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Prenatal Metal Exposure Effects on Newborn Neurobehavior and Epigenetic Modifications in a U.S. Birth Cohort Study

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An abstract of
A dissertation submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy in Environmental Health Sciences
2022

Abstract

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By Pei Wen Tung

Abstract

The placenta plays an integral role in programming newborn health and its functions may be affected by environmental exposure. Prenatal toxic metal exposure can contribute to detrimental neurodevelopmental outcomes in children. Moreover, epigenetic mechanisms have been postulated as an underlying mechanism between toxicant exposures and developmental implications. Utilizing the Rhode Island Child's Health Study (RICHS), this work investigated prenatal metal exposures alone and in combination and the associated newborn neurobehavior and epigenetic effects.

To determine the impacts of individual and a mixture of placental metal(s) on neurobehavior, we classified RICHS newborns into five neurobehavioral profiles based on their NICU Network Neurobehavioral Scale performance. We observed placental cadmium and detectable Pb were associated with higher odds of newborns belonging to the atypical neurobehavior profile. Using quantile g-computation, we demonstrated increased odds of newborns belonging to the atypical neurobehavior profile as all metal levels in the mixture increase by one quartile, and cadmium was suggested as the driving factor for the overall placental metal mixtures' neurobehavioral impact.

To examine the associations between prenatal lead and placental epigenetic modifications, we applied an epigenome-wide association study (EWAS) and conducted overrepresentation analysis. EWAS indicated prenatal lead exposure was associated with differential placental DNA methylation and hydroxymethylation. Likewise, overrepresented pathways enriched among differential methylation or hydroxymethylation of genes were involved in developmental, calcium transport and regulation, and cell signaling functions.

Overall, this work illustrates prenatal metal exposure, both individually and as a mixture, adversely impacted neurobehavior. Our results emphasize the importance of understanding joint impacts of environmental exposures on neurobehavior and suggest the need of comprehensive mixtures approach to address distinct combinations of environmental stressors for their influences on children's health. Additionally, placental functions susceptible to toxicants are highlighted as we established that prenatal lead exposure modulated placental epigenetics which may contribute to dysregulated placental functions and in turn developmental consequences. These findings warrant additional research in larger cohorts to further characterize placental epigenetic profiles, and to elucidate the underlying mechanisms relevant to prenatal toxicant exposures, epigenetic mechanisms, and early developmental outcomes.

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Acknowledgements

I would like to start by thanking my advisor, Dr. Carmen Marsit, for the continued support and encouragement throughout my doctoral training. Thank you for allowing me to formulate and develop my own research goals from the start, and for providing sound advice whenever I needed it. The pandemic has affected so many aspects of academic research, but your guidance has made these changes less daunting. You have always been approachable and would always make sure resources needed for the projects were available. Thank you for always being very generous with your time and knowledge, and for showing and including me in a kind, genuine and supportive mentoring environment.

Thank you to my committee members, Dr. Caudle, Dr. Everson, Dr. Gaskins, Dr. Liang and Dr. Smarr, for lending me your time and offering your support and feedback. I have sincerely enjoyed working with and learning from all of you over the past couple of years.

I would also like to thank the Marsit Lab members for your support over the years. I officially joined the group during the pandemic, and our weekly lab meetings have been so important in helping me structure this dissertation and understand the data, and overall in making sure I get to carry out my projects smoothly. It has been so much fun working with such a supportive group of scientists.

I would like to thank the EHS program and faculty members; everyone that I had the chance to work with have been so integral in shaping me into a public health researcher. Thank you to previous and current EHS students, it has been such a welcoming and nice experience being a part of the EHS family. A special thanks to my PhD cohort; starting from when we were

new (and young) PhD students, I feel very lucky to have you as my peers as we all explored our research interests and focuses and figured out a healthy PhD work-life balance.

I would like to thank my wonderful group of college friends back home in Taiwan. Thank you all for staying in touch and for including me in all your big milestones in life. Your messages and FaceTime calls (despite our 12-hour time difference) would always make my day and have never failed to put a smile on my face after long workdays. It's been more than two years since we all got together, but thank you for your steady support and for being just one message away, and I will always cherish our friendship that started from our freshman year of college. A very special thanks to my dearest friends Ann, Tiffany and Kelly. I would also like to thank my friends from different academic stages that have always been supportive throughout the years. We are all pursuing different goals now but this journey would not be the same without your company. Thank you especially to Vicki, Annissa, Tzu-Jung and Sara for sharing parts of my academic years and for all the nice memories we made during our downtime.

Most importantly, I would like to thank my family for their unconditional support and encouragement as I navigate through graduate school. My sisters, for always seeing the bright side of things and made sure I still experienced fun things and had good food in my free time. My grandparents, for being my biggest supporters and would always tell me to remember to take care of myself before attending to anything else. And my parents, for supporting my academic endeavors and for instilling in me the importance of a strong work ethic and a love of learning. They would lend a listening ear when things got stressful, and would always be the first to cheer me on as I reach milestones throughout the years. I am forever grateful for their guidance and patience and for the opportunities they gave me, and I would not be where I am now without their continued support and love. Thank you, Mom and Dad.

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Chapter 1: Introduction

Developmental origins of health and disease (DOHaD)

The developmental origins of health and disease (DOHaD) concept postulates that exposure to environmental factors during the prenatal and/or early postnatal periods may contribute to adverse health outcomes and diseases later in childhood and adult life (Gluckman, Buklijas, and Hanson 2016). Experimental animal studies have provided evidence on the association between hostile environmental conditions and suboptimal growth and health trajectories in the offspring (Vickers et al. 2000; Woodall et al. 1996). In the epidemiology field, the DOHaD concept first originated from a 1986 study by Barker and Osmond, in which they observed a strong association between infant mortality and ischemic heart disease (Barker 2007; Barker and Osmond 1986). In the study region of England and Wales, researchers were perplexed by the observation of higher mortality rates from cardiovascular disease in less prosperous areas, as opposed to in more affluent geographical areas where the population had access to unhealthier (i.e., high fat proteins, trans fats, etc.) dietary choices. The authors suggested their findings may be due to nutritional differences in the prenatal period, where undernutrition *in utero* adversely altered the physiology and metabolic systems regarding nutritional intake, making individuals in economically disadvantaged areas more susceptible to cardiovascular diseases later in life. This phenomenon may also be linked to the developmental plasticity concept, which described programming and priming the developing fetus for the intrauterine environment, but likely at the expense of them unable to properly adapt to variations of postnatal environment perturbations later in life (Bateson et al. 2004).

The 1944-1945 Dutch Winter Famine was a pivotal event that further characterized the link between *in utero* nutritional status and perinatal and pediatric outcomes in relation to the DOHaD

theory. Offspring born to women pregnant during the famine were found to have poor growth and developmental outcomes (Lumey, Stein, and Susser 2011; Painter et al. 2006; Smith 1947; Susser, Hoek, and Brown 1998). Exposure during critical windows of development during gestation was also emphasized through the famine, with evidence showing maternal undernutrition in the last trimester was associated with lower birth weight (Roseboom, de Rooij, and Painter 2006). In addition to nutrition-associated influences, epidemiological studies have also explored how intrauterine exposure to chemical stressors, such as air pollution, endocrine disrupting chemicals and metals, may contribute to detrimental effects on fetal growth and neurodevelopment later in childhood (Haugen et al. 2015; Heindel et al. 2017).

Throughout the decades, DOHaD research, brought on by the Dutch Famine cohort, has since expanded to understand the intergenerational and epigenetic effects of adverse *in utero* environment and developmental outcomes later in life. Epigenetics is the study of heritable gene function and expression modifications that are not attributed to DNA sequence changes (Moore, Le, and Fan 2013) (see *Epigenetic marks* section below). Emerging epigenetic concepts have been applied to advance DOHaD studies and researchers suggested epigenetic modifications may serve as a defense mechanism for the developing fetus to respond to early life environmental perturbations. One of the best studied types of epigenetic modification, DNA methylation, was found to be related to maternal dietary choices during pregnancy and childhood adiposity (Godfrey et al. 2011). Likewise, in the context of environmental toxicants such as metals, the integration of epigenetic mechanisms with the DOHaD framework may elucidate the association of prenatal metal exposures with the predisposition of adverse neurodevelopmental and behavioral outcomes in children.

