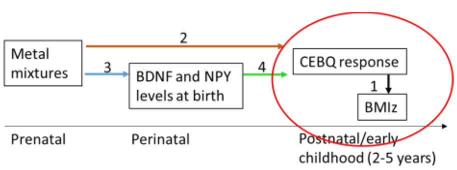


Figure 2. Regression of BMIz at 5 years on six CEBQ eating domains
Estimates of regression coefficients (open red circles) with 95% CIs.

Table 2. Satiety and appetite regulation and BMIz at 5 years



Outcome: **BMIz age 5 years**
Co-variables: **maternal BMI, smoking, education, race*, sex***

	Satiety responsiveness (SR)					Slowness in eating (SE)					Combined SRSE			
	n	$\hat{\beta}$	se	p-value		n	$\hat{\beta}$	se	p-value		n	$\hat{\beta}$	se	p-value
All	107	**-0.608**	**0.175**	**0.0008**	**All**	107	**-0.431**	**0.181**	**0.019**	**All**	107	**-0.750**	**0.206**	**0.0004**
Females	55	**-0.940**	**0.286**	**0.002**	**Females**	55	**-0.661**	**0.288**	**0.026**	**Females**	55	**-1.122**	**0.327**	**0.001**
Males	52	-0.299	0.223	0.186	**Males**	52	-0.233	0.241	0.339	**Males**	52	-0.392	0.270	0.154
AA	49	**-0.677**	**0.292**	**0.025**	**AA**	49	-0.619	0.342	0.078	**AA**	49	**-1.012**	**0.373**	**0.010**
W	49	**-0.622**	**0.251**	**0.018**	**EA**	49	-0.301	0.246	0.228	**EA**	49	**-0.635**	**0.287**	**0.033**

AA: African American | W: White
*Not in model when stratified by the variable
Analyses with CEBQ were limited to AA and W given small sample sizes for other race/ethnicities

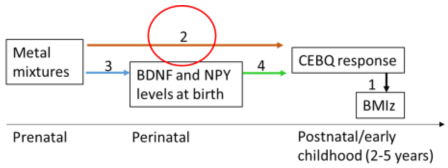
The estimate of the regression coefficient ($\hat{\beta}$), standard error (se), and p-values are presented for analyses run altogether and by sex and race.

Metal mixtures associate with satiety and appetite response and are sex specific

To determine the extent to which these obesity-related eating behaviors were associated with prenatal exposure to metal mixtures, we evaluated the association between the two CEBQ domains, satiety responsiveness (SR), slowness in eating (SE), and the combined SRSE, and metal combinations in Factor A and B, previously identified to be associated with increased BMI (Chapter Three) on (Table 3a-c). The most significant associations were between Factor A comprised of barium, calcium, cobalt, copper, and strontium and SR [$\hat{\beta}$: -0.33, se: 0.09, p=0.0004]. This association was stronger when we considered the combined domain SRSE [$\hat{\beta}$: -0.25, se: 0.08, p=0.004].

These inverse associations indicate that prenatal exposure to this metal combination is associated with lower satiety response and the association with slowness in eating may be less consistent. As identified in the analysis between SR and BMIz, the associations between metal combination (Ba, Ca, Co, Cu, Sr) and satiety response or combined SRSE may be stronger in females (SR [$\hat{\beta}$: -0.50, se: 0.10, p<0.0001] and SRSE [$\hat{\beta}$: -0.39, se: 0.10, p=0.0008]). While Factor B comprised of mercury and tin was also associated with SR and SRSE in females, these associations are less robust. As seen in Table 3, the inverse associations with Factor A appear to be similar in African Americans and Whites, especially with respect to satiety response.

Table 3. Metal mixtures and satiety and appetite regulation



3a. Metal mixtures and satiety responsiveness

Outcome: Satiety responsiveness

Co-variables: maternal BMI, smoking, SES, race*, sex*

	Factor A					Factor B			
	n	β	se	p-value		n	β	se	p-value
All	69	**-0.328**	**0.087**	**0.0004**	**All**	69	-0.130	0.112	0.253
Females	32	**-0.499**	**0.102**	**<.0001**	**Females**	32	-0.300	0.191	0.129
Males	37	-0.184	0.138	0.194	**Males**	37	0.050	0.171	0.769
AA	22	**-0.531**	**0.139**	**0.002**	**AA**	22	**-0.466**	**0.205**	**0.037**
W	45	**-0.221**	**0.106**	**0.044**	**W**	45	0.134	0.144	0.355

Analyses with CEBQ were limited to AA and W given small sample sizes for other race/ethnicities

3b. Metal mixtures and slowness in eating

Outcome: Slowness in eating

Co-variables: maternal BMI, smoking, SES, race*, sex*

	Factor A					Factor B			
	n	β	se	p-value		n	β	se	p-value
All	69	-0.150	0.104	0.155	**All**	69	-0.141	0.124	0.258
Females	32	**-0.253**	**0.141**	**0.084**	**Females**	32	**-0.378**	**0.196**	**0.066**
Males	37	-0.072	0.162	0.659	**Males**	37	-0.115	0.194	0.555
AA	22	-0.219	0.190	0.265	**AA**	22	-0.359	0.224	0.129
W	45	-0.120	0.133	0.372	**W**	45	-0.127	0.173	0.469

Analyses with CEBQ were limited to AA and W given small sample sizes for other race/ethnicities

3c. Metal mixtures and satiety responsiveness + slowness in eating

Outcome: **Combined SRSE**

Co-variables: **maternal BMI, smoking, SES, race*, sex***

	Factor A					**Factor B**			
	n	**β**	**se**	**p-value**		**n**	**β**	**se**	**p-value**
All	69	**-0.250**	**0.084**	**0.004**	**All**	69	-0.134	0.104	0.204
Females	32	**-0.392**	**0.103**	**0.0008**	**Females**	32	**-0.334**	**0.169**	**0.060**
Males	37	-0.135	0.134	0.322	**Males**	37	-0.023	0.163	0.889
AA	22	**-0.397**	**0.147**	**0.016**	**AA**	22	**-0.417**	**0.191**	**0.044**
W	45	-0.177	0.105	0.100	**W**	45	0.018	0.141	0.897

Analyses with CEBQ were limited to AA and W given small sample sizes for other race/ethnicities

BDNF and NPY levels at birth associate with metal groupings

Because higher BDNF and lower NPY levels are associated with increased satiety and appetite suppression, we sought to evaluate if the mechanism by which prenatal exposure to trace metals [measured in blood obtained at median gestation age 12 weeks] alters eating behavior at age 2-5 years may be due in part to altered BDNF and NPY concentrations as early as birth. To accomplish this, we evaluated the association between the two metal mixtures (Factor A and B) and BDNF and NPY levels at birth. As seen in Table 4a & b, we found near significant associations between Factor A comprising barium, calcium, cobalt, copper, and strontium and lower BDNF [$\hat{\beta}$: -35.31, se: 17.95, p=0.051]; interestingly, this association may be stronger in males [$\hat{\beta}$: -51.21, se: 27.43, p=0.066] than females [$\hat{\beta}$: -21.21, se: 24.46, p=0.388]; however, the standard errors were large. The metal mixture comprised of tin and mercury was also associated with lower concentrations of BDNF at birth [$\hat{\beta}$: -45.16, se: 23.45, p=0.056] and this appears to be largely driven by males as well [$\hat{\beta}$: -76.11, se: 34.98, p=0.033] as compared to females [$\hat{\beta}$: -10.66, se: 35.82, p=0.767].

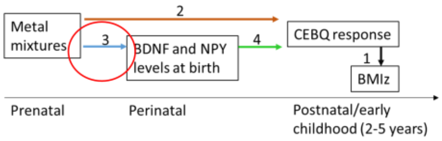
We also determined whether these associations varied by race since African Americans and Hispanics have, on average, higher concentrations of these toxic metals than Whites.

Interestingly, the strongest association was with Factor B and Whites [$\hat{\beta}$: -97.16, se: 49.02, p=0.053]. We did not find any associations with NPY.

These associations suggest that lower concentrations of Ba, Ca, Co, Cu, and Sr, as well as Hg during pregnancy may increase BDNF levels at birth. These associations indicate that the factors comprising BaCaCoCuSr and HgSn reduce BDNF levels that are evident at birth, and satiety response at age 5 years.

To our knowledge, these are the first data finding that the metal mixture comprising BaCaCoCuSr in the first trimester may be important in regulating obesity development via appetite and satiety regulation. To determine if the non-normal distributions of BDNF and NPY may have unduly influenced our findings, we dichotomized the BDNF and NPY concentrations at the upper 95% mean and employed logistic regression models. Doing so did not materially alter the findings of an inverse association between the maternal metal mixture Factor A [p=0.109], or Factor B [p=0.089] and BDNF levels at birth. No associations were found with Factor A or B and NPY.

Table 4. Metal mixtures and BDNF or NPY levels



4a. Metal mixtures and BDNF

Outcome: BDNF

Co-variables: maternal BMI, smoking, SES, race*, sex*

	Factor A					Factor B			
	n	β	se	p-value		n	β	se	p-value
All	177	**-35.31**	**17.95**	**0.051**	All	177	**-45.16**	**23.45**	**0.056**
Females	92	-21.21	24.46	0.388	Females	92	-10.66	35.82	0.767
Males	85	**-51.21**	**27.43**	**0.066**	Males	85	**-76.11**	**34.98**	**0.033**
AA	65	-39.83	30.35	0.195	AA	65	-4.49	34.96	0.898
H	44	-37.78	35.60	0.295	H	44	-17.20	52.44	0.745
W	61	-44.90	29.93	0.139	W	61	**-97.16**	**49.02**	**0.053**

4b. Metal mixtures and NPY

Outcome: NPY

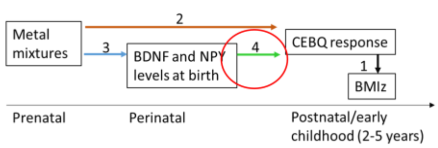
Co-variables: maternal BMI, smoking, SES, race*, sex*

	Factor A					Factor B			
	n	β	se	p-value		n	β	se	p-value
All	204	0.092	0.608	0.879	All	204	0.124	0.806	0.878
Females	102	-0.766	0.871	0.381	Females	102	-0.991	1.213	0.416
Males	102	0.999	0.870	0.254	Males	102	0.890	1.174	0.451
AA	72	-0.933	0.970	0.340	AA	72	0.167	1.185	0.888
H	50	0.353	1.115	0.753	H	50	-0.776	1.612	0.633
W	73	1.428	1.146	0.217	W	73	-0.076	1.856	0.968

BDNF levels associate with satiety responsiveness and slowness in eating domains

Regressing eating behavior responses on BDNF and NPY levels identified near significant associations between BDNF and satiety responsiveness, slowness in eating, and combined SRSE (Table 5a-c). These associations appear to be largely driven by females (Satiety Responsiveness [$\hat{\beta}$: 0.001, se: 0.0007, p=0.114], Slowness in Eating [$\hat{\beta}$: 0.002, se: 0.0006, p=0.009], and the combined variable SRSE [$\hat{\beta}$: 0.0014, se: 0.0006, p=0.022]. No significant associations between NPY and eating behavior domains were found. Our data indicating that increased BDNF levels at birth associates with increased satiety response and eating more slowly in early childhood are consistent with the hypothesis that the appetite suppressing functions of BDNF may modulate eating behaviors related to satiety regulation.