Prenatal metal exposure and children's neurodevelopment

Exposure to ubiquitous environmental contaminants is an important public health concern especially among the susceptible children's population. Toxicants such as metals are persistent in the environment from both natural and anthropogenic activities and the general population is often exposed, voluntarily and involuntarily, through ambient air, drinking water, dietary or industrial sources. With reference to the DOHaD hypothesis, the developing fetus is most sensitive to environmental metal disturbances (Gluckman, Buklijas, and Hanson 2016). Therefore, it is plausible that exposure to toxic metals during the critical prenatal period impacts normal developmental trajectories and will lead to lasting health effects throughout the life course (Sanders, Henn, and Wright 2015).

Cadmium (Cd) is a non-essential metal classified as carcinogenic to humans (Group 1) by International Agency for Research on Cancer (IARC), which indicates there is sufficient evidence of Cd and Cd compound carcinogenicity to humans (IARC 1993). For the general, non-smoking population, the dominant exposure source of Cd is dietary intake (Järup and Åkesson 2009). When exposed prenatally, Cd can be detected in cord blood and fetal tissues (Z. Chen et al. 2014; Espart et al. 2018; Gundacker and Hengstschläger 2012). Relevant to the gestational period, the placenta modulates toxicant transmission between mother and fetus. In the case of Cd exposure, the placenta has been found to sequester Cd, thus acts at least as a partial barrier to the metal (Wier et al. 1990). Cd in placenta can be particularly detrimental to the developing fetus, with evidence suggesting that Cd may affect fetal development through impacts on placental functions (Geng and Wang 2019). Epidemiologic studies have also demonstrated associations between prenatal Cd exposure and decreased verbal and performance IQ in children (Kippler et al. 2012; Sanders, Henn, and Wright 2015; Taylor, Golding, and Emond 2016; Tian et al. 2009).

Lead (Pb) is an extensively studied neurotoxicant, with children as the targeted population of concern due to high exposure (i.e., hands-to-mouth behavior and Pica), high absorption, and high vulnerability (incomplete blood-brain barrier and prenatal susceptible windows during brain development) (Gorini, Muratori, and Morales 2014). During the prenatal period, sources of Pb exposure may include air, drinking water, lead-based paint in older residences, or consumer products (ATSDR 2020b). The Centers for Disease and Control Prevention (CDC) has recently revised the blood Pb reference value from 5 µg/dL to 3.5 µg/dL in 2021 (LEPAC 2021), given that even considerably low levels of Pb have been shown to negatively impact neurodevelopment in children. Various studies have established associations between blood Pb exposure and disrupted normal development, including deficits in cognitive skills and IQ, behavioral problems and increased risks of neuropsychological outcomes (Gump et al. 2017; Hong et al. 2015; Joo et al. 2018; Jianghong Liu et al. 2014; Taylor et al. 2017).

On the other hand, some essential metals are of great interest to researchers as they explore the delicate balance of homeostasis of such metals in regulating physiological processes. Manganese (Mn) is an essential metal that is critical for maintaining normal biological and cellular functions. It is instrumental to human organisms for its regulatory features in enzyme systems, digestion, reproduction and growth (Aschner and Aschner 2005; L. Li and Yang 2018). Mn also plays an important role in neuronal, metabolic and antioxidant defense processes (Horning et al. 2015). As one of the most abundant elements on earth, the drinking water, soil and deposited dust are potential Mn exposure sources (ATSDR 2012b). Mn also occurs naturally in edible food sources, such as nuts, grains and leafy vegetables, which may be a major exposure source for the general population (Lucchini et al. 2017). However, excessive levels may result in detrimental outcomes. Studies suggested that prenatal or early postnatal exposure to Mn will affect

neurodevelopment given the sensitivity of the exposure windows (Lin et al. 2013; Chung et al. 2015; Yu et al. 2014). As an essential element with potential neurotoxic properties, some research have reported U-shaped associations between blood Mn levels and mental development scores (Bhang et al. 2013; Claus Henn et al. 2010), indicating a possible toxic threshold of Mn.

Aside from Mn, suboptimal levels of essential elements during the prenatal period have also been postulated to influence neuropsychological development in children. While copper (Cu) deficiency is uncommon in the U.S. population, Cu toxicity has been suggested to lead to preterm birth (S. S. Kim et al. 2018), neurological impairments (Crisponi et al. 2010), and early developmental risks (Uriu-Adams et al. 2010). Iron (Fe) is particularly important in the prenatal period where for its' role in promoting normal fetal growth and central nervous system development. The literature is less consistent in the association between Fe and neurodevelopment (Iglesias, Canals, and Arija 2018); some studies have suggested links between Fe dysregulation and impaired mental and psychomotor development (Tamura et al. 2002), while others observed a positive association between Fe intake and executive functioning in children at age 7 (Arija et al. 2019).

Selenium (Se) is another crucial element for normal fetal growth and nervous system development (Rayman 2012). Se deficiencies have been linked to pregnancy complications (i.e., preeclampsia, and miscarriages) and adverse birth and developmental outcomes (Amorós et al. 2018; Pieczyńska and Grajeta 2015). Notably, studies have also reported that Se protects against Cd- and Mn-induced neurotoxicity (X. Yang et al. 2014; Zwolak 2020). There is also evidence that demonstrated positive associations between prenatal Se and motor skills (Polanska et al. 2017) and cognitive functions in children (Skröder et al. 2017). Zinc (Zn) is a micronutrient involved in developmental and neurotransmission processes (Adamo and Oteiza 2010). Gestational Zn

deficiency has been associated with fetal brain malformations, and the disrupted brain growth may consequently increase risks of psychomotor and behavioral disorders later in life (Keen et al. 1993; X. Yang et al. 2013).

Cobalt (Co) is a naturally occurring element and can be most commonly found in the environment combined with other elements like arsenic and sulfur (ATSDR 2004). Molybdenum (Mo) is also naturally present in food (i.e., legumes, milk, meat) and available as supplements (ATSDR 2020a). Both Co and Mo are trace elements essential to metabolism and enzymatic processes, but they are far less studied for their neurodevelopmental-related features, especially in the human population.

Although the relationship between several metals and children's neurodevelopmental outcomes has since been widely studied, the magnitude of effects and direction of associations remain inconclusive across studies. While there are more commonly used or gold-standard biomarkers for certain metals (i.e., assessing Pb exposure in blood) (Barbosa et al. 2006), utilizing distinct biomarkers, such as placental tissue and toenails that represent varying exposure time points throughout gestation may expand our understanding on the associations between prenatal metal exposure and neurodevelopment and behavioral outcomes in the offspring.

Assessing environmental metal exposures as a mixture

Health effects contributed by individual exposures to metals have been well established, yet it is unlikely that the population is exposed to only a single metal at any given period. In recent years, the importance of evaluating environmental exposures as a mixture has gained attention due to real-life scenarios where toxicants co-exist and likely concurrently exert adverse health outcomes in the exposed population. Accumulating evidence has shown the effects of combined

metal exposures on birth size, reproductive outcomes, and cognitive impairments (Deyssenroth et al. 2018; Freire et al. 2018; Gollenberg et al. 2010; Horton et al. 2018; Kordas et al. 2015; McDermott et al. 2011; Valeri et al. 2017). Specifically, a majority of studies have focused on combined effects of two to three metals on neurodevelopmental outcomes. For instance, exposures to Pb and Cd have been linked to cognitive issues in children at 6 months of age (Y. Kim et al. 2013), and co-exposure to Pb and Mn were associated with increased detrimental effects on cognitive and language development in children at 2 years of age (Lin et al. 2013; Y. Kim et al. 2009).

Depending on exposure levels, different interactions between metals may change. Antagonistic effects were observed at very low levels of both Pb and Cd in maternal blood, while synergistic effect between Pb and Cd was documented when Cd levels were above the median (Y. Kim et al. 2013). Proposed reasons of the observed synergistic effect of multiple metals include their abilities to induce oxidative stress (Lee et al. 2006; Domingo-Relloso et al. 2019) or disrupt neuroendocrine homeostasis (Ishitobi et al. 2007). Moreover, co-exposure to Pb and methylmercury (MeHg) (Boucher et al. 2012; Yorifuji et al. 2011), Pb and Mn (Claus Henn et al. 2012), and arsenic (As) and Mn (Valeri et al. 2017; Wright et al. 2006) during major windows of vulnerability have all been highlighted for the potentially aggravated effects on cognitive and motor development and behavior.

Nevertheless, there remains countless different combinations of metals that may pose threat to human health, and limited research has investigated the combined effects of exposure to larger number of metals on child development. Recent statistical modeling advancements that are more accommodating to high-dimensional and complex combinations of exposure variables are enabling researchers to address environmental mixtures research questions. Given the nature of

concomitant exposure to multiple metals and trace elements, utilizing suitable modeling techniques to characterize the combined effects of prenatal metal exposures, and considering shared pathways of these metals on disrupting normal developmental and behavioral progresses is needed.

The use of the NICU Network Neurobehavioral Scale (NNNS)

At the turn of the century, neonatal assessments mainly involved the development of primitive reflex models to evaluate infant functioning (Sherrington 1906). Followed by research exploring more generalized motor functioning (Saint-Anne-Dargassies 1955) and disentangling the concept of state (Prechtl 1974), the field had evolved into more comprehensive examinations on infants' active ability to modulate behavioral functioning in response to stimuli (Lester and Tronick 2004). In the 1950s, competent infants, as termed by researchers, referred to infants with organized motor abilities and were able to regulate their own states (Tronick and Lester 2013). With the advancement in infant assessment theories and studies, the Neonatal Behavioral Assessment Scale (NBAS) was developed in 1973 and became the dominant assessment tool in the field (Brazelton 1973). Although it included assessments for infant's ability to self-regulate and to interact with stimuli, the NBAS did not have normed instruments, and this could lead to incomparability to other studies or inadequate characterization of the infant's neurobehavioral state (Tronick and Lester 2013).

Developed by Lester and Tronick, the Neonatal Intensive Care Unit Network Neurobehavioral Scale (NNNS) draws on several previous behavioral assessment tools including NBAS (Brazelton 1973), Neurological Examination of the Full-Term Newborn Infant (Prechtl 1977), the Neurologic Examination of Maturity of Newborn Infants (Amiel-Tison 1968), the

Neurobehavioral Assessment of the Preterm Infants (Korner et al. 1991), and the Assessment of Preterm Infants Behavior (Als et al. 2005). The NNNS implemented standardized scales to examine the full range of newborns' neurobehavioral performances which included information on neurologic integrity, behavioral functioning and signs of stress (Tronick and Lester 2013; Lester, Tronick, and Brazelton 2004; Boukydis, Bigsby, and Lester 2004). Initially designed for the National Institute of Child Health and Human Development (NICHD) Neonatal Intensive Care Unit Research Network, the NNNS was applied in study settings to understand the effects of prenatal drug exposure on children's behavioral performance for the multisite maternal lifestyle study (Lester, Tronick, and Brazelton 2004). It can be procedurally applied once infants are stable and enables examiners to describe the infants' overall developmental maturation and behavioral states. In addition, the NNNS has also been proven to be applicable to healthy full-term infants (Provenzi et al. 2018; J. Liu et al. 2010; Fink et al. 2012), and has been utilized in estimating infants' neurobehavior associated with different environmental exposures (Maccani et al. 2015; Paquette et al. 2014).

The NNNS assessment includes 13 summary scores associated with infant's neurobehavior, reflex and stress: habituation, attention (orientation), arousal, self-regulation, handling, quality of movement, excitability, lethargy, non-optimal reflexes, asymmetry reflexes, hypertonicity, hypotonicity, and stress/abstinence. Higher summary scores indicate a higher level of the specific construct was observed in the infant, whereas lower scores indicate a lower level of the assessed construct. From a large random sample of clinically healthy infants, researchers were able to define normative performance and proposed NNNS summary scores between 10^{th}-90^{th} percentiles as normal performance, and scores below the 10^{th} percentile or over the 90^{th} percentile as poor neurobehavior (Fink et al. 2012). As their study population had a typical variation of demographic

and medical status, the generated summary score cut-offs are commonly adapted and referenced by other study samples.

Previous studies have proposed using latent profile analysis (LPA) to incorporate NNNS assessment in their investigations on determinants of newborn adverse neurobehavioral performance (J. Liu et al. 2010; Sucharew et al. 2012). LPA assumes there are underlying types or groups with different personal attributes or features in the population. The goal of utilizing LPA is to identify such patterns and generate discrete profiles with minimized heterogeneity within a profile and maximized heterogeneity across different profiles. Researchers may reference several model fit statistics, such as lower Akaike information criterion (AIC), Bayesian information criterion (BIC) and log-likelihood values (Berlin, Williams, and Parra 2014), and adequate profile sizes to decide on an optimal number of discrete profiles that would best represent all types of NNNS scale patterns in the study population.

Disrupted placental functions in response to prenatal metal exposures

The placenta is an organ unique to the gestational period; it is the master regulator of nutrient and gas exchange between the maternal and fetal systems and oversees a myriad of biological mechanisms that are crucial to maintaining pregnancy and promoting normal fetal growth and development. Given the indispensable role of placenta during gestation, perturbations from environmental exposures, such as neurotoxic metals, to placental physiology and functions have been shown to lead to a wide range of prenatal, perinatal and birth outcomes, such as preeclampsia, intrauterine growth restriction, preterm birth, and low birth weight (Caserta et al. 2013; Geng and Wang 2019; F. Wang et al. 2014).

During gestation, the placenta demonstrates neuroendocrine features and is in charge of the production and transfer of hormones and neurotransmitters between maternal and fetal circulations (Pasca and Penn 2010; Rosenfeld 2020). Biological relevance and mechanisms of placental-originated hormones, growth factors and neurotransmitters are integral to the DOHaD theory, as they can act as regulators for fetal growth and are often instrumental to the development of fetal organs such as the fetal brain (Gluckman et al. 2008; Pasca and Penn 2010). Studies have suggested that the neurotransmitter serotonin is mostly exclusively synthesized by the placenta, and then supplied to the fetal system to modulate brain programming and development (Bonnin et al. 2011; Bonnin and Levitt 2011b; Gaspar, Cases, and Maroteaux 2003). Animal model findings have indicated that placenta-originated serotonin is in charge of hypothalamus development at mid-gestation (Bonnin et al. 2011). Thus, it is probable that the placenta is involved in neurodevelopment at least partially through the serotonergic system. A number of prenatal perturbations have been associated with adverse neurophysiological endpoints through altered placental serotonin activities. Maternal inflammation, of which the placenta is most susceptible to, led to disrupted fetal neurodevelopment through interfering with the serotonin-dependent neurogenic process (Goeden et al. 2016). Exposure to endocrine disrupting chemicals bisphenol A (BPA) and bisphenol S (BPS) was linked to decreased placental serotonin concentrations (Mao et al. 2020). Additionally, serotonin level changes have been postulated as a potential factor leading to autism spectrum disorder (ASD) and anxiety-like behaviors (Chugani et al. 1999; Sodhi and Sanders-Bush 2004; Whitaker-Azmitia 2001).

On account of these previous findings, prenatal exposure to chemical and/or non-chemical stressors may affect placental physiology and indirectly affect neurotransmitter (i.e., serotonin) or other neuroactive factor synthesis, release or uptake, which could impact the development in

regions critical in facilitating learning and memory, and give rise to adverse neurodevelopmental outcomes (Bonnin and Levitt 2011a; Rebuli and Patisaul 2016; Rock and Patisaul 2018; Schug et al. 2015). The term placenta-brain-axis mentioned by Rosenfeld further emphasized the crucial role of placenta in gatekeeping the prenatal environment to ensure normal neurodevelopment (Rosenfeld 2021). Consequently, placental malfunctions may put infants at risk and potentially result in increased susceptibility to neurodevelopmental disorders throughout the life course.

The placenta acts as a partial barrier to several non-essential metals, allowing some metals to partially pass and accumulate in fetal tissues while sequestering others in the placental tissue itself (Gundacker and Hengstschläger 2012; Osman et al. 2000). Some studies have reported exogenous metal compounds in the placenta affect placental pathology through mechanisms such as inducing oxidative stress and inflammatory responses (Al-Saleh et al. 2015; Rehman et al. 2018; Z. Wang et al. 2012), and on a molecular level, modifying epigenetic mechanisms (Appleton et al. 2017; Everson et al. 2019; Vilahur, Vahter, and Broberg 2015). Considering the interrelation between the placenta and brain, disruptions to these mechanisms by placental metal exposures would be plausible explanations for subsequent adverse (neuro)developmental outcomes in children. Yet, there is limited evidence from human studies linking placental metal exposures and neurodevelopmental and behavioral outcomes, or detailing the compromised placental mechanisms in response to prenatal metal exposures that may also contribute to impaired behavioral development (O'Connor, Miller, and Salafia 2019). Owing to the multifaceted interface between maternal and fetal systems during the gestational period, providing robust evidence to assess whether and if so, how placental metal exposures act on placental functions to impact children's neurodevelopment is crucial.