Table 5. BDNF or NPY levels and eating behavior



5a. BDNF or NPY and satiety responsiveness

Outcome: Satiety responsiveness
Co-variables: maternal BMI, smoking, SES, race*, sex*

	BDNF					NPY			
	n	β	se	p-value		n	β	se	p-value
All	**53**	**0.0008**	**0.0004**	**0.076**	**All**	59	0.005	0.011	0.672
Females	26	0.0011	0.0007	0.114	**Females**	26	-0.011	0.021	0.606
Males	27	0.0006	0.0006	0.337	**Males**	33	0.011	0.013	0.432
AA	19	0.0009	0.0008	0.310	**AA**	20	-0.018	0.023	0.461
W	33	0.0006	0.0006	0.275	**W**	38	0.011	0.013	0.392

Analyses with CEBQ were limited to AA and W given small sample sizes for other race/ethnicities

5b. BDNF or NPY and slowness in eating

Outcome: Slowness in eating
Co-variables: maternal BMI, smoking, SES, race*, sex*

BDNF					NPY				
	n	β	se	p-value		n	β	se	p-value
All	53	**0.0008**	**0.0005**	**0.078**	**All**	59	0.005	0.011	0.645
Females	26	**0.0018**	**0.0006**	**0.009**	**Females**	26	-0.008	0.021	0.707
Males	27	0.0003	0.0007	0.726	**Males**	33	0.009	0.014	0.546
AA	19	**0.0013**	**0.0007**	**0.095**	**AA**	20	-0.017	0.023	0.458
W	33	0.0007	0.0006	0.285	**W**	38	0.009	0.014	0.508

Analyses with CEBQ were limited to AA and W given small sample sizes for other race/ethnicities

5c. BDNF or NPY and satiety responsiveness + slowness in eating

Outcome: Combined SRSE
Co-variables: maternal BMI, smoking, SES, race*, sex*

BDNF					NPY				
	n	β	se	p-value		n	β	se	p-value
All	53	**0.0008**	**0.0004**	**0.046**	**All**	59	0.005	0.010	0.621
Females	26	**0.0014**	**0.0006**	**0.022**	**Females**	26	-0.010	0.018	0.607
Males	27	0.0005	0.0006	0.456	**Males**	33	0.010	0.013	0.441
AA	19	0.0010	0.0007	0.155	**AA**	20	-0.017	0.021	0.411
W	33	0.0007	0.0005	0.223	**W**	38	0.010	0.012	0.397

Analyses with CEBQ were limited to AA and W given small sample sizes for other race/ethnicities

Discussion

To gain mechanistic insights linking prenatal exposure to trace metal mixture that comprises BaCaCoCuSr to obesity in children at age 5 years, we examined associations between metal mixtures, biomarkers of appetite and satiety regulation, and eating behavior responses (satiety and eating rate). Our key findings were that the metal mixtures, previously found to be associated with increased BMIz scores in children, were associated with lower BDNF levels and reduced satiety and increased eating speed, consistent with the idea that prenatal exposure to environmental chemicals may affect satiety function. Our findings necessitate examining the neurotoxic effects of metals in the context of obesity risk. Specifically, our findings highlight the need to assess the relationships between co-occurring metal exposure during pregnancy and the altered regulation of satiety and appetite.

Metals such as iron and zinc are essential factors or co-factors for normal brain development whereas metals including arsenic, cadmium, lead, and mercury are neurotoxic both in development and adulthood (7, 16, 17). These heavy metals have been shown to affect different parts of the brain (18, 19). Moreover, depending on dose, there are essential metals that can also be toxic (e.g., copper, manganese). While neurotoxic effects of these metals in cognitive, motor, and behavioral development and neurodegeneration is well-documented, these metals are increasingly being implicated as environmental obesogens (20-22). The effects of prenatal exposure to metals on the neurocircuitry involved in regulating appetite, hunger, and satiety function are less studied. The role of metals and how they may alter the interplay of the gut-brain axis is a developing area of research (8, 23).

In our study, we focused on two markers of appetite and satiety regulation: brain derived neurotrophic factor (BDNF) and neuropeptide Y (NPY). Although BDNF plays key roles in brain development related to synaptic plasticity, placental and fetal development, neuroinflammation, and neurobehavioral function (24-26), the expression and levels of this neurotrophic factor are also associated with appetite suppression and/or improved satiety response. In support of these functions, data from humans and model systems indicate that the reduction of BDNF expression is associated with hyperphagia and obesity (10, 27-29). If these findings are confirmed in larger studies, they would support that lower levels of BDNF in umbilical cord blood plasma may identify children with a higher likelihood of obesity, for early interventions when obesity is still easier to treat.

NPY also has important roles in multiple processes including the regulation of mood, immune function, and behavior (11, 30, 31). NPY is an orexigenic peptide that stimulates the appetite and reduces satiety. It is involved in the crosstalk of the adipose-gut-brain axis (11, 32). Both BDNF and NPY have been measured as markers of different neurodevelopmental outcomes. Altered levels of both have been found associated with metal exposures. For example, arsenic and lead was associated with reduced BDNF levels while cadmium has been associated with increased NPY levels (33-35). In our WGBS study examining cadmium exposure and differential DNA methylation, we identified significant hypermethylation of *BDNF* in mothers with higher cadmium levels (36).

We selected these markers to approximate appetite and satiety dysregulation based on the review of the literature and our own findings, however, we note that there are additional markers including leptin, insulin, pro-opiomelanocortin, and inflammatory cytokines that should and can be evaluated to further elucidate our observations. While our study identified relationships between co-occurring chemicals, biomarkers of appetite regulation, and eating behavior and obesity, the data should be interpreted in the context of the limitations. One limitation is that we may have been under-powered, especially for the stratified analyses. For example, although there were sex differences, evaluating these differences within race/ethnic strata would have greatly diminished power. While we identified associations between metal mixtures and BDNF levels and eating behavior response, *in vitro* or *in vivo* studies to recapitulate these findings is needed given the potential roles of unmeasured confounding present in epidemiologic analyses. We also note that BDNF and NPY levels were measured only once, at birth. These relationships may be associated with unmeasured maternal factors, including non-chemical stressors, and thus, analysis with these factors, when available, could clarify these associations. Ideally, BDNF and

NPY measurements in maternal blood during pregnancy and in the offspring at multiple time points between birth and the age at which BMI and CEBQ were measured, would clarify the nature of these associations. Additionally, there are methodological considerations associated with BDNF quantitation. We measured BDNF levels in plasma, however, some protocols emphasize the need to measure BDNF in platelet-poor plasma or serum. Meanwhile, one study found that the heterogeneity of BDNF measurements was not prevented when utilizing platelet-poor plasma as compared to plasma (37-40). This highlights the need to further evaluate and interpret how, in the context of its role as an appetitive marker, BDNF levels may or may not vary in the different blood components. Therefore, measurements at multiple time points and in different biological samples (such as serum and saliva) would strengthen and clarify our findings.

These limitations notwithstanding, our study utilized pre-, peri-, and post-natal time points to examine the associations of how prenatal exposure to metal mixtures can modulate markers of appetite and satiety at birth, and subsequent eating behaviors, contributing to an increased risk of obesity. Our study begins to provide mechanistic insights into how environmental exposures may contribute to obesity via the alteration of eating related neurocircuitry that can be tested in model systems.

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CHAPTER FIVE

Analysis of Umbilical Cord Blood Differential DNA Methylation in African Americans and Whites

This manuscript is currently in preparation for journal submission

Abstract

Background: The incidence of chronic human diseases, including cardiovascular disease, Type 2 diabetes mellitus, and certain cancers, are increasing and affecting African Americans (AA) and other ethnic minorities disproportionately. While genetics have clarified some potential mechanistic links behind increased onset and disparate outcomes, informative epigenetic data are limited.

Objective: Our objective was to identify methylated CpG sites associated with African Americans (AA) when compared to Whites (W) that are distinct as early as birth.

Methods: Pregnant women enrolled at median gestation 12 weeks (n=367 [AA: n=197, W: n=170]) were followed to delivery, where parturition data and specimens were collected. DNA extracted from umbilical cord whole blood was analyzed using Infinium HumanMethylation450K arrays, and *ACKR1* (Duffy blood group) pyrosequencing and genotyping. Regression models were used to identify CpG site methylation associated with AA, adjusting for maternal cigarette smoking, parity, maternal age, pre-pregnancy obesity, sex, gestational age, socioeconomic status, and cell proportions in whole blood leukocytes.

Results: After Bonferroni correction, the methylation at 4,579 CpG sites mapping to 1,942 genes were significantly associated with race/ethnicity (adjusted $p<0.05$). Gene enrichment analysis suggests that the top terms include stroke, myocardial infarction, and BMI. The top two CpG sites (cg04922029 & cg18552413) associated with AA (24.7% and 7.5% higher methylation, respectively) map to *ACKR1*, and are associated with the African ancestral C allele of rs2814778, shown to be associated with differences in inflammatory response.

Conclusion: We identified DNA methylation profiles in umbilical cord blood that associate with African Americans. Several of the identified differentially methylated CpGs map to genes related

to inflammation signaling, which may contribute to disparities in inflammation-related chronic diseases such as cardiovascular disease and Type 2 diabetes mellitus.

Introduction

Non-communicable chronic conditions, including Type 2 diabetes, cardiovascular disease, and neoplasms, remain the leading causes of death globally. In the US, the incidence and mortality of these chronic conditions also disproportionately affect ethnic minority populations (1, 2). Although the past 20 years have seen a reduction in the life expectancy gap between African Americans (AAs) and Whites (W), disparities in morbidity and mortality remain (3). The geographic variation in incidence of these diseases, where the preponderance of chronic diseases such as cardiovascular disease exist in the southeastern US, and the higher risk for individuals of African descent, point to a complex relationship between genetic and environmental factors. Although genetic factors that contribute to these outcomes are well-documented, it is becoming increasingly clear that the contribution of environmental factors (comprised of both chemical and non-chemical exposures) to these disparate outcomes may be larger than previously appreciated (4). Elucidating the potential mechanisms underlying these disparities, especially those linked to early life when preventive measures may be taken (e.g., lower birthweight), is an important public health need.

The Developmental Origins of Health and Disease (DOHaD) hypothesis posits that environmental exposures during the perinatal period play a critical role for outcomes (5, 6). Data accumulated over the last fifteen years from model systems and human studies support the hypothesis that epigenetics links the early environment to adult disease susceptibility. Epigenetic responses to environmental factors can cause long-term changes to gene pathways. Epigenetic mechanisms underpinning disease risk and development is a key area of research in terms of functional characterization and accessibility.

DNA methylation at cytosines of CpG dinucleotides is one of the most studied epigenetic mechanisms regulating chromatin structure and gene expression in humans. Altered CpG methylation has been linked to both chemical and non-chemical exposures in adults and children (7-9). Multiple CpG sites associated with AA status have been identified in DNA obtained from adults (10, 11). However, whether differential methylation at these sites is also associated with early indicators of chronic disease has not been extensively studied. These risk factors include lower birth weight or preterm birth, which also disproportionately affect African Americans. Elucidating epigenetic associations to these outcomes may provide mechanistic insights linking early life to adult-onset disease, and point to novel pathways that may be amenable to intervention (12, 13).

In adults, a growing number of studies have analyzed CpG methylation using genome-scale approaches (e.g., the Illumina 450K or EPIC arrays) to identifying race/ethnicity associated DNA methylation differences (14). Biological processes, conditions, and diseases studied include inflammation (10), immune function (11), metabolic syndrome (15), and daytime sleepiness (16), as well as responses to environmental exposures such as air pollution (17) and cigarette smoking (18). To our knowledge, only a few studies have examined these differences in early life, which the DOHaD hypothesis posits is a critical time window for these alterations to occur. One longitudinal analysis examined methylation throughout the pregnancy course and at birth (13), one multi-cohort study characterized and generated prediction metrics on race/ethnicity differences using placental DNA methylation data (19), and another multi-cohort study examined interactions of genetics and environment on newborn cord blood DNA methylation (12). In a similar approach, one study examined DNA methylation differences in African American and White newborns using the 27K array (45). The purpose of this present study was to identify differential methylation of CpG sites associated with AA status among newborns, from array data comprising more than 450,000 CpG sites, and to determine baseline methylation differences that may contribute to these disparate outcomes.

Methods

Study participants

Participants for the study derived from the Newborn Epigenetics Study (NEST), a prospective pre-birth cohort of mothers and their offspring, were enrolled between 2005 and 2009. The protocol for accruing study participants and collecting specimens has been described in detail (20, 21). Briefly, ~1,100 pregnant women attending Duke Medicine prenatal clinics were approached and ~900 consented to participate. To be eligible for inclusion, participants had to be ≥18 years, English speakers, and plan to deliver the index offspring at Duke Obstetrics to allow for collection of maternal and offspring specimens and parturition data. Among these participants, we excluded women who planned to put the index offspring up for adoption, as this would limit future follow-up, and those known to be HIV positive because of unknown effects of HIV treatment on the epigenome. The present analyses are limited to the first ~400 in whom we measured CpG methylation using the Infinium 450K Human Methylation Beadchip (21-24) and to African American and White participants.