Epigenetic marks: DNA methylation and hydroxymethylation

From the molecular aspect, the developing epigenome may be especially susceptible to exogenous environmental signals that could alter the epigenome during the dynamic phases of programming. In contrast to hereditary genetic changes of gene expression and functions as consequences of deletion, mutation, insertion or translocation to the DNA sequence, epigenetics is the study of changes in gene activity that do not involve alterations to the DNA sequence itself (Moore, Le, and Fan 2013).

Among the diverse types of epigenetic mechanisms, DNA methylation is one of the most extensively studied. Breakthroughs in acknowledging DNA methylation as an epigenetic factor came from several studies in the late 1970s, where researchers identified that DNA methylation patterns will maintain through cell cycles and DNA methylation modification is linked to gene expression and functions (Compere and Palmiter 1981; Holliday and Pugh 1975; Razin and Riggs 1980; Riggs 1975). DNA methylation in the mammalian genome occurs predominantly (70-75%) at cytosine residues within CpG (cytosine-phosphate-guanine) dinucleotides (Laird 2003; Moore, Le, and Fan 2013). DNA methyltransferases (DNMTs) catalyze the process of the covalent addition of a methyl group from S-adenyl methionine (SAM) to the fifth carbon (C5) position of the cytosine residue to form 5-methylcytosine (5mC) (Bestor 2000; P. A. Jones 2012). In the DNMT enzyme family, DNMT1 is in charge of copying and the maintenance of the DNA methylation patterns from the paternal DNA strand during cell mitosis (Hermann, Goyal, and Jeltsch 2004; P. A. Jones 2012), while DNMT3a and DNMT3b are known as *de novo* methyltransferases for their roles in setting up new DNA methylation patterns (Okano, Xie, and Li 1998; Okano et al. 1999). These DNMTs act together and complement one another to establish

and maintain DNA methylation patterns for embryo development and differentiation (Bird 2002; P. A. Jones and Liang 2009; Walsh and Bestor 1999).

The field of epigenetics has broadened and discovered additional epigenetic marks. One of which is hydroxymethylation, generated during demethylation and involving a hydroxymethyl group replacing the hydrogen atom at the C5 position of the cytosine. The process of converting 5mC to 5hmC is catalyzed by the ten-eleven-translocation (TET) family of methylcytosine dioxygenases (Ito et al. 2010; Tahiliani et al. 2009). Discovered in 1972, 5-hydroxymethylcytosine (5hmC) was initially recognized exclusively as an intermediate product of the demethylation process, and its' epigenetic characteristics in the human genome had not been well established until several decades later (Hashimoto et al. 2012; Tan and Shi 2012; H. Wu and Zhang 2011). 5hmC is highly tissue specific, most abundantly observed in the brain, and can be found in locations such as bodies of actively transcribed genes and at transcription factor binding sites and enhancer regions (Pastor et al. 2011; Stroud et al. 2011; Szulwach, Li, Li, Song, Han, et al. 2011). Findings that indicate 5hmC as more than an intermediate in the demethylation process include the presence of high levels of 5hmC in post-mitotic brain (Globisch et al. 2010; Kriaucionis and Heintz 2009), and that 5hmC was differentially associated with gene activity during neuronal differentiation (Hahn et al. 2013; Wen et al. 2014). Thus, emerging evidence is beginning to support the epigenetic functional significance of 5hmC, along with identifying the critical role of 5hmC in regulating chromatin structure and gene expression pertaining to developmental programming processes (Santiago et al. 2014; Szulwach, Li, Li, Song, Wu, et al. 2011).

A growing body of literature has shown that epigenetic modifications may be one of the underlying mechanisms linking environmental metal exposures and developmental programming ramifications. Studies have linked early exposure to arsenic, cadmium, lead, and mercury and

adverse birth and developmental outcomes through gene-specific and genome-wide DNA methylation assessed from various tissues, including maternal blood and cord blood (Appleton et al. 2017; Kippler et al. 2013; Maccani et al. 2015; Meakin et al. 2019; Pilsner et al. 2009). For hydroxymethylation, experimental studies have also suggested associations between 5hmC and toxic metals (Cardenas et al. 2017; Tellez-Plaza et al. 2014). With the relatively low 5hmC levels across tissues and detection limitations in distinguishing between 5mC and 5hmC, human studies have only gradually begun to examine potential effects of prenatal environmental exposures on 5hmC patterns. For metals, an earlier birth cohort study showed prenatal lead (Pb) exposure was associated with peripheral blood 5hmC changes in four candidate genes involved in neurodevelopment (Rygiel et al. 2021). Building on their findings, expanding research to assess genome-wide 5hmC patterns would provide more comprehensive understandings on 5hmC-mediated epigenetic mechanisms in relation to prenatal Pb influences.

Given the placenta's essential role as a programming factor during the prenatal period, epigenetic changes in placental tissue may serve as a potential mechanism for prenatal toxic metal exposure and adverse (neuro)developmental and behavioral outcomes in children. Further research is needed in this field as findings on genome-wide DNA methylation changes in response to certain metals remain inconclusive in human studies. Furthermore, there exist research gaps in assessing metal-related methylation variations in placental functions that may be crucial to development and behavior. Likewise, more research dedicated to understanding the hydroxymethylation profiles in human tissues, particularly the placenta, is also warranted.

Dissertation overview

Environmental perturbations can exert detrimental effects on newborn development and behavior during the prenatal period. Considering the population is most likely subject to concurrent exposure to multiple toxicants (i.e., metals and trace elements), determining environmental mixtures' effect on health outcomes is a key advancement in public health research. The placenta plays a fundamental role in the dynamic developmental programming period. Therefore, understanding epigenome changes through placental DNA methylation and hydroxymethylation may help identify the disrupted epigenetic mechanisms and thereby the dysregulated biological functions, in relation to prenatal toxicant exposure.

This dissertation aims to investigate how prenatal metal exposures associate with newborn neurobehavior and placental epigenetic variations by utilizing environmental epidemiological and molecular data from a U.S. based birth cohort. The primary aims of this work are:

Aim 1: Investigate the association between placental metal exposure and newborn neurobehavioral performances in the Rhode Island Child Health Study (RICHS). *Hypothesis*: Prenatal exposure to cadmium (Cd), manganese (Mn) and lead (Pb), quantified through placental tissue, will be associated with newborn neurobehavioral performances indicated through the NICU Neurobehavioral Scales (NNNS) profiles.

Aim 2: Understand the mixture effect of prenatal metal exposures on newborn neurobehavioral performances in RICHS. *Hypothesis*: Prenatal exposure to multiple placental metals as a mixture will be associated with newborn neurobehavioral performances indicated through NNNS profiles.

Aim 3: Evaluate epigenetic effects of prenatal lead (Pb) exposure through placental DNA methylation and hydroxymethylation. *Hypothesis*: Prenatal Pb exposure, quantified through

distinct biomarkers (toenails and placenta), will be associated with differential placental DNA methylation and hydroxymethylation, and the observed epigenetic modifications will be involved in biological functions and pathways related to developmental processes.

These aims are addressed in the following dissertation chapters, two of which have been published as stand-alone manuscripts, and the third is currently under review for publication. Chapter 2, previously published in *Environmental Research* (Tung et al. 2022b), addresses the hypothesis for Aim 1 with a latent profile analysis to generate NNNS neurobehavioral profiles, followed by individual regression models associating profile assignment with prenatal Cd, Mn and Pb exposure. Chapter 3, previously published in *Environmental Epidemiology* (Tung et al. 2022a), addresses the hypothesis for Aim 2 with quantile g-computation to model the overall mixture effect of a total of eight placental metals and trace elements on neurobehavioral profiles in RICHS newborns. Chapter 4 addresses the hypothesis for Aim 3 with an epigenome-wide association study of prenatal Pb exposure in distinct biomarkers and placental DNA methylation and hydroxymethylation, followed by an overrepresentation analysis to identify the associated biological pathways. Chapter 5 summarizes the research conclusions and discusses future directions.

Figure

Figure 1-1. Graphical summary of dissertation aims.
Adapted from the DOHaD concept, toxicant exposure during the gestational period may lead to adverse childhood health outcomes. Examining prenatal metal exposures individually (Aim 1, blue) and in combination (Aim 2, green) may provide additional insights on the associated impacts on newborn neurobehavior performances indicated through NNNS profiles. Prenatal metal exposure, for instance lead (Pb), may also affect the *in utero* environment. Investigating the associations between prenatal Pb and placental DNA methylation and hydroxymethylation (Aim 3, purple) may elucidate the underlying mechanisms of how Pb-induced placental epigenetic modifications influence placental functions and lead to potential developmental implications.
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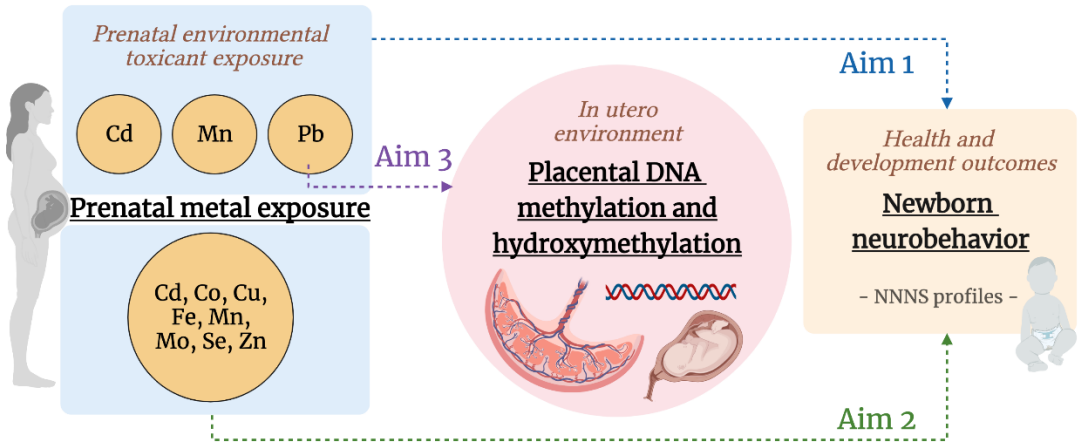


Chapter 2: Impacts of placental cadmium, lead and manganese exposure on newborn neurobehavioral performances

Published in a different format as:

Tung, Pei Wen, Amber Burt, Margaret Karagas, Brian P. Jackson, Tracy Punshon, Barry Lester, and Carmen J. Marsit. 2022b. "Association between Placental Toxic Metal Exposure and NICU Network Neurobehavioral Scales (NNNS) Profiles in the Rhode Island Child Health Study (RICHS)." *Environmental Research* 204 (March): 111939. https://doi.org/10.1016/j.envres.2021.111939.

Abstract

Prenatal exposure to heavy metals has been linked to a variety of adverse outcomes in newborn health and later life. Toxic metals such as cadmium (Cd), manganese (Mn) and lead (Pb) have been implicated to negatively affect newborn neurobehavior. Placental levels of these metals may provide additional understandings on the link between prenatal toxic metal exposures and neurobehavioral performances in newborns. To evaluate associations between placental concentrations of toxic metals and newborn neurobehavioral performance indicated through the NICU Network Neurobehavioral Scales (NNNS) latent profiles. In the Rhode Island Child Health Study cohort (n=625), newborn neurobehavioral performance was assessed with NNNS, and a latent profile analysis was used to define five discrete neurobehavioral profiles based on summary scales. Using multinomial logistic regression, we determined whether increased levels of placental toxic metals Cd, Mn and Pb associated with newborns assigned to the profile demonstrating atypical neurobehavioral performances. Every doubling in placenta Cd concentration was

associated with increased odds of newborns belonging to the atypical neurobehavior profile (OR: 2.72, 95% CI [1.09, 6.79]). Detectable placental Pb also demonstrated an increased odds of newborns assignment to the atypical profile (OR: 3.71, 95% CI [0.97, 13.96]) compared to being in the typical neurobehavioral profile. Toxic metals Cd and Pb measured in placental tissue may adversely impact newborn neurobehavior. Utilizing the placenta as a prenatal toxic metal exposure biomarker is useful in elucidating the associated impacts of toxic metals on newborn health.

Introduction

Exposure to environmental contaminants is an important public health concern especially among children. Toxicants, such as heavy metals, are persistent in the environment from both natural and anthropogenic activities. The general population is often exposed, voluntarily and involuntarily, through ambient air, drinking water, dietary or industrial sources. Due to the ubiquity of heavy metals in the environment, exposure to these contaminants is often inevitable in the susceptible children's population.

A growing body of evidence has documented heavy metals contributing to a wide variety of adverse outcomes including decreased fetal growth and length of gestation, low birth weight, and cognitive and behavioral deficits in children (Sanders, Henn, and Wright 2015; S. S. Kim et al. 2018; Kordas et al. 2015; Sabra et al. 2017; Taylor, Golding, and Emond 2016). As exposure to even low levels of toxic metals during critical developmental windows have been linked to detrimental neuropsychological outcomes, it is imperative that we investigate the underlying association between prenatal heavy metal exposure and neurobehavioral outcomes in newborns at the earliest time point, as earlier prevention and intervention strategies can be the most successful (Ris et al. 2004; Claus Henn et al. 2010; Lanphear et al. 2005).

Although much work on the mechanistic toxicology of toxic metals has focused, appropriately, on impacts to the developing brain, there is a growing body of literature suggesting an important role for the placenta and its functions in neurodevelopment (Lester and Marsit 2018; Shallie and Naicker 2019; Bale 2016). Besides its role in nutrient, waste, gas, and water transport, the placenta also produces a variety of neurotransmitters and acts to metabolize maternal hormones in support of healthy fetal brain development (Rosenfeld 2021). These data also suggest that stressors that can impact fetal neurodevelopment may do so, at least in part, through impacts on the placenta (Nugent and Bale 2015; O'Donnell, O'Connor, and Glover 2009; Tomlinson et al. 2019).

Cadmium (Cd) is considered a toxic metal and has been widely studied for its adverse impacts on human health (ATSDR 2012a). Aside from the commonly known effects on kidney functions and attribution to various types of cancer, Cd exposure has also been linked to neurologic, developmental and cognitive impairments in the vulnerable children's population (ATSDR 2012a; Sanders, Henn, and Wright 2015; Rodríguez-Barranco et al. 2013). Cd is known to accumulate in the placenta, and that its pregnancy-related toxicity is due to impacts on the placenta including eliciting oxidative stress, interfering with the transfer of essential metals to the fetus and impairing the developmental progress of the fetus during the critical gestational period (Geng and Wang 2019; Gundacker and Hengstschläger 2012; Levin et al. 1981; Z. Wang et al. 2012). This would also suggest that utilizing the placenta as a biomarker when examining its health effects can provide important information.

Lead (Pb) is an extensively studied developmental toxicant and children are the targeted population of concern due both to higher exposure scenarios and the susceptibility of the developing brain to the exposure (Charney et al. 1983; Gorini, Muratori, and Morales 2014;

ATSDR 2020b). Even at relatively low levels, exposure can lead to detrimental effects on children's development, thus suggesting that there may be no "safe" limit for Pb exposure (Bellinger 2008; Canfield et al. 2003; Taylor et al. 2017). Although Pb can be transferred through the placenta to fetal circulation, detected levels in placenta may indicate high *in utero* Pb exposure and result in detrimental effects to the developing fetus.(Goyer 1990; Schell et al. 2003).

Manganese (Mn) is an essential element and is commonly found in the environment (ATSDR 2012b). Aside from natural sources, dietary sources such as rice and whole grains contain the highest Mn levels (ATSDR 2012b; Aschner and Aschner 2005; Horning et al. 2015). However, Mn is additionally recognized as a toxicant with excessive Mn targeting the developing central nervous system (CNS) and contributing to neurological disorders (Bjørklund, Chartrand, and Aaseth 2017; Horning et al. 2015; Yu et al. 2014). During pregnancy, Mn can accumulate in the placenta and through impacts to the placenta may elicit adverse effects on the developing CNS of the fetus.

Numerous studies have investigated the association between prenatal Cd, Pb or Mn exposure and neurobehavioral outcomes in young children. However, with different approaches of neurobehavioral assessments and varying biological matrices available for measuring prenatal heavy metal exposure, the exact relationship between metal exposure and neurobehavioral-related outcome remains uncertain. The placenta can serve as a useful biomarker for measuring prenatal exposure, and to our best knowledge, there are still research gaps in associating placental metal levels and neurobehavioral deficits (Rodríguez-Barranco et al. 2013). Therefore, our study goal is to quantify Cd, Mn and Pb levels in the placenta and examine the hypothesis that placental trace metal concentrations are related to atypical neurobehavioral performance identified at birth.