Data and specimen collection

A risk factor questionnaire was completed by pregnant women who agreed to participate in the study. The questionnaire included anthropometric, lifestyle, and sociodemographic factors previously shown to influence methylation profiles and/or vascular, metabolic and neurodevelopmental outcomes. Race/ethnicity was self-reported from categories that included non-Hispanic Black, non-Hispanic White, Hispanic, American Indian/Alaska Native, Asian, and

Other. Maternal age was categorized based on whether at the time of delivery, women were of advanced maternal age (≥35 years) or not (<35 years). Body mass index (BMI) before pregnancy was categorized by BMI <30 kg/m^2 and BMI ≥30 kg/m^2. Cigarette smoking was categorized into whether or not women smoked before or during pregnancy. Parity was categorized as nulliparity (0), primiparity (1) or multiparity (≥2). The composite measure of socioeconomic status (SES) was derived from education and insurance information.

At delivery, parturition data were abstracted from medical records using a standardized form, and included gestational age of the offspring (in weeks), sex, and birth weight (in grams). Umbilical cord blood was gravity drained from the umbilical vein into an EDTA tube. Within two hours, these specimens were transported to the laboratory where they were centrifuged to separate plasma, buffy coat, and red cell fractions, and aliquots were frozen at -80ºC.

Quantifying DNA methylation in umbilical cord blood

DNA was purified from buffy coat and 500 ng aliquots were submitted to Duke Genome Sciences Core Laboratory for processing. Methods for bisulfite conversion and methylation analyses have been detailed elsewhere (25, 26). Briefly, purified genomic DNA was bisulfite-converted using the EZ-96 DNA Methylation Kit (Zymo Research) according to manufacturer's instructions. Methylation data from the 450K array was analyzed in R. The *Minfi* (27) package (version 1.28.0) was used to calculate the methylation fractions. Raw microarray intensities were normalized by *preprocessQuantile* function in the *minfi* package. Bead counts were extracted by the *beadcount* function in wateRmelon (28) package. We excluded probes with detection p-value >0.01 or bead counts <3 in >5% of samples. The *Combat* function in *SVA* (29) package was used to adjust for batch effects. We excluded probes containing a SNP with minor allele frequency >5% at the site of CpG interrogation or at the single nucleotide extension. Based on PCA analysis, we removed samples with mis-matched sex. We also removed samples from participants who were not African American or White. After the quality control procedure, a total 475,384 probes in 367 samples remained for association analysis. We estimated cell type proportion using Houseman's reference-based method (30).

Evaluation of association between methylation and ethnicity and pathway and gene-set enrichment analysis

We employed multiple linear regression analysis with CpG site methylation as the outcome and the predictor variables of self-reported race/ethnicity (AA or W) and the following covariates: smoking before or during pregnancy, parity, maternal age, pre-pregnancy obesity, sex, gestational age, socioeconomic status, and cell proportions in whole blood. We applied a Bonferroni correction to control for multiple testing. For our pathway and gene-set enrichment analyses, we utilized the Ingenuity Pathway Analysis (Qiagen) and Enrichr tools (31, 32).

Genotyping for *ACKR1/DARC* rs2814778

Among the n = 367 individuals included in the regression models, DNA from cord blood was available or extracted from frozen buffy coat and genotyped for the *ACKR1* SNP rs2814778. An M13 biotinylated primer was used for SNP genotyping using pyrosequencing: 5'-[biotin]-CGCCAGGGTTTTCCCAGTCACGAC-3' (44), and genotyping primers for rs2814778 were designed using PyroMark Assay Design Software (Qiagen): FYN fwd (with M13 sequence underlined) 5'-<u>CGCCAGGGTTTTCCCAGTCACGAC</u>GATGGCCCTCATTAGTCCTTG-3'; FYN rvs 5'-CTGTCAGCGCCTGTGCTT-3'; and FYN seq 5'- GCGCCTGTGCTTCCA-3'. In total, n = 250 (AA: 136 | W: 114) were genotyped. We employed multiple linear regression analysis with the topmost significant differentially methylated CpGs in our analysis *ACKR1* cg04922029 and cg18552413 as the outcome variables, and the predictor variables of rs2814778 genotype with the aforementioned covariates. For validation, an additional 62 umbilical cord blood DNA samples (32 AA and 30 W) that were not part of the n=367 were also genotyped for rs2814778.

Bisulfite PCR and pyrosequencing for the *ACKR1/DARC* significant CpGs

Using Pyromark Assay Design Software (Qiagen), we designed bisulfite primers for pyrosequencing assays to cover the region with the most significantly methylated CpG sites. Two primer sets were designed; one to capture cg18552413, and the other to capture cg04922029 along with three proximal CpG sites within 50 bases (Figure 1). These assays were run on bisulfite converted DNA samples from the validation dataset (n = 62). DNA was bisulfite converted using the EpiTect bisulfite conversion kit (Thermo Fisher) for pyrosequencing analysis.

```
Sequence is 5' to 3'

GTAAAATGCCCACTTTCTGGTCCCCACCTTTTTCCTGAGTGTAGTCCCAA
CCAGCCAAATCCAACCTCAAAACAGGAAGACCCAAGGCCAGTGACCCCCA
TAGGCCTGAGGCTTGTGCAGGCAGTGGGCGTGGGGTAAGGCTTCCTGATG
CCCCCTGTCCCTGCCCAGAACCTGATGGCCCTCATTAGTCCTTGGCTCTT
ATCTTGGAAGCACAGGCGCTGACAGCCGTCCCAGCCCTTCTGTCTGCGGG
CCTGAACCAAACGGTGCCATGGGGAACTGTCTGCACAGGGTGAGTATGGG
GCCAGGCCCCAGAGTCCCTTATCCCTATGCCCCTCATTTCCCGTGCTGTT
TGCCCCTCAGTCTTTATATCTCTTCCTTTTCCTCCTCATCTTTTCTCCCT


Primers for cg18552413:

DARC pyro Bt RVS-2: Btn-CCCCCATAAACCTAAAACTTATACAAAC
DARC pyro FWD-2: AGGATTAATGAGGGTTATTAGGT
DARC pyro SEQ-2: ATAGGGGGTATTAGGAA

Primers for cg04922029:

DARC pyro Bt RVS-1: Btn-CCCTATCCCTACCCAAAACCTAATAA
DARC pyro FWD-1: GATAAGGGATTTTGGGGTTTGGTTTTATA
DARC pyro SEQ-1: ATTTTGTGTAGATAGTTTTTTATG


Other annotations in the sequence:
Additional CGs (no cg ID)
SNP rs2814778
ATG
```

Figure 1. Primer sequences for the validation of *ACKR1* methylation

Evaluation of epigenetic age

We utilized the epigenetic clock calculator (33) to estimate the epigenetic age of the cord blood DNA samples between African Americans (n = 164) and Whites (n = 142). Given that these samples were obtained at the time of birth, we had a reliable measure of age (i.e., 0 years).

Statistical analysis

Data were analyzed in GraphPad Prism 8, JMP Pro 14, or R.

Results

Distribution of covariates among study subjects

The distribution of key characteristics for the 197 AA and 170 W pregnant women and their offspring are presented in Table 1. African American women were significantly more likely to smoke than White women (41.1% vs. 26.5%, p-value <0.05). They also were significantly more likely to be multiparous, deliver preterm and lower birth weight infants, be obese prior to pregnancy, and of lower socioeconomic status ($p<0.05$). In contrast, White women were significantly more likely to be of advanced maternal age. We found no significant differences in the distribution of sex and cell proportions, but all the factors were included in the analysis.

Race/ethnicity, CpG methylation differences, and pathway analysis

When comparing offspring born to African American and White women, 4,579 differentially methylated CpG sites were identified (Bonferroni adjusted p-value <0.05) mapping to 1,942 genes. These associations were adjusted for cell proportions whose expected distribution are provided in Table 1. Gene-set enrichment analysis of the 1,942 genes using Ingenuity Pathway Analysis (IPA) identified significant ($p<0.05$, right-tailed Fisher's exact test) canonical pathways related to cytokine signaling, inflammation, and cancers including breast and colon. The top 25 most significant terms are presented in Table 2a. Other organ/tissue specific pathways and terms include neuronal signaling and cardio-metabolic pathways (e.g., leptin signaling in obesity, Type 2 diabetes signaling, and cardiovascular related processes). We also input the 1,942 genes into Enrichr to identify top diseases and pathways through dbGaP and GWAS 2019. Top diseases and pathways comprised cardio- and cerebro-vascular terms including body mass index, myocardial infarction, heart rate, and stroke. The top 20 most significant terms are presented in Table 2b & 2c. Notwithstanding the broad scope of these analyses, consistent terms related to inflammatory conditions were generated. The fact that these conditions disproportionately affect African Americans suggests disparate risk may exist as early as birth.

Table 3 provides the top 20 genes that contain the most significant methylation differences for offspring of African American women when compared to White offspring (AA individuals were the reference population) after adjusting for cell proportions. The two most significantly differentially methylated CpG sites map to the Atypical Chemokine Receptor 1 (*ACKR1*), previously known as *DARC* and annotated as such in the 450K array and in Table 3. These two CpG sites, cg04922029 and cg18552413 are proximal to the SNP rs2814778, a regulatory variant in the 5'UTR of *ACKR1*. We also identified a third CpG site, cg20003179, that also maps to *ACKR1* but its significance and magnitude of methylation change was considerably lower. For cg04922029 and cg18552413, methylation differences were 24.7% higher (adjusted p-value= 1.7E-61) and 7.5% (adjusted p-value = 9.9E-43) respectively, in offspring born to African American mothers compared to those born to White mothers. However, White newborns had 1.2% higher methylation of the CpG site cg20003179, adjusted p-value = 0.012.

Six CpG sites in the neighboring gene Cell Adhesion Molecule 3 (*CADM3*) have significant differences between African Americans and Whites. The two CpG sites with closest proximity to *ACKR1* (i.e., within 10kb of *ACKR1*), were also hypermethylated in AA (~5% higher, adjusted p-value <E-16). The remaining four CpGs were all hypomethylated in AA (~3.5% lower). *CADM3* and *ACKR1* are members of a conserved linkage group, along with *AIM2* and *FCER1A* (34), the latter two did not significantly differ between African Americans and Whites.

Several other genes with multiple CpG sites were also significantly associated with AA status. These include CpG sites mapping to *ARTN* (n=8), *RNF135* (n=8), and *SEPT8* (n=10). We also identified seven CpG sites mapping to seven separate genes with >25% difference in methylation, and thus higher percentage differences than *ACKR1*. Two were hypomethylated in AA newborns (*LPHN3*, 28.2%, adjusted p-value =1.3E-11 and *SLC25A21*, 25.1%, adjusted p-value = 0.0013) and five were hypermethylated in AA newborns (*ACOT11*, 31.9%, adjusted p-value = 6.41E-14, *MXRA7*, 31.5%, adjusted p-value = 4.32E-20, *FHIT*, 29.7%, adjusted p-value = 5.25E-12, *TOX2*, 26.7%, adjusted p-value = 2.66E-12, and *CCS*, 25%, adjusted p-value = 2.7E-8).

ACKR1 genotype and associations with methylation

From our genotyping of *ACKR1* rs2814778, we found that the percentage of African American newborns (n = 136) with the CC, TC and TT genotypes were 58.8%, 33.1%, and 8.1% respectively. In White newborns (n = 114), the percentages were 1.7%, 5.3% and 93% for the CC, TC and TT genotypes, respectively. Methylation levels for cg04922029 and cg18552413 at the CC, TC and TT genotypes are presented in Figure 2.