Methods

Study Population

The infants included in this analysis are from the Rhode Island Health Study (RICHS), a hospital-based birth cohort established at the Women and Infants Hospital in Providence, Rhode Island, USA. The RICHS cohort recruited mothers of term infants (≥ 37 weeks) oversampled for infants that were SGA (small for gestational age) and LGA (large for gestational age), while recruiting AGA (adequate for gestational age) infants matched on sex, gestational age (within 3 days), and maternal age (within 2 years) to those at the extremes. Exclusion criteria of RICHS included mothers younger than 18 years of age, pregnancies resulting in preterm birth (< 37 weeks), or infants born with congenital or chromosomal abnormalities. A total of 840 mother-infant pairs were enrolled into the study. Medical records were used to collect anthropometric and clinical data, including birth weight, gestational age, depression and anxiety/panic/obsessive compulsive disorder history. Interviewer-administered questionnaires were used to obtain information on sociodemographic, behavioral, and exposure histories.

NNNS Assessment

The NICU Network Neurobehavioral Scale (NNNS) is a standardized assessment aimed to comprehensively assess newborns' neurologic integrity, behavioral functioning and signs of stress (Lester and Tronick 2004). The assessments results in 13 summary scores, including habituation, attention (orientation), arousal, self-regulation, handling, quality of movement, excitability, lethargy, non-optimal reflexes, asymmetry reflexes, hypertonicity, hypotonicity, and stress/abstinence.

In RICHS, NNNS was administered after the first 24 hours of life, and prior to discharge by certified psychometrists. Of the 840 enrolled infants in RICHS, 625 infants (74%) were assessed with NNNS (**Figure 2-1**). For the habituation construct, as the newborn was required to be asleep for assessment, information was not collected for 54.9% of the infants (Lester, Tronick, and Brazelton 2004). As a result, we only included the remaining 12 summary scores of NNNS for the statistical analyses.

Metal Assessment

Placental parenchyma tissue suitable for trace metals analysis was collected from 192 participants in RICHS within 2 hours of delivery (**Figure 2-1**). Biopsies from approximately 1-2cm from the cord insertion site, free of maternal decidua, were excised, rinsed, and stored in trace element-free tubes until further examination. Placental concentrations of twenty-four metals, including Cd, Mn, and Pb were quantified at the Dartmouth Trace Elements Analysis Core using ICP-MS protocols as described in detail elsewhere (Punshon et al. 2016). Briefly, after samples were transferred and brought to room temperature, HNO_3/HCl (Optima^{TM}) solution was added based on sample wet weight. Following EPA method 3050B, samples were then digested via microwave, and 0.25-0.35 ml of H_2O_2 was added to each sample tube. Quality control measures included the analysis of standard reference material (NIST 1566b, oyster tissue), initial and continuing calibration verification, and the use of fortified blanks, analytical duplicates and spikes. The detection limits (LOD) for placenta Cd, Mn and Pb were 2.12 ng/g, 10.61 ng/g and 2.12 ng/g, respectively.

Statistical Analysis

We compared demographic characteristic differences between the RICHS study population, and the subset of participants with both placental heavy metal and NNNS data with chi-square test and t-test.

Descriptive statistics for all NNNS summary scales were reported, with higher score on each scale indicating a higher level of the measured construct. Depending on the measured aspect of neurobehavior, higher scores may either indicate better or worrisome performance. For instance, newborns with higher scores in the attention scale were more alert and able to follow visual stimulations with eyes and head during assessment. Higher level of the quality of movement scale indicated predominantly smooth movements and little jerkiness or startles. On the contrary, newborns with high scores in the lethargy or stress/abstinence scales exhibited more signs of the measured construct, which are less optimal.

We used latent profile analysis (LPA) to understand the underlying NNNS summary score patterns in the RICHS study population. According to Liu et al., LPA assumes the study population consists of several subgroups that can be labeled as latent profiles or classes (J. Liu et al. 2010). The purpose of using LPA for the NNNS scores was to produce discrete profiles with minimized heterogeneity within a profile and maximized heterogeneity across different profiles. In order to obtain an optimal number of profiles based on the NNNS summary scores in the study population, we fitted multiple models with different number of profiles, and the model with the most appropriate fit statistics was chosen as the number of profiles for RICHS. Referring to information-criteria based fit statistics, the model with the lowest log-likelihood and Bayesian Information Criteria (BIC) values was preferred. Entropy value greater than 0.8 is ideal as this would indicate greater classification accuracy of the subjects. Additionally, profile size will also be taken into

consideration, as the smallest profile should still include at least 5% of the study population (Berlin, Williams, and Parra 2014).

Heavy metal levels and NNNS summary scores were compared across profiles with ANOVA analyses. The association between placental heavy metal levels and newborn neurobehavioral performances indicated through profiles was assessed using multinomial logistic regression. Samples with Cd levels below the detection limit were assigned a value of LOD divided by the square root of 2. We applied $\log_2$-transformed Cd and Mn levels in the models for approximation to normal distribution, and for interpretation purposes. Alternatively, 51.6% samples were flagged as <LOD for Pb levels, so we then treated placental Pb as a binary variable (non-detect vs. detectable) in the regression models. Regression models were further adjusted for covariates collected from medical records and in-person questionnaires administered by interviewers and were *a priori* determined in reference to previous RICHS studies. Adjusted covariates included infant sex, maternal age, maternal race (white or not white), maternal body mass index, and educational attainment (dichotomized into obtained high school or less versus more than high school education). We also ran sensitivity analysis to test whether model results were robust when tobacco smoking status during pregnancy was included.

Statistical analyses were conducted using R version 3.5.1, and LPA analysis was performed with Mplus version 8.4.

Results

Study population

Demographic and gestational characteristics of the RICHS cohort are shown in **Table 2-1**. Of the 625 RICHS newborns with NNNS information, 51.4% were females and 57% were born under the adequate for gestational age (AGA) birth weight category. Maternal participants were predominantly white (70.6%) and had obtained at least some post high school education (73.4%). The subset of infants with both NNNS and placental heavy metal information (n=192) showed similar distribution for most of the demographic characteristics. 47.4% of the subset of infants were females, 73.4% mothers were white, and 75.5% had some post high school education. Average gestational age in the RICHS study population and subset were both around 39.3 weeks. Birth weight category distribution was significantly different between the full cohort and sub-cohort (p=0.001), and birth weight in the subset (mean=3644 g) was significantly higher than the entire study population (mean=3477 g; p=0.003).

NNNS profiles

Descriptive statistics of the summary scales of NNNS are shown in **Supplemental Table 2-1** and were used to identify potential outliers. Extreme values were found for stress abstinence, non-optimal reflexes, and asymmetric reflexes scales, but no individuals in this study population were consistently outliers for all of the NNNS summary scales.

To determine the most appropriate number of profiles in this study population, we fit LPA models with 2 to 6 profiles. As the fitted profile numbers increased from 2 to 6 profiles, BIC values decreased (**Supplemental Table 2-2**). Entropy values for all the models were all greater than 0.8, which was preferred, and showed better accuracy in classifying participants into different profiles. Additionally, profile size was used to determine the optimal number of profiles as it is recommended that the smallest profile should not be smaller than 5% of the study population.

Despite having the lowest BIC value, one of the profiles generated from the 6-profile model consisted of only 29 participants (4.6%). Based on fit statistics, profile size, and average class probabilities, the 5-profile model showed the best fit for the RICHS cohort.

Figure 2-2 demonstrated distinct NNNS patterns of the five profiles based on the standardized summary scores as the original scores were not on the same scale. Mean and standard deviation of the 12 summary scores by profiles are shown in **Supplemental Table 2-3**. The largest profile (Profile 2) consisted of 172 (27.5%) participants, while the smallest profile (Profile 5) included 45 (7.3%) subjects. Compared to other profiles, Profile 5 infants showed the most extreme scores, indicating atypical neurobehavior compared to infants in other profiles. Infants in this profile were characterized with the highest arousal, excitability, hypertonicity and stress abstinence signs, and the lowest quality of movement, regulation and non-optimal reflexes. Profile 4 (N=124, 19.8%) infants showed more signs of lethargy, hypotonicity, non-optimal reflexes and asymmetric reflexes, and lowest attention and arousal. Compared to Profiles 4 and 5, Profile 3 infants (N=157, 25.1%) required more handling, but behaved on average for most of the summary scores. The largest group, Profile 2, showed relatively average performances for most of the summary scores with the exception of the lethargy scale, which was the lowest across all profiles. Infants in Profile 1 (N=127, 20.3%) showed the highest attention, quality of movement, and regulation, and lowest stress abstinence signs, along with less handling, excitability, and hypertonicity. Given the summary scale pattern indicating most scales around the mean, in the following regression models, Profile 2 served as the reference profile.