Validation of *ACKR1* methylation by pyrosequencing and identification of additional hypermethylated CpGs

Using pyrosequencing, we measured *ACKR1/DARC* methylation of cg04922029 and cg18552413 in cord blood DNA from the n = 62 validation dataset (32 AAs and 30 Ws). Both CpG sites are located in the 5'UTR region of *ACKR1* with cg18552413 located 72bp upstream and cg04922029 located 45bp downstream of rs2814778. We were able to validate the results of the array at cg04922029 and cg18552413 by showing that associations between AA status and methylation levels persisted. Figure 3 shows the pyrosequencing results analyzed by maternal self-reported race or rs2814778 genotype. Intriguingly, methylation data revealed three additional significantly hypermethylated CpGs in proximity (within 30bp) to cg04922029, also downstream of rs2814778 and just upstream of the start codon. These three CpGs do not have cg IDs as they are not present in either the 450K or EPIC arrays. In the validation dataset, African American newborns were ~23-40% hypermethylated ($p<0.0001$, t-test) at four CpG sites: cg04922029 and the additional three CpGs not present in the 450K or EPIC arrays. At cg18552413, African American newborns were ~10% hypermethylated compared to White newborns ($p<0.0005$, t-test) assayed. We also genotyped the newborns in the validation dataset. The genotype distributions at the *ACKR1* variant rs2814778 by maternal self-reported race were similar to the primary dataset, although there was a higher percentage of African American individuals who were homozygous for the C allele (81.3%), and White individuals who were homozygous for the T allele (100%). Overall, we were able to validate the findings from the 450K analysis at the top two most significantly differentially methylated CpG sites using pyrosequencing methods in a validation dataset.

Determination of epigenetic age

We also examined whether there was mitotically heritable accelerated aging among AAs, utilizing the Horvath epigenetic age calculator (33). We found that African Americans had a higher DNA methylation predicted age (mean = 0.86 years, SD = 3.86) than Whites (mean = 0.39 years, SD = 0.22), but this difference was not significant (p=0.12).

Discussion

Leveraging the 450K Methylation array data from the NEST cohort, we identified 4,579 CpG sites corresponding to 1,942 genes which were differentially methylated between AA and W individuals. Of these, 1,513 CpG sites were hypomethylated and 3,066 were hypermethylated in African American newborns. The magnitude of these methylation differences ranged from 0.5%-28.2% hypomethylated and 0.6%-31.9% hypermethylated in AAs. Pathway analysis of genes corresponding to the differentially methylated CpGs suggests an enrichment for terms related to inflammatory diseases and processes, conditions that also disproportionately affect African Americans.

It has been hypothesized that the increased risk in common chronic diseases such as cardiovascular disease, as well as some cancers, is due in part to accelerated epigenetic aging, and exposures to a disproportionate burden of chemical and non-chemical stressors among African Americans may be a contributing factor (35-38). However, evidence supporting accelerated aging as a potential explanation for the differential methylation and perhaps subsequent chronic disease susceptibility, was modest (higher epigenetic age in AA) and not significant. Analyses on a larger sample set are needed for extensive interpretation of epigenetic aging in race/ethnicity differences.

Another key finding was that the top two most significant differentially methylated CpGs between African Americans and Whites mapped to *ACKR1*. These two CpGs are proximal to the SNP rs2814778 located in the 5'UTR region of *ACKR1*. The T>C substitution at this SNP results in an absence of Duffy antigen on erythrocytes (Duffy-null) and is associated with the white blood cell count quantitative trait locus 1, resulting in reduced absolute neutrophil counts, also known as benign ethnic neutropenia (39-42, 46). Moreover, this genetic variant is related to African ancestry and resistance to, and modulation of *Plasmodium vivax* parasitemia (43). The SNP-CpG association between rs2814778 and cg04922029 in *ACKR1* has been previously related to African ancestry in a 450K analysis (10). In a study using the EPIC methylation array in monocytes from adult males (11) and in a study using the 27K methylation array in umbilical cord blood, cg18552413 was also significantly associated with African ancestry (45).

While the present study identified both CpGs, the 27K (45) and EPIC (11) arrays do not contain the cg04922029 site. Meanwhile, cg18552413 was not associated with ethnicity in the 450K analysis (10) in whole blood despite being represented on this array. Further analysis into considerations including methodological approaches, admixture mapping, as well as targeted sequencing is needed to clarify these findings. In all, three CpG sites in *ACKR1* using the 450K array were significant after p-value adjustment. Upon validation using pyrosequencing in an independent set of individuals, we identified three additional CpG sites that were consistently hypermethylated in AA individuals. These are not annotated in either 450K or EPIC arrays but are proximal to the SNP rs2814778 and cg04922029, and the start codon of *ACKR1*.

Mechanisms by which *ACKR1* genotype alters methylation have been hypothesized. An early study (42) reported that the T>C substitution resulted in the improper binding of the erythroid specific transcription factor GATA1 to its binding site, leading to null expression of

ACKR1 on erythrocytes. The lack of binding of GATA1 to initiate transcription could lead to aberrant recruitment of demethylases or methyltransferases at this site, thus, the hypermethylation that is observed in the most proximal CpG sites around the SNP is likely a consequence of the variant allele. This consistent methylation pattern in this region suggests the genotype-epigenotype link. Further characterization as well as larger studies are needed.

ACKR1 deficiency, a consequence of SNP genotype, leading to null expression on the surface of red blood cells, is most widely associated with resistance to malarial infection, due to the lack of ACKR1 as a site for *P. vivax* and *P. knowlesi* binding and subsequent entry. However, its role has been explored in the context of multiple chronic diseases as well (47). The function of ACKR1, a part of the Atypical Chemokine Receptor (ACKR) family, is as a receptor for chemokine binding and as a regulator of expression for other chemokine receptors. However, unlike canonical chemokine receptors, ACKRs do not couple to G-proteins and signal cell migration (48). It has been hypothesized that ACKR1 serves as a reservoir to sequester chemokines. In support, a lack of *ACKR1* expression has been associated with increased inflammation (increased or maintained circulation of chemokines) (49, 50). With its role in regulating inflammation, the function of ACKR1 in modulating diseases associated with aberrant or sustained inflammation such as cancer, have been reported. ACKRs have been shown to regulate the expression of tumor suppressing genes, thus inhibiting proliferation, promoting tumor senescence, and, with its sequestration of chemokines, modulating tumor neovascularization, and thus metastasis (47, 51, 52). In Ackr1 deficient mice, increased adiposity and insulin resistance were observed (53). The SNP rs2814778 in *ACKR1* has been associated with modulating acute lung injury (54) and HIV susceptibility (55).

Of the 1,942 significant and correspondent genes identified by ethnic-origin DMRs in our dataset, *MAD1L1*, *ARTN*, *RNF135*, and *SEPT8* are among the top 20 genes with the highest number of significant CpG sites. *MAD1L1* has been identified as related to population differences (56). *ARTN* encodes for Artemin, a member of the GDNF (glial derived neurotrophic factor) family. Artemin has roles in the survival of peripheral neurons including dopaminergic neurons and in promoting tumorigenesis, metastasis, and drug resistance in breast cancer (57). *RNF135* has been identified to be related to tumor specific ethnicity associated CpGs (E-CpGs) in four different types of tumor tissue (breast, head/neck, colon, and uterine). This same study also identified ethnicity-associated CpGs in ADME (absorption, distribution, metabolism, and excretion) related genes including *DHRS4* and *DHRS7*. Of the four *DHRS4* E-CpGs reported in their analysis of breast tumor tissue, one CpG site in *DHRS4*, cg01878807 was identified in our dataset; in both our study and their study, this CpG site was hypermethylated in African Americans with a 12% methylation increase, adjusted p-value = 9.7E-23, in our dataset. The identification of *DHRS4* cg01878807 in different tissues (breast vs blood) as well as a concordant change in methylation direction highlights the importance of evaluating *DHRS4* in the context of environmental health disparities (58). *SEPT8* encodes for Septin-8, part of the Septin family of nucleotide binding proteins. Differential methylation of *SEPT8* was identified in a study examining population differences (14). *SEPT8* methylation differences were also reported in a study looking at cognitive impairment in Mexican American individuals (59) and the role of Septins as tumorigenic or tumor suppressive in several cancer types, with *SEPT8* expression being upregulated in cancers including bladder, liver, lung, and pancreas, was reviewed in (60). Our finding of methylation differences in genes and regions associated with cancer in an accessible tissue/cell source such as umbilical cord blood, may provide clues into early indicators of chronic disease development.

When we evaluated CpG sites with the largest percent methylation differences, we found that seven CpG sites (mapping to seven unique genes) had >25% difference in methylation percentage. Two CpG sites were hypomethylated (mapping to *LPHN3*, *SLC25A21*) and five were hypermethylated (*ACOT11*, *MXRA7*, *FHIT*, *TOX2*, *CCS*) in African American newborns compared to White newborns. Several of these genes are considered to be potential tumor markers in multiple cancers. Multiple studies have identified associations between dysregulated expression of *ACOT11* and lung cancer (61, 62) as well as bladder cancer (63). *TOX2* has been associated with multiple cancer types including colorectal (64), glioma (65), lung (66, 67) and breast (66) cancers and may serve as a marker for the tumor microenvironment. *LPHN3* may have a role in breast cancer development (68), and *FHIT* may serve as a marker for cervical cancer screening (69). Many of these aforementioned cancer types disproportionately affect African American individuals, often with poorer prognostic outcomes. Several of these genes also have roles in other disparate chronic conditions that also disproportionately affect African Americans including Alzheimer's disease (*SLC25A21*, (70)), obesity (*ACOT11*, (71-73)), and increased blood pressure (*TOX2*, (74)). For example, increased expression of *ACOT11* is associated both with lung cancer and obesity risk. We observed a 31.9% increase in methylation at one CpG site in *ACOT11* in AA individuals (the largest difference in our dataset). However, functional studies are needed to determine how differential methylation at specific CpG sites modulate expression.

In summary, we set out to identify CpG sites that were differentially methylated between African American and White individuals in hopes of identifying environmentally induced pathways with ethnic-specific responses. These pathways could then be assessed for preventive measures and early detection of common chronic diseases as early as birth, particularly diseases with ethnic disparities in incidence and mortality. Our data suggest that epigenetic marks related to inflammation and dysregulation of immune function are associated with African Americans. They also confirm that genetic variations such as rs2814778 can contribute substantially to CpG methylation levels, as in the case for *ACKR1*. Most standard pipelines looking at methylation differences by ethnicity filtered out the CpG methylation marks that were likely to be associated with genetic variants, in order to address inherent ancestry/population confounding tied to outcomes. Future studies to assess additional ethnicities and increasing sample size, and expanding tissue analytes beyond mixed whole blood are needed. Further analysis on the role of *ACKR1* in disparate chronic diseases outcomes, including cardio-metabolic conditions and cancers, is strongly warranted, and analyses into the functional implications of the identified hypo- and hypermethylated marks are needed. Limitations notwithstanding, we were able to identify epigenetic marks in newborn cord blood that have been previously reported to be associated with cancer and other chronic conditions. These CpG sites and their corresponding genes may be candidates for assessing longitudinal disease risks.

Table 1. Participant characteristics

	AA (N = 197)		W (N = 170)	
	N (%)	Mean (SD)	N (%)	Mean (SD)
Maternal cigarette smoking				
Yes	81 (41.1)		45 (26.5)	
No	112 (56.9)		121 (71.2)	
Missing	4 (2.0)		4 (2.3)	
Parity				
0	52 (26.4)		65 (38.2)	
1	60 (30.5)		57 (33.5)	
≥2	85 (43.1)		40 (23.5)	
Missing			8 (4.7)	
Gestational age (weeks)				
Overall		38.3 (2.13)		38.8 (2.08)
<37	40 (20.3)		23 (13.5)	
≥37	157 (79.7)		147 (86.5)	
Delivery age (years)				
overall		27.1 (6.34)		30.6 (5.84)
<35	164 (83.2)		128 (75.3)	
≥35	33 (16.8)		42 (24.7)	
Pre-pregnancy BMI				
Overall		31.3 (10.30)		25.1 (7.14)
<30	91 (46.2)		138 (81.2)	
≥30	75 (38.1)		29 (17.1)	
Missing	31 (15.7)		3 (1.8)	
Socioeconomic status(SES)[a]				
Low	81 (41.1)		19 (11.2)	
Otherwise	102 (51.8)		146 (85.9)	
Missing	14 (7.1)		5 (2.9)	
Offspring sex				
Female	96 (48.7)		86 (50.6)	
Male	101 (51.3)		84 (49.4)	
Birth weight (grams)				
Overall		3038 (632)		3302 (586)
<2500g	36 (18.2)		15 (8.8)	
≥2500g	160 (81.2)		154 (90.6)	
Missing	1 (0.5)		1 (0.6)	
Estimated cell proportions[b]				
B-cell		0.116 (0.045)		0.109 (0.036)
CD4 T-cell		0.179 (0.073)		0.175 (0.066)
CD8 T-cell		0.143 (0.052)		0.142 (0.054)
Granulocyte		0.364 (0.130)		0.388 (0.128)
Monocyte		0.101 (0.033)		0.097 (0.036)
Natural killer cell		0.008 (0.017)		0.015 (0.027)
Nucleated red blood cell		0.135 (0.099)		0.118 (0.098)

[a] Socioeconomic status was identified by combining medical insurance type and education level, categorized as low if medical insurance type is "Public insurance" and education level is "less than high school". [b] Cell proportions were estimated by Houseman's method. AA: African American | W: White

Table 2. Pathway enrichment analyses

2a. Top 25 canonical pathways from Ingenuity Pathway Analysis

Rank	Ingenuity Canonical Pathway	-log(p-value)	Gene IDs
1	Sperm Motility	7.11E+00	MAP2K5, PRKAR2B, PLCB2, PRKCQ, PRKG2, PRKAG2, PDE4B, EPHB1, TYRO3, ZP3, NPPB, TIE1, FYN, LCK, FLT1, ITPR1, FGFR4, ZAN, PRKCZ, CATSPER2, PRKCE, TNK1, JAK2, PTK6, PRKG1, ALK, INSR, ROR2, JAK1, EPHB2, NPPC, DSTYK, PTK7, LMTK3, PLA2G4C, ITPR3, CSK, SRMS, PLA2G12A, EPHA8, PRKCG
2	GP6 Signaling Pathway	6.22E+00	COL7A1, PRKCQ, TLR9, COL5A3, LAMA5, COL26A1, FYN, PIK3CD, LAMB3, GRB2, ITPR1, FGFR4, COL25A1, PRKCZ, LAMA4, PRKCE, COL6A3, LAMA1, PIK3R4, PIK3C2B, COL5A1, COL18A1, COL23A1, COL16A1, GP6, RAP1B, COL9A2, PRKCG
3	IL-15 Production	5.08E+00	MAP2K5, TNK1, JAK2, PTK6, ALK, INSR, ROR2, JAK1, EPHB2, IL6, DSTYK, EPHB1, PTK7, LMTK3, TYRO3, TIE1, FYN, LCK, CSK, FLT1, SRMS, FGFR4, PRKCZ, EPHA8
4	Virus Entry via Endocytic Pathways	4.58E+00	FLNB, PRKCE, ITGAL, PIK3R4, PRKCQ, ITGA6, TLR9, PIK3C2B, DNM1, ITGB7, RASD2, FYN, PIK3CD, RALA, RAP1B, GRB2, CD55, FLNC, HLA-C, HLA-B, FGFR4, PRKCZ, PRKCG
5	Dopamine-DARPP32 Feedback in cAMP Signaling	4.57E+00	KCNJ2, PRKAR2B, PLCB2, PRKCQ, PRKG2, CSNK1G2, PRKAG2, ADCY3, CREBBP, ITPR1, CREB3L3, CAMKK2, PRKCZ, PPP1R1B, PRKCE, ADCY4, PRKG1, GRINA, PPP1CA, PPM1L, CAMKK1, PAWR, PPP2R2A, ITPR3, CACNA1C, DRD4, ADCY9, PRKCG
6	Axonal Guidance Signaling	4.53E+00	TUBB3, PRKAR2B, PLCB2, SRGAP3, PRKCQ, GNB1, SLIT3, PRKAG2, BMP2, ADAMTS2, PIK3CD, ADAMTS17, FGFR4, BDNF, PRKCE, HERC2, PIK3R4, ADAM19, PIK3C2B, TUBB8, EPHB2, SRGAP1, ARPC2, NTNG2, RAP1B, BCAR1, EPHA8, PDGFD, MMP9, PTCH1, NRP2, SEMA4C, RHOD, TLR9, FZD7, EPHB1, DOCK1, FYN, ADAMTS15, SEMA7A, GRB2, GIT1, GNG13, PRKCZ, MMP28, EFNA2, PLXNA2, EPHA10, LIMK2, ADAM23, WNT5B, GLI2, UNC5A, NFATC1, RASSF5, FARP2, RASD2, NGFR, RALA, MAP2K2, PDGFA, PRKCG, MMP14
7	Gap Junction Signaling	4.41E+00	MAP2K5, GAD1, TUBB3, PRKAR2B, PLCB2, GJC2, PRKCQ, PRKG2, TLR9, CSNK1G2, PRKAG2, GJB2, PIK3CD, ADCY3, MAPK7, GRB2, ITPR1, FGFR4, PRKCZ, HTR2A, PRKCE, ADCY4, PIK3R4, PRKG1, PIK3C2B, TUBB8, RASD2, ITPR3, RALA, MAP2K2, RAP1B, ADCY9, PRKCG
8	Synaptogenesis Signaling Pathway	4.32E+00	RASGRF1, DAB1, PRKAR2B, TLR9, PRKAG2, SHF, EPHB1, LRP8, FYN, VTI1A, PIK3CD, FARP1, ADCY3, LCK, GRB2, STX1B, CREBBP, ITPR1, CREB3L3, CDH23, GRM1, FGFR4, BDNF, EFNA2, CDH13, PRKCE, NRXN2, EPHA10, ADCY4, UNC13A, CDH9, PIK3R4, GRINA, PIK3C2B, EPHB2, ARPC2, RASD2, RALA, RAP1B, CPLX1, SYT9, CACNB3, ADCY9, MARCKS, EPHA8
9	Endocannabinoid Developing Neuron Pathway	4.02E+00	MAP2K5, ADCY4, BRCA1, PRKAR2B, PIK3R4, GNB1, TLR9, PIK3C2B, PRKAG2, RASD2, PIK3CD, ADCY3, RPTOR, RALA, MAP2K2, MAPK7, RAP1B, GRB2, CREBBP, MLST8, CREB3L3, FGFR4, ADCY9
10	CCR3 Signaling in Eosinophils	4.00E+00	PRKCE, LIMK2, PLCB2, PIK3R4, PRKCQ, GNB1, TLR9, PIK3C2B, MPRIP, PLA2G4C, RASD2, PIK3CD, ITPR3, RALA, MAP2K2, MYLK, RAP1B, GRB2, PLA2G12A, ITPR1, GNG13, FGFR4, PRKCZ, PRKCG
11	fMLP Signaling in Neutrophils	3.97E+00	PRKCE, PLCB2, PIK3R4, PRKCQ, GNB1, TLR9, PIK3C2B, NOX4, NFATC1, NOX3, ARPC2, RASD2, PIK3CD, ITPR3, RALA, MAP2K2, RAP1B, GRB2, ITPR1, GNG13, FGFR4, PRKCZ, PRKCG
12	Endocannabinoid Cancer Inhibition Pathway	3.93E+00	MAP2K5, PRKAR2B, TRIB3, TLR9, PRKAG2, VIM, PIK3CD, ADCY3, GRB2, CREBBP, MLST8, CREB3L3, CAMKK2, FGFR4, CASP2, ADCY4, CASP10, SMPD3, PIK3R4, PIK3C2B, NOS2, RPTOR, MAP2K2, TRPV1, SMPD4, ADCY9
13	Renin-Angiotensin Signaling	3.92E+00	PRKCE, ADCY4, JAK2, PRKAR2B, PIK3R4, PRKCQ, TLR9, PIK3C2B, PRKAG2, SHF, RASD2, PIK3CD, ADCY3, ITPR3, RALA, MAP2K2, RAP1B, GRB2, ITPR1, FGFR4, PRKCZ, ADCY9, PRKCG
14	Breast Cancer Regulation by Stathmin1	3.88E+00	TUBB3, PRKAR2B, PLCB2, PRKCQ, GNB1, TLR9, PRKAG2, ARHGEF16, PIK3CD, ADCY3, GRB2, ITPR1, GNG13, FGFR4, PRKCZ, PRKCE, ADCY4, LIMK2, PIK3R4, PPP1CA, PPM1L, PIK3C2B, TUBB8, PPP2R2A, RASD2, ITPR3, ARHGEF1, RALA, MAP2K2, RAP1B, ADCY9, PRKCG
15	Molecular Mechanisms of Cancer	3.88E+00	CDK11A, LRP5, CDK11B, PRKAR2B, PLCB2, PRKCQ, PRKAG2, BMP2, PIK3CD, ADCY3, FGFR4, PRKCE, ADCY4, JAK2, PIK3R4, TGFB2, JAK1, PIK3C2B, TGFBR2, RAP1B, RASGRF1, PTCH1, RHOD, AXIN1, CDK10, TLR9, MAX, FZD7, ARHGEF16, FYN, GRB2, CREBBP, PRKCZ, FNBP1, BRCA1, CASP10, WNT5B, CDK9, ZBTB17, RALGDS, CTNNA2, RASD2, ARHGEF1, RALA, MAP2K2, CDK15, RB1, HIPK2, ADCY9, PRKCG, DIABLO
16	Corticotropin Releasing Hormone Signaling	3.85E+00	PRKCE, ADCY4, CACNA2D4, GAD1, PTCH1, PRKAR2B, PRKCQ, GLI2, PRKAG2, NOS2, ADCY3, ITPR3, MAP2K2, CACNA1C, RAP1B, CREBBP, ITPR1, CREB3L3, UCN3, PRKCZ, BDNF, CACNB3, ADCY9, PRKCG
17	P2Y Purigenic Receptor Signaling Pathway	3.85E+00	PRKCE, ADCY4, PRKAR2B, PLCB2, PIK3R4, PRKCQ, GNB1, TLR9, PIK3C2B, PRKAG2, RASD2, PIK3CD, ADCY3, RALA, MAP2K2, RAP1B, GRB2, CREBBP, CREB3L3, GNG13, FGFR4, PRKCZ, ADCY9, PRKCG
18	Role of NFAT in Cardiac Hypertrophy	3.71E+00	HDAC10, PRKAR2B, PLCB2, PRKCQ, GNB1, TLR9, PRKAG2, IL6, PIK3CD, ADCY3, GRB2, ITPR1, GNG13, FGFR4, HDAC4, PRKCZ, HDAC1, PRKCE, ADCY4, CACNA2D4, PIK3R4, TGFB2, PIK3C2B, TGFBR2, RASD2, ITPR3, RALA, MAP2K2, CACNA1C, RAP1B, CACNB3, ADCY9, PRKCG
19	Ceramide Signaling	3.65E+00	SMPD3, TNFRSF1A, PIK3R4, PPM1L, TLR9, PIK3C2B, PPP2R2A, ENPP7, RASD2, PIK3CD, NGFR, CERK, RALA, RAP1B, GRB2, FGFR4, PRKCZ, SMPD4, DIABLO
20	NF-κB Activation by Viruses	3.52E+00	PRKCE, EIF2AK2, ITGAL, PIK3R4, PRKCQ, ITGA6, TLR9, PIK3C2B, RASD2, PIK3CD, LCK, RALA, RAP1B, GRB2, FGFR4, PRKCZ, RIPK1, PRKCG
21	VEGF Family Ligand-Receptor Interactions	3.47E+00	PRKCE, NRP2, PIK3R4, PRKCQ, TLR9, PIK3C2B, PLA2G4C, RASD2, PIK3CD, RALA, FLT1, MAP2K2, RAP1B, GRB2, PLA2G12A, FGFR4, PRKCZ, PRKCG
22	CDK5 Signaling	3.44E+00	ADCY4, LAMA1, PRKAR2B, PPP1CA, ITGA6, PPM1L, PRKAG2, LAMA5, PPP2R2A, RASD2, ADCY3, NGFR, RALA, MAP2K2, MAPK7, RAP1B, PPP1R1B, BDNF, ADCY9
23	Thrombopoietin Signaling	3.44E+00	PRKCE, JAK2, PIK3R4, PRKCQ, TLR9, PIK3C2B, RASD2, PIK3CD, RALA, MAP2K2, RAP1B, GRB2, FGFR4, PRKCZ, PRKCG
24	Neuropathic Pain Signaling In Dorsal Horn Neurons	3.42E+00	PRKCE, TACR1, PRKAR2B, PLCB2, PIK3R4, PRKCQ, GRINA, TLR9, PIK3C2B, PRKAG2, KCNQ2, PIK3CD, ITPR3, GRB2, ITPR1, GRM1, FGFR4, PRKCZ, BDNF, PRKCG
25	Colorectal Cancer Metastasis Signaling	3.41E+00	LRP5, MMP9, PRKAR2B, TNFRSF1A, RHOD, IL6R, GNB1, AXIN1, TLR9, PRKAG2, IL6, FZD7, PIK3CD, ADCY3, GRB2, GNG13, FGFR4, MMP28, FNBP1, ADCY4, JAK2, PIK3R4, WNT5B, TGFB2, JAK1, PIK3C2B, RALGDS, TGFBR2, RASD2, NOS2, RALA, MAP2K2, RAP1B, GRK3, ADCY9, MMP14