Placental heavy metal concentrations

The mean, standard deviation, minimum and maximum values of placental Cd, Mn and Pb are shown in **Supplemental Table 2-4**. We further examined log_2-transformed placental metals concentration and detectable placental Pb distribution by the five NNNS profiles (**Figure 2-3**). Placental Cd and Mn did demonstrate an elevation in Profile 5, although the differences across profiles were not considered statistically significant. We also observed a greater proportion of detectable Pb amongst placenta from individuals classified in Profile 5.

Association between heavy metal and profiles

In line with the bi-variate results, in the unadjusted multinomial logistic regression results (**Table 2-2**), detectable placental Pb also demonstrated an increased odds of newborns belonging to the atypical Profile 5 (OR: 3.12, 95% CI [0.89, 10.97]) with Profile 2 as the comparison group. In adjusted regression models, compared to belonging in Profile 2 with typical neurobehavior, there were increased odds of newborns being placed in the atypical neurobehavior Profile 5 with every doubling increase in placenta Cd level (OR: 2.72, 95% CI [1.09, 6.79]). Additionally, newborns with detectable placental Pb levels also demonstrated an increased odds of belonging to Profile 5 (OR: 3.71, 95% CI [0.97, 13.96]), although in all models the 95% CI are wide suggesting potentially unstable results. Alternatively, increased placenta Mn level was not associated with increased odds of newborns belonging to the atypical Profile 5 (OR: 1.16, 95% CI [0.28, 4.92]). As a sensitivity analysis, we fit models with maternal smoking status as a covariate (**Table 2-2**: Adjusted[b]), noting that the number of smokers in this population is small and the number of smokers within any profile was limited. These models indicated some attenuation of the effect size for the associations between placental Cd and Pb with Profile 5. In models examining membership in Profile 5 versus all other profiles (**Supplemental Table 2-5**), we observed an association

between detectable placental Pb levels and increased odds of atypical neurobehavioral performances in newborns (OR: 3.94, 95% CI [1.15, 13.46]), as well as increased odds for Profile 5 membership associated with increasing Cd concentrations (OR: 2.39, 95% CI [1.03, 5.52]).

Discussion

Our study explored placental concentrations of putative neurotoxic trace metals and neurobehavior assessed by NNNS profiles. By categorizing our study population based on the NNNS using the LPA method, and after controlling for multiple covariates, we observed an association between increased placental Cd levels and higher odds of newborns belonging to the atypical neurobehavior profile (Profile 5) compared to them being placed in the typical Profile 2. Detectable placental Pb was also associated with increased odds of newborns being placed in the atypical neurobehavior profile compared to those with placental Pb levels below the detection limit.

Metal concentrations measured in this present study were generally consistent with levels detected in other study populations. Cd levels measured in the RICHS placenta samples were towards the lower end of the reported range of levels worldwide (Esteban-Vasallo et al. 2012). A review reported that the average concentration of placental Cd was around 4 ng/g in non-exposed environments (Esteban-Vasallo et al. 2012; Iyengar and Rapp 2001). Our reported arithmetic placental Cd mean at 4.56 ng/g (±SD 2.58) was lower compared to levels measured in some countries outside the US, but slightly greater than levels measured in a study in North Carolina (Al-Saleh et al. 2011; Laine et al. 2015; Kippler et al. 2010). The range of placental Mn level in RICHS was much wider than the detected range in a birth cohort study in Spain (Freire et al. 2018). Amongst those with detectable Pb levels, placental concentrations averaged at 4.49 ng/g (±SD 3.89), which was lower than the reported value from several study populations across the world,

but comparable to the value reported in the New Hampshire Birth Cohort study (Punshon et al. 2016; Esteban-Vasallo et al. 2012; Al-Saleh et al. 2011; Singh et al. 2010).

Cd is a known developmental toxicant as established by numerous studies, and common sources of Cd exposure are diet, smoking during pregnancy or industrial contamination in residential environment. The RICHS population had relatively low prevalence of women who smoked during pregnancy (10.2%), and the study setting was not occupational in nature, thus the main source of Cd exposure measured in the placenta was most likely due to diet (K. Kim et al. 2019). However, further understanding of dietary Cd sources in RICHS is limited as we did not obtain dietary information from the participating women.

Proper placental functions are crucial for normal fetal growth and development. During pregnancy, the placenta acts as a barrier and is thought to protect the developing fetus from Cd exposure by limiting transfer to fetal circulation. Nonetheless, Cd levels were detectable in cord blood and newborn serum in previously published studies (Z. Chen et al. 2014; Carrillo-Ponce et al. 2004). In our study, we investigated prenatal Cd exposure using the placenta as a biomarker and observed a significant association between Cd and the atypical Profile 5, characterized by poorer neurobehavioral performances, such as increased signs of stress and excitability. The MOCEH study in Korea found no association between Cd measured in maternal blood and neurodevelopment measured at 6 months (Y. Kim et al. 2013). The HOME study also did found no significant association between maternal urinary Cd and cognitive and behavioral outcomes in 1–8-year-old children (W. Yang et al. 2020). Additionally, no relationship between blood Cd and neurodevelopment was observed in children 2 years of age in the TLC study (Y. Cao et al. 2009). Limited numbers of epidemiologic studies have investigated the potential link between prenatal Cd exposure and fetal development through impacts on the placenta. However, in line with our

findings, other studies have identified inverse associations between prenatal Cd exposure measured through maternal blood, cord blood or urine, and verbal and performance IQ in children (Sanders, Henn, and Wright 2015; Kippler et al. 2012; Tian et al. 2009). As we further explore prenatal Cd exposure in the placenta, the present finding adds to the understanding of the potential for adverse impacts from prenatal Cd exposure on fetal development.

We further observed an association between detectable Pb and assignment to the atypical profile for newborns in the RICHS population. Pb is well-documented for its developmental neurotoxicity in children (Ris et al. 2004; ATSDR 2020b; Osman et al. 2000). Our results are in agreement with a series of studies that demonstrated the negative effects on neurodevelopment from early-life Pb exposure. Postnatal dentine Pb was linked to increased behavioral problems in Mexican children between the ages of 8-11 (Horton et al. 2018). In addition, prenatal and early postnatal exposure to Pb have also been found to be associated with decrements in IQ and compromised neuropsychological function in children (Lanphear et al. 2005; Gorini, Muratori, and Morales 2014; Bellinger 2008; Lidsky and Schneider 2003; Wasserman et al. 2000). Contrary to these findings, Freire et al. measured placental Pb in the INMA cohort as we did in the present study and found no association with neurodevelopmental outcomes at 4-5 years of age (Freire et al. 2018). Additionally, Taylor et al. (2017) did not observe association between placenta Pb levels and motor skills in 7-year-old children (Taylor et al. 2017). Among infants, the MOCEH study also found no association between Pb exposure measured early in pregnancy and mental (MDI) and psychomotor (PDI) development index scores, but Pb levels during late pregnancy period was found to be linked to lower MDI scores assessed at 6-month of age (Y. Kim et al. 2013). Thus, whether early indicators of neurodevelopmental performance among newborns associate with placental Pb will need to be confirm or refuted by further studies.

We also investigated Mn exposure and newborn neurobehavior in this study population but did not find a significant association between increased placental Mn levels and higher odds of newborns belonging to the atypical Profile 5. Unlike Cd and Pb that are classified as toxic metals, Mn is recognized as an essential nutrient crucial for development and growth, though excess levels have been linked to cognitive and behavioral issues early in life, at 6 months in the MOCEH study and in children at 2 years of age from a study in Taiwan (ATSDR 2012b; Horning et al. 2015; Chung et al. 2015; Lin et al. 2013). A meta-analysis found a 50% increase in Mn levels was associated with decreased IQ points in children aged 6-8 years (Rodríguez-Barranco et al. 2013). The CHAMACOS cohort also linked increased Mn exposure to poorer behavioral performance (Mora et al. 2015). In contrast, the same study found improved memory and cognitive function in older boys to be associated with higher Mn levels. In agreement with our study, a French study found no association between Mn exposure measured in the placenta and cognitive scores at age 3 or age 6 (Takser et al. 2003). The mean Mn level in our study cohort was similar to the French study (0.095 ug/g vs. 0.10 ug/g), thus it is possible that placental Mn at this level was not high enough to negatively impact neurobehavior.