1,942 unique gene IDs were input into the IPA software tool. This corresponded to 1,717 gene symbols available for analysis

2b. Top 20 terms from Enrichr analysis of the GWAS 2019 catalog

Term	Overlap	P-value	Adj. P-value	Gene IDs
Red vs. brown/black hair color	8/20	3.36E-04	0.584	*PKHD1; HERC2; TUBB3; FANCA; SPIRE2; CDK10; SIK1; CBFA2T3*
Brown vs. black hair color	11/36	4.26E-04	0.370	*AKAP12; OCA2; SLC45A2; HERC2; PPFIBP2; ZBTB38; TMTC3; CDK10; ZNF831; EZR; TPCN2*
Waist-to-hip ratio adjusted for BMI (adjusted for smoking behaviour)	16/68	6.49E-04	0.376	*EYA4; HLA-C; CMIP; PIGC; BMP2; MACROD1; BBX; HOXC4; PEMT; TRIB1; MSC; FGFR4; ZNF664; KCNJ2; LEKR1; HOXC6*
Waist circumference adjusted for BMI in active individuals	18/85	0.0012	0.506	*LRRC36; TGFB2; ATAD5; ZBTB38; PTCH1; HMGA1; LOXL1; NPPC; BMP2; VPS53; HOXC5; PRKG2; HOXC4; FGFR4; LEKR1; CPA5; DIS3L2; HOXC6*
Waist circumference adjusted for body mass index	24/128	0.0012	0.428	*LRRC36; KCTD19; TGFB2; ATAD5; ZBTB38; PTCH1; HMGA1; LOXL1; NPPC; BMP2; VPS53; ADAMTS17; HOXC5; HIST1H3G; HOXC4; PRKG2; ANKS1A; ZNF423; MSC; FGFR4; CPA5; LEKR1; HOXC6; DIS3L2*
Waist circumference adjusted for BMI (joint analysis main effects and physical activity interaction)	18/87	0.0015	0.446	*LRRC36; TGFB2; ATAD5; ZBTB38; PTCH1; HMGA1; LOXL1; NPPC; BMP2; VPS53; HOXC5; PRKG2; HOXC4; FGFR4; LEKR1; CPA5; DIS3L2; HOXC6*
Waist circumference adjusted for BMI (adjusted for smoking behaviour)	17/81	0.0017	0.433	*KCTD19; TGFB2; ZBTB38; PTCH1; HMGA1; LOXL1; BMP2; ADAMTS17; HOXC5; PRKG2; HIST1H3G; ANKS1A; ZNF423; MSC; FGFR4; LEKR1; DIS3L2*
Takayasu arteritis	18/88	0.0018	0.383	*HLA-J; ATP8A1; NOTCH4; OR2J3; HLA-B; HCG4; HLA-C; FRMD4A; HLA-G; NCRNA00171; DUSP22; IL6; A2BP1; HIST1H3G; CLNK; FAM19A5; HLA-DPA1; OPCML*
Pulmonary function	14/61	0.0018	0.344	*TRIM71; HDAC4; WWOX; TGFB2; DDX1; PTCH1; C10ORF11; AGER; PITPNC1; THSD4; ADAM19; DAAM2; IL6R; TNS1*
Chronic hepatitis B infection	6/15	0.0019	0.334	*CD40; NOTCH4; HLA-DPB1; HLA-C; CFB; HLA-DPA1*
Waist circumference adjusted for BMI in non-smokers	15/69	0.0022	0.349	*KCTD19; TGFB2; ZBTB38; PTCH1; HMGA1; LOXL1; BMP2; HOXC5; PRKG2; HIST1H3G; ZNF423; MSC; FGFR4; LEKR1; DIS3L2*
Response to citalopram treatment	5/11	0.0024	0.348	*EGFLAM; UBE3C; LAMA1; FHIT; EMID2*
Hip circumference adjusted for BMI	19/99	0.0029	0.385	*SUZ12; TGFB2; CRADD; ATAD5; ZBTB38; INSR; PTCH1; HMGA1; BMP2; FAM101A; GMDS; ADAMTS17; PITPNM2; MACROD1; PRKG2; CORO7; C16ORF55; KCNJ2; DIS3L2*
Waist circumference adjusted for BMI (joint analysis main effects and smoking interaction)	16/79	0.0034	0.426	*KCTD19; TGFB2; ZBTB38; PTCH1; HMGA1; LOXL1; BMP2; HOXC5; PRKG2; HIST1H3G; ANKS1A; ZNF423; MSC; FGFR4; LEKR1; DIS3L2*
Alzheimer's disease progression score	5/12	0.0038	0.439	*BCAR3; AKAP12; DAB1; RPS6KA2; STK32B*
Response to TNF inhibitor in rheumatoid arthritis (change in tender 28-joint count)	5/12	0.0038	0.412	*ZNF595; ZNF718; MRPL36; NAV2; DOCK1*
Visceral fat	11/46	0.0038	0.391	*FANK1; TLE1; MYO3B; PEBP4; EYA4; CRY2; MYCBPAP; NCAM2; CDH9; GPC6; TP73*
Attention deficit hyperactivity disorder	17/90	0.0055	0.532	*OCA2; C21ORF34; CCDC46; MYT1L; GRIK4; TRPT1; FHIT; CLYBL; DNM1; ANO3; SV2C; DLC1; CDH13; CHMP7; GPC6; ARSB; ST3GAL3*
Blond vs. brown/black hair color	26/160	0.0062	0.564	*HDAC4; OCA2; SLC45A2; TMTC3; TMEM91; SYNE2; AKAP12; C1ORF127; SYNGR1; DSTYK; HERC2; ERMP1; CHL1; PEBP4; SDF4; ZBTB38; FZD7; TBCD; PEX14; TPCN2; IL6; PPFIBP2; CDK10; SIK1; ZNF831; EZR*
Low tan response	7/24	0.0063	0.549	*OCA2; SLC45A2; HERC2; FANCA; PDE4B; SPIRE2; TPCN2*

2c. Top 20 terms from Enrichr analysis on dbGaP

Term	Overlap	P-value	Adj. P-value	Odds Ratio	Gene IDs
Lymphocytes	24/44	1.11E-13	3.82E-11	5.617	*PCDHGA8; NGFR; PCDHGA7; PCDHGB7; PCDHGA6; PCDHGB6; PCDHGA5; PCDHGB5; PCDHGA4; MYO10; PCDHGB4; PCDHGA3; PCDHGB3; PCDHGA2; PCDHGB2; PCDHGA1; PCDHGC3; PCDHGA9; PCDHGA10; PCDHGA11; PCDHGA12; EGFLAM; PCDHGB1; ARSB*
Stroke	50/289	3.97E-05	0.0069	1.782	*PCSK2; GALNT12; DPP10; SRP19; GRIK4; ADARB2; PITPNC1; SHF; KIAA1549; ACTR1B; PDE4B; C19ORF57; SAMD12; EPHB1; PRKG1; TEAD3; BCAS3; KCNH5; STARD9; ANXA13; FRMD4A; NAV2; STON1-GTF2A1L; RNF144A; CTTN; PCCA; ITGA6; ZNF831; BCAT1; RIN2; LPHN3; DOCK1; NTM; RNF8; CACNA1C; SPTY2D1; AGER; FHIT; JAKMIP3; PAPSS1; MTHFD1L; CUX1; MGAT5; NACC2; ERI3; CAMTA1; SRGAP3; CMTM8; ESR2; SP4*
Body Mass Index	68/437	6.39E-05	0.0074	1.603	*ALK; VIPR2; MEGF11; IL5RA; ADARB2; PARK2; GALNT10; ADAMTS2; ITGAX; BBX; ROPN1L; FAM19A5; RALGPS1; BCAS3; TLE1; VWF; GRID1; COL23A1; FBXO17; SORCS1; TNNI3K; PDGFRL; ANO3; ADCY9; MYO3B; DAAM2; ELMO1; CDH13; DGKI; RALA; EPAS1; LAMA1; LAMA4; ITPR1; ADCY3; FHIT; DDO; DPP6; MTHFD1L; GBX1; MGAT5; CAMTA1; SLIT3; CTNNA2; SRGAP1; MAP2K5; OPCML; KLHL29; WWOX; CMTM8; BDNF; SLC16A12; PLEKHA5; FAM189A1; NFATC1; TRAPPC9; FOXN3; TPD52L1; ESR1; TMEM154; EXT1; DAB1; CRYGB; SMOC1; AGBL2; CCDC88C; CCDC3; SPRN*
Hemoglobins	30/157	2.32E-04	0.0200	1.968	*ALK; CTBP2; ZNF492; CCDC146; AGAP1; CACNA1C; FGF1; FBLN2; THSD4; WDR66; DPP6; PRDM16; SLC25A21; FAM19A5; GPC6; ARSB; IMPG1; LHPP; CMTM8; UNC13A; FRMD4A; PDGFRL; GALNS; MARCH1; NFASC; PPFIBP2; PCCA; MYO3B; OR2L13; RIN3*
Hair Color	6/11	2.50E-04	0.0172	5.617	*OCA2; SLC45A2; HERC2; FANCA; CDK10; TPCN2*
Myocardial Infarction	39/229	3.71E-04	0.0213	1.754	*PRR5; ZNF492; SLC35F3; ATP8A1; MEGF11; TSHZ2; LPCAT1; LRP5; MIA3; PLOD1; HSD17B12; CELSR2; FHIT; CMIP; CALCR; CUX1; CDH23; PLXNA2; C9ORF171; FLNB; DIP2C; FAM19A5; WWOX; CRADD; VWF; FOXN3; NAV2; INADL; EXT1; VANGL1; SMOC2; SDK1; MARCH1; GJB2; ELMO1; CDH13; PRKCQ; VWA2; MAD1L1*
Longevity	10/32	6.34E-04	0.0312	3.218	*GABRB3; C7ORF50; ABCC4; COL5A1; ZNF516; COL6A3; NCAM2; GPR133; MAD1L1; ST3GAL3*
Schizophrenia	18/82	7.48E-04	0.0322	2.261	*RERE; WWOX; HS3ST3A1; NOTCH4; AGAP1; CACNA1C; FHIT; LRP8; ANO3; TSPAN18; CDH13; ARSG; PGPEP1; PLCB2; MAD1L1; SPTBN1; SDCCAG8; OPCML*
Heart Rate	27/155	0.0020	0.0758	1.794	*ALK; ANKRD33B; ATP8A1; SLC35F3; LAMA1; TSHZ2; RASGRF1; ANTXR1; DPP6; C1ORF229; CTNNA2; PAG1; PRKG1; OPCML; TGFB2; COL25A1; ST8SIA2; FBXO15; CACNA2D4; VWA3B; TMEM232; ARHGAP10; WBSCR17; ZNF516; ELMO1; TP73; MCPH1*
Albumins	11/43	0.0022	0.0746	2.635	*MARCH1; PTPRN2; LRCH1; GMDS; CREB3L3; MGRN1; DAAM2; SLC2A3; TNRC18; LCT; FHIT*
Tunica Media	20/104	0.0022	0.0694	1.981	*WWOX; EIF4E3; NTM; ANXA5; PRKAG2; CACNA1C; CMIP; ADAM19; SDK1; MARCH1; COL5A1; SLC7A8; MTHFD1L; PRDM16; TBC1D7; ALPL; CDH13; TUBGCP3; TACC2; OPCML*
Triglycerides	38/244	0.0024	0.0686	1.604	*RB1; HDAC4; TAGLN; LAMA1; NOTCH4; NTM; TSHZ2; ITPR1; JMJD1C; LPL; CELSR2; ARNTL; PKHD1; SV2C; PEBP4; FYN; SRGAP3; NCAM2; DIP2C; PCNT; DLGAP2; ZNF664; SLC12A8; RABGAP1L; GRID1; GALNT2; PCIF1; ESRRG; SYT9; KIAA1217; MARCH1; WBSCR17; PFKL; DAB1; SIK3; CLNK; XKR6; ASTN1*
Coronary Artery Disease	33/205	0.0026	0.0693	1.658	*FANK1; ALK; MEGF11; MIA3; CELSR2; FHIT; PARK2; PAPSS1; TRIM9; MTHFD1L; PEBP4; CAMTA1; FYN; CTNNA2; SRGAP3; ANKS1A; EPHB1; IL6R; ARSB; WWOX; ZBTB16; ACOT11; MKL1; KIAA1462; SHROOM3; C10ORF11; SORCS2; ANO3; SH3RF3; PCCA; DLC1; HHIPL1; CDH13*
Neutrophils	13/58	0.0032	0.0790	2.308	*DPP10; NGFR; DSCAM; MYO10; ABCB9; EGFLAM; FAM184A; OGFOD2; PITPNM2; RGR; MAD1L1; ARSB; RAB7A*
Erythrocyte Count	21/115	0.0033	0.0770	1.881	*CMTM7; ABCC4; PPM1L; ZAN; ST8SIA2; C10ORF11; FHIT; ZNF709; NFASC; PPFIBP2; PCCA; OR2L13; DAAM2; DLC1; SMOC1; PRDM16; ZNF423; LGR6; RIN2; PRKG1; RAB7A*
Body Height	54/385	0.0038	0.0812	1.444	*GABRB3; DPP10; ALK; SLC35F3; KNDC1; ANTXR1; MEOX1; CMIP; BAI1; SLC12A6; GPR133; PRKG1; DIS3L2; TNS1; PRKCG; VWF; ZBTB38; MKL1; SORCS2; ETV6; NCOR2; ARHGAP10; MYO3B; TRIM14; COL6A3; CDH13; ARHGEF1; CCDC40; TNXB; LAMA1; NTM; TSHZ2; PON1; ITPR1; RAP1B; ADAP2; FAM184A; ADAMTS17; HMGXB3; LHPP; WWOX; PTCH1; PLEKHA5; TBCD; HMGA1; ESRRG; FOXN3; SULF1; NTAN1; EFR3B; ESR1; EXT1; WDR60; XKR6*
Insulin	17/89	0.0049	0.0996	1.967	*ALK; WWOX; TRRAP; RBM19; ITPR1; SORCS2; PARK2; CAPZB; NACC2; CCDC88C; CDH23; FYN; EPHB2; BCAT1; PRKG1; KCNK3; RALGPS2*
Hemoglobin A, Glycosylated	22/128	0.0058	0.1107	1.770	*DPP10; ALK; WWOX; DSCAM; GRID1; PVT1; PBX2; SPG7; ESR2; B3GNTL1; SYNE2; HK1; SMC1B; ETV6; SHF; WBSCR17; DPP6; ZEB1; CHL1; PRDM16; ERI3; RIBC2*
Insulin Resistance	12/58	0.0089	0.1609	2.131	*ALK; BFSP2; CAPZB; TRRAP; CCDC88C; ITPR1; UBE2N; FYN; CTNNA2; EPHB2; BCAT1; PARK2*
Lipoprotein(a)	6/21	0.0125	0.2164	2.942	*TULP4; NOX3; EZR; PARK2; PRKG1; AGPAT4*

Table 3. Top 20 genes with the most significant differentially methylated CpGs

Annotated gene ID	# of CpG sites	Chromosome	Methylation Difference (min, max)	Adjusted P-value (min, max)
DARC	3	chr1	(-24.72%, 1.19%)	1.70E-61, 1.23E-02
ADAP2	2	chr17	(-6.14%, 11.47%)	1.41E-32, 9.74E-22
CNN1	4	chr19	(-13.76%, -4.72%)	5.16E-29, 5.54E-12
H1FOO	1	chr3	7.42%	6.25E-27, 6.25E-27
RNF135	8	chr17	(1.04%, 17.56%)	1.13E-24, 2.51E-05
ARTN	8	chr1	(-6.78%, 4.88%)	1.36E-24, 2.78E-03
SEPT8	10	chr5	(1.85%, 13.12%)	1.09E-23, 3.24E-05
TRPV3	3	chr17	(-7.51%, -1.35%)	3.58E-23, 2.56E-02
DHRS4	3	chr14	(-12.00%, -1.51%)	9.67E-23, 2.27E-02
C14orf167	3	chr14	(-12.00%, -1.51%)	9.67E-23, 2.27E-02
CADM3	6	chr1	(-5.56%, 6.30%)	1.96E-22, 6.78E-03
APBA2	2	chr15	(-6.41%, -1.49%)	2.01E-21, 9.74E-04
SORD	1	chr15	13.25%	2.11E-21, 2.11E-21
TLE1	2	chr9	(-5.69%, -5.68%)	2.57E-21, 1.57E-03
S100A2	1	chr1	-7.77%	7.61E-21, 7.61E-21
MXRA7	1	chr17	-31.53%	4.32E-20, 4.32E-20
SRMS	5	chr20	(-7.34%, -2.84%)	5.87E-20, 1.22E-04
LMF1	3	chr16	(-5.51%, 13.62%)	1.04E-19, 2.79E-03
DHX58	5	chr17	(-3.84%, 9.47%)	1.24E-19, 1.70E-03
LRRC24	6	chr8	(-6.20%, 6.84%)	3.40E-19, 3.85E-03

Methylation was regressed on race/ethnicity (AA or W) and the following covariates: maternal cigarette smoking, parity, maternal age, pre-pregnancy obesity, sex, gestational age, socioeconomic status, and cell proportions in whole blood. African Americans were the referent population for this table. In the text, methylation differences are reported with Whites as the referent population. For example, a -10% methylation difference in this table indicates a 10% methylation increase in African Americans

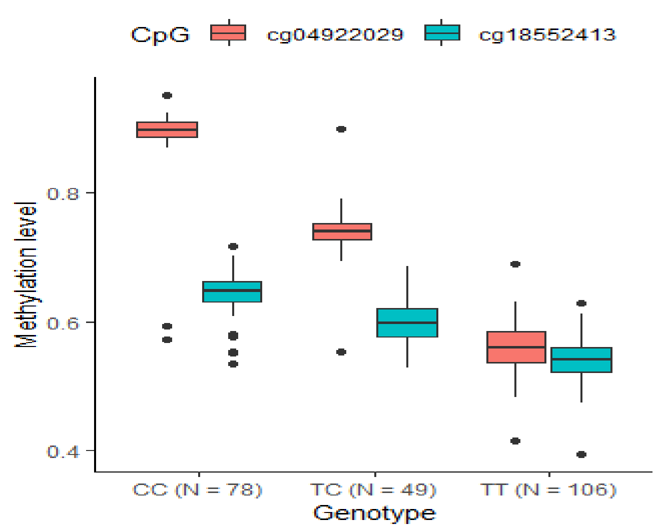

Figure 2. *ACKR1* methylation by rs2814778 genotype
Methylation fractions on cg04922029 or cg18552413 from the 450K array were regressed on rs2814778 genotype (number of C alleles) and covariates (both p-values< 2.2E-16)

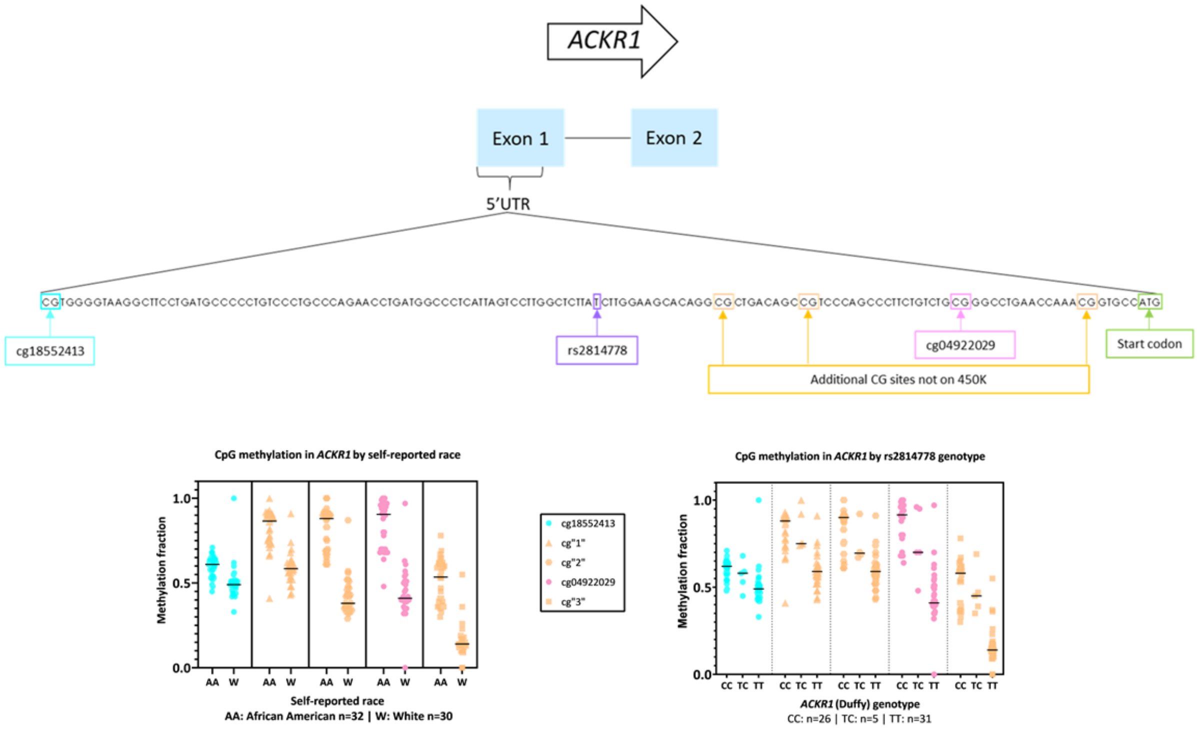

Figure 3. Validation of *ACKR1* methylation via pyrosequencing
A simplified diagram of the 5'UTR region in *ACKR1* containing the SNP rs2814778, the two most significant differentially methylated CpG sites (cg04922029 and cg18552413), and three additional CpG sites not on the 450K array. Plots show methylation fractions (from pyrosequencing assays) by self-reported race/ethnicity (left) or *ACKR1* rs2814778 genotype (right). The two exons (represented as simple rectangles) are not to scale.

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CONCLUSIONS

The main objective of this project was to identify and clarify environmental determinants *in utero* that may modulate offspring development and growth. Per the DOHaD hypothesis, this altered growth trajectory increases the risk of chronic conditions such as obesity in later life. The onset of obesity and its cluster of correlates including insulin resistance, NAFLD, and hypertension, largely present in adults, are increasingly being found in children. Thus, our outcome of interest focused on childhood obesity. We also examined blood pressure, as hypertension is a co-morbid condition that clusters with obesity. Our project utilized data and samples from the NEST cohort and for Chapter Three, we also utilized data from a smaller cross-sectional study whose participants resided in the same geographic area as the NEST participants. In Chapters Three and Four, we examined the predictors of prenatal metal mixture exposure (Chapter Three) and metals and satiety response (Chapter Four) on the outcome of childhood obesity. In Chapter Five, we explored epigenetic (DNA methylation) differences between African American and White individuals at birth. We hoped to gain insights into molecular phenotypes in early life that may set a differential trajectory for the development of chronic diseases including cancer and cardiovascular conditions.

In Chapter Three, we identified a group of metals that together may increase the risk of obesity development in female children. Using Factor Analysis, an unsupervised dimension reduction technique, we reported 8 factors identified from the 24 metals. When BMIz and systolic or diastolic (SBP or DBP) blood pressure outcomes were regressed on each factor, the strongest association was between a factor comprised of Ba, Ca, Co, Cu, and Sr, with BMIz in females. We applied two other statistical methods to support and clarify the findings from Factor Analysis. We utilized Partial Least Squares (PLS) regression, a supervised dimension reduction technique, and Adaptive Elastic Net, a variable selection technique. Overall, these methods support a potential role of the Ba, Ca, and Co grouping on increased obesity risk in females. While our study objective was to identify metal combinations associated with weight or blood pressure outcomes, we also identified single metals including As, Cr, and Se with consistent associations with blood pressure (As, Se), and weight (Cr). These associations were largely sex-specific.

In Chapter Four, our study examined the potential mechanism of prenatal metal exposures on altering the neurocircuitry regulating appetite and satiety, thus leading to increased obesity risk. We linked measurements made at 3-4 different time points during the life course: 1) metal levels in women during the first trimester of pregnancy; 2) levels of the appetite and satiety related markers BDNF and NPY at birth; 3) parental responses to appetitive traits using the Childhood Eating Behavior Questionnaire (CEBQ) around 2-5 years; and 4) BMIz scores at 5 years of age. We ran our regression analyses in all participants to enhance power, but also stratified by sex and race. Our key findings were that the domains of satiety responsiveness (SR) and slowness in eating (SE), pertaining to appetite and satiety regulation, were inversely associated with BMIz at 5 years of age overall but with stronger associations in females. A reduced SR suggests that more caloric intake may be needed to feel full while reduced SE suggests that the eating rate is increased—which has previously been associated with obesity (1). We regressed SR and SE on the top two metal groupings (factors) related to BMIz (identified through Factor Analysis in Chapter Three), and found inverse associations between the top factor (Ba, Ca, Co, Cu, Sr) and satiety responsiveness and eating speed. This suggests that increased levels of this metal grouping might decrease feelings of fullness. When we evaluated relationships between the levels of these factors with BDNF and NPY, we identified inverse associations between the factors and BDNF, whose role in the context of appetite regulation is to suppress the appetite by increasing satiety. We identified modest associations between BDNF levels at birth with slowness in eating. Overall, we identified potential relationships between early life metal exposure and dysregulated satiety response in childhood. These associations appear to be sex and race specific. The findings in this study support the hypothesis that prenatal metal exposures may alter the neurocircuitry involved in satiety regulation. However, larger sample sizes and functional studies are needed to confirm these findings.

In Chapter Five, we identified CpG methylation differences in umbilical cord blood samples between African American (AA) and White (W) individuals at birth. Our objective was to determine whether epigenetic differences at birth between African Americans and Whites might provide molecular insights into the disparities in chronic disease prevalence and mortality. We used an epigenome wide array and identified differential methylation of 4,579 CpG sites corresponding to 1,942 genes. Twice as many CpG sites were hypermethylated in African American newborns (3,066 CpGs) than White newborns (1,513 CpGs). The largest methylation percent differences (>25%) between AA and W mapped to seven genes associated with several cancers that have disproportionate prevalence and mortality in AA individuals including breast, lung, and colon cancers. The most significant differentially methylated CpG sites cg04922029 and cg18552413 (hypermethylated in AA), mapped to Atypical Chemokine Receptor *ACKR1,* a receptor for chemokine binding that functions as a chemokine reservoir and thus plays an important role in regulating inflammation. We identified strong associations with the C allele of the *ACKR1* SNP rs2814778 (associated with African ancestry and resistance to malarial infection due to its null expression on erythrocytes), and hypermethylation of both cg04922029 and cg18552413. Upon further analysis and validation using pyrosequencing, we identified hypermethylation of three nearby additional CpG sites not on the array in AA. Previous reports identified associations with either cg04922029 and cg18552413 and African ancestry (2-4). Our identification of both of these CpG sites and an additional three CpG sites not annotated on epigenome-wide arrays, along with functional studies showing that rs2814778 (homozygous C) inhibits binding of the erythroid transcription factor GATA1 leading to an ACKR1-null phenotype on red blood cells (5), strongly suggest that the C allele of the SNP rs2814778 leads to the hypermethylation of all nearby CpG sites. However targeted analysis and functional studies are needed to elucidate the mechanisms that may contribute to these observations.

We note overall limitations of our project. One recurring limitation is the issue of sample size. An increased sample size would contribute towards clarifying or validating identified associations. Furthermore, given the inherent and unmeasured confounding of epidemiological studies, causation is difficult to infer. Therefore, *in vivo* and *in vitro* studies recapitulating our findings will be important for clarification.

Environmental determinants during prenatal development include but are not limited to chemicals, nutrition, stress, and sociocultural factors. With this in mind, an important consideration in study design and analysis is in evaluating interactions and possible effect modifications between multiple determinants (e.g., chemical, genetic, social) on health outcomes. This is our current and developing research goal.

Overall, our project provides some insights into environmental determinants during pregnancy and child health outcomes.

PERSPECTIVES

Deciduous teeth: biological archives and portents?

In epidemiological studies, accessible tissues (e.g., hair, saliva, deciduous teeth) that can serve as biological proxies for less accessible tissues (e.g., biopsies), contribute to increased sample size and availability. A growing interest in deciduous (baby) teeth goes beyond its availability as an accessible tissue. Teeth can serve as an archive of both pre- and post-natal environmental exposure. Deciduous teeth develop during gestation and the first deciduous tooth erupts from the gum at six months of age. Deciduous teeth fall out between 6-12 years of age to accommodate the eruption of permanent teeth. At the end of the first trimester, the dentin and enamel of deciduous teeth begin to develop, and analogous to tree rings, form incremental layers. In deciduous teeth, approximately one layer or ring is formed daily. The process of birth forms the neonatal line which demarcates the prenatal dentin or enamel from the postnatal dentin or enamel. Using laser-ablation mass spectrometry, environmental chemicals that may deposit in bone, including metals such as lead and manganese, can be measured and the timing of exposure can also be determined using the incremental layers. Thus, deciduous teeth can serve as a *retrospective temporal exposome* as coined by others (6, 7).

The structure and phenotype of the tooth itself can also serve as a biomarker of maternal and early childhood environmental influences. Maternal age, low birth weight, and female sex have been linked to the delayed eruption of the first deciduous tooth (8). Gestational diabetes mellitus has been associated with enamel hypoplasia (9). Maternal obesity and smoking are linked to an increased risk of dental caries in pre-school aged children (10), while maternal calcium supplementation was linked to decreased risk of dental caries (11). Teeth in some regards can serve as indicators of bone and overall health. For example, periodontal disease is linked to inflammatory conditions including arteriosclerosis, diabetes, rheumatoid arthritis, and obesity (12).

Currently, deciduous teeth are being collected in the NEST cohort with the primary and initial purpose of measuring and timing toxic metal exposure prenatally and postnatally. This will involve sectioning and processing the tooth for analysis. As this process involves destruction of the tooth, we sought to catalog tooth morphology, appearance, and structure prior to "exposome analysis". Our long term goal was to generate a "tooth compendium" that may serve as an informative metric for the effects of early life environmental determinants on bone health. For example, if X metal is found at high levels in some individuals, we might examine whether in these same individuals, there was also dysregulated mineralization or altered thickness of the enamel. Conversely, with additional follow-up data becoming available in now adolescent participants in NEST, the morphological and structural data on the deciduous tooth may serve as an earlier life biomarker. For example, we might assess whether there are differences in enamel or dentin mineral density, or prevalence of dental caries, with increased BMI in adolescents.

In collaboration with dental researchers at the NIDCR and NIAMS, we identified the teeth, noted the presence of any fillings, chipping, caries, or discoloration, and scanned the teeth using micro-computed tomography (micro-CT). A non-destructive technique, micro-CT analysis quantitates volumes, densities, and thickness of teeth, and provides high resolution structural data and visualization of the layers of the teeth (e.g., enamel, dentin, pulp).

Herein, we briefly present and discuss preliminary findings in our analysis on deciduous teeth. As other studies have identified a modulatory role of maternal nutrition on tooth development, we examined the relationships between maternal nutrition and enamel and dentin thickness and density as well as presence of dental caries/fillings.

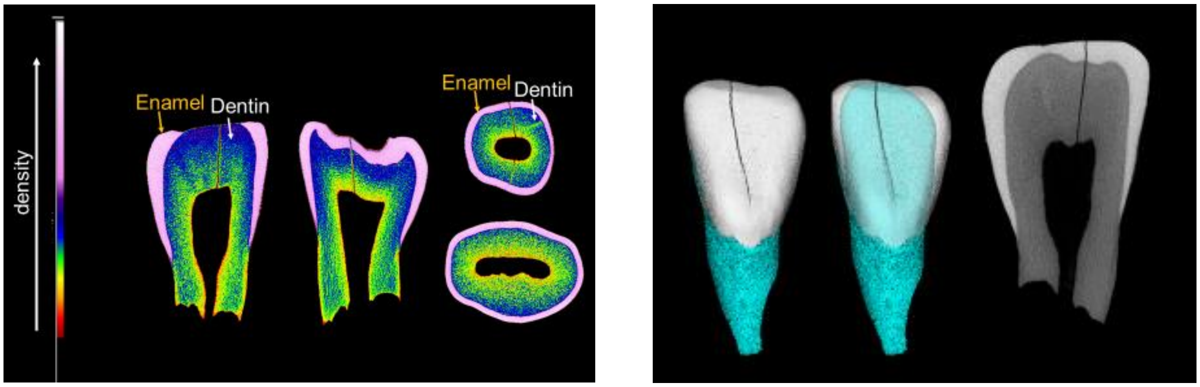

Figure 1. micro-CT images of deciduous teeth from NEST
Left: Color map depicting bone mineral density throughout segments of the tooth. Enamel is generally the most dense and of relatively uniform density (lilac) while dentin mineral density varies. These color maps can be used to measure density. *Right:* 3-D image of the tooth (white = enamel and cyan = dentin). Enamel and dentin thickness can be measured using these images.

Preliminary results

To date, approximately 100 teeth have been scanned, with ~25 teeth reconstructed and analyzed for bone thickness and density. In these analyses, we had n = 11 unique individuals with available measurements and maternal levels of plasma vitamin B6 (pyridoxal phosphate and pyridoxic acid), B9 (folate), B12, and vitamin D (all in units of ng/mL). Due to differences in thickness and density by tooth number, we limited our present analysis to central or lateral incisors and canines and did not use molars. When we regressed enamel thickness on pyridoxal phosphate or pyridoxic acid and the following covariates (sex, maternal BMI, race), we identified positive associations with both pyridoxal phosphate ($p = 0.06$) and pyridoxic acid ($p<0.0005$). We did not find any other significant associations.

When we assessed relationships between individuals who had data on the presence or absence of either dental caries or fillings in their tooth/teeth (n = 99) and maternal factors, we identified significant associations by race: African American and Hispanic individuals were more likely to have dental caries/fillings than Whites $p<0.05$. We also identified significant associations between low vitamin D levels and higher prevalence of caries $p<0.05$. No other significant associations were identified.

Future directions

We interpret these results with caution. Sample sizes on scanned teeth are very low. Additionally, vitamin D levels are strongly correlated with race: African American individuals have lower vitamin D levels in our study and in other studies. We were unable to stratify the vitamin D analysis by race, as all individuals in the “low” vitamin D category were African American. Increased sample size may help clarify these findings. Notwithstanding these limitations, we highlight that to our knowledge, this study may be the first in terms of available number of deciduous teeth ($\geq$100 yet to be scanned/analyzed), measured maternal and offspring variables, and utilization of micro-CT scanning to conduct in-depth structural and quantitative analyses on bone thickness and density.

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