While our findings on placental Cd and Pb and atypical neurobehavioral phenotype generally agreed with prior studies, discrepancies may have arisen from the type of biologic matrices used. Maternal urine, maternal blood, cord blood, hair and toenails have all been used to determine the link between Cd, Pb or Mn levels and children's neurobehavior, with fewer studies of placenta. Thus, the metals' characteristics, such as Cd's accumulative nature in the placenta and Pb's ability to pass through the placenta along with the time point in which biological matrices were collected, or the study population's exposure environment and cultural patterns, could have

resulted in differences in the direction and strength of association between neurodevelopment and metal exposures across studies.

We were able to distinguish NNNS score pattern differences across five profiles and identified typical and atypical profiles based on neurobehavior characteristics. Initially designed to study effects of prenatal drug exposure on child outcomes, the NNNS assessment utilizes standardized scales to examine newborns' neurobehavioral performances (Lester, Tronick, and Brazelton 2004; Tronick and Lester 2013). From analyzing NNNS results from a large random sample of clinically healthy newborns, Fink et al. showed that NNNS scales ranging between the 10^{th} to the 90^{th} percentile would indicate normative neurobehavior (Fink et al. 2012). Researchers have since successfully applied NNNS assessments to understand neurobehavior potentially associated with different environmental exposures in healthy full-term infants.

Like others, we used the LPA approach to analyze NNNS data. We observed similarities in the identified profiles to those previously of Liu et al. in the Maternal Lifestyle Study (MLS), a multicenter longitudinal study designed to understand effects of illicit drugs during pregnancy on the mother, fetus and infant (J. Liu et al. 2010; Bauer et al. 2002). The similarities of the NNNS patterns across the profiles of these two populations provides evidence that NNNS assessment and profiling method can be used to characterize neurobehavioral performances in low-risk, healthy infants and to investigate the determinants of these profiles.

NNNS scores evaluated within 24-72 hours of birth have prospectively predicted infant temperament and neuropsychological characteristics in toddlers and preschool-aged children (J. Liu et al. 2010; Sucharew et al. 2012; Sheinkopf et al. 2006). Thus, the application of the NNNS measurements may allow early insight in the newborns' neurobehavioral performances which

could inform ongoing monitoring or early interventions to those categorized into the atypical profiles. It is possible that by ascertaining the newborn's neurobehavioral performance before hospital discharge, rather than waiting for adverse neurobehavior to manifest, we would be able to provide interventions within the early developmental period.

Advantages of this study include understanding prenatal metal exposure using the placenta as a biomarker. As prenatal metal exposures are known to elicit adverse impacts on the developing fetus, measuring placental levels of Cd, Mn and Pb may offer new understandings on prenatal exposure characteristics and associated neurobehavioral performance within days of life. With the LPA method, we were able to generate discrete neurobehavioral profiles, and the relatively large cohort size (N=625) in RICHS allowed adequate profile sizes with distinct NNNS patterns comparable to prior work in another study population, suggesting our findings are generalizable beyond the study region. We were able to identify atypical neurobehavior if newborns were assigned to Profile 5, though the profile size is considerably smaller than the other profiles. This is likely to due to RICHS being comprised of primarily low-risk and healthy participants, so there are relatively fewer extreme cases of neurobehavioral issues among the newborns.

For this present study, we analyzed individual metal's contribution to atypical NNNS score patterns, but it is likely that the population was concurrently exposed to multiple metals. To better address how these developmental toxicants impact child's neurobehavior, additional research considering metal exposures as a mixture and identifying the major contributor(s) driving the impacts of metal mixture on neurobehavior is needed. Another limitation of this study is the lack of information on exposure source of the participants. Other than smoking, a common source of exposure to Cd in the general population is dietary intake, such as shellfish or other Cd-contaminated foods (ATSDR 2012a). The general population is also primarily exposed to Mn

through food and water. It is likely that with the lack of dietary data, we are restricted to fully address the association between metal exposure and neurobehavior, as certain dietary components may lead to increased metal exposure, but are also considered beneficial towards development. Aside from dietary intake, inhalation of particulate matter containing Mn or dermal contact with Mn-contaminated air, water, and soil are all possible sources of Mn exposure (ATSDR 2012b). While the overall metal exposure levels in RICHS are lower compared to other studies, future work examining metal levels based on the different exposure sources could further clarify the adverse impacts of metals on neurobehavior.

Conclusion

In this study, we found that placental toxic metals including Cd and Pb negatively affected neurobehavioral performance in newborns in a generally healthy population as indicated through the NNNS. Our findings also highlight the importance of the placenta in newborn health and the utility of measuring of placental metal concentrations to evaluate child health outcomes.

Tables

Table 2-1. Demographic and gestational characteristics.

Characteristic	***Newborns with NNNS data (N=625)***	***Newborns with NNNS and placental metal data (N=192)***
	n (%)	
Infant Gender		
Female	321 (51.4%)	91 (47.4%)
Male	304 (48.6%)	101 (52.6%)
Birth Weight Category[a]		
SGA	123 (19.7%)	29 (15.1%)
AGA	356 (57.0%)	93 (48.4%)
LGA	146 (23.4%)	70 (36.5%)
Delivery Method		
Cesarean section	317 (50.7%)	110 (57.3%)
Vaginal	308 (49.3%)	82 (42.7%)
Maternal Race		
White	441 (70.6%)	141 (73.4%)
Other	167 (26.7%)	47 (24.5%)
Unknown	17 (2.7%)	4 (2.1%)
Infant Race		
White	408 (65.3%)	123 (64.1%)
Other	201 (32.2%)	64 (33.3%)
Unknown	16 (2.6%)	5 (2.6%)
Maternal Education Status		
No more than high school	166 (26.6%)	47 (24.5%)
Some post-high school	459 (73.4%)	145 (75.5%)
Maternal Smoking Status		
Yes	64 (10.2%)	26 (13.5%)
No	555 (88.8%)	165 (85.9%)
Unknown	6 (1.0%)	1 (0.5%)
	Mean ± SD	
Birth weight (grams)	3477 ± 664.75	3644 ± 680.37
Gestational age (weeks)	39.34 ± 0.96	39.31 ± 0.95
Maternal age (years)	29.48 ± 5.49	29.79 ± 5.63
Maternal BMI (kg/m^2)	26.71 ± 6.96	27.48 ± 7.14

[a] SGA: small for gestational weight; AGA: adequate for gestational weight; LGA: large for gestational weight
HS: High school

Table 2-2. Odds ratio (95% CI) from multinomial regression models.
(Reference group: Profile 2)

	Profile 1	**Profile 3**	**Profile 4**	**Profile 5**
Unadjusted				
log_2 Cd	1.30 (0.74, 2.31)	0.95 (0.55, 1.64)	0.90 (0.51, 1.57)	1.70 (0.78, 3.66)
log_2 Mn	0.64 (0.24, 1.70)	0.97 (0.39, 2.45)	0.87 (0.34, 2.25)	2.38 (0.65, 8.63)
detectable Pb	0.73 (0.31, 1.68)	0.91 (0.41, 2.03)	0.90 (0.39, 2.05)	**3.12 (0.89, 10.97)***
Adjusted [a]				
log_2 Cd	1.55 (0.82, 2.92)	1.02 (0.57, 1.84)	1.08 (0.59, 1.98)	**2.72 (1.09, 6.79)****
log_2 Mn	0.57 (0.19, 1.66)	0.79 (0.29, 2.15)	0.92 (0.33, 2.58)	1.16 (0.28, 4.92)
detectable Pb	0.93 (0.38, 2.29)	0.96 (0.41, 2.21)	0.88 (0.37, 2.09)	**3.71 (0.97, 13.96)****
Adjusted [b]				
log_2 Cd	1.57 (0.83, 2.99)	1.03 (0.57, 1.86)	1.11 (0.60, 2.03)	**2.27 (0.90, 5.70)***
log_2 Mn	0.57 (0.20, 1.67)	0.80 (0.30, 2.16)	0.93 (0.33, 2.63)	1.05 (0.24, 4.61)
detectable Pb	0.95 (0.38, 2.35)	0.97 (0.42, 2.25)	0.91 (0.38, 2.20)	**3.42 (0.88, 13.32)***

[a] Adjusted for infant gender, maternal age, maternal race, maternal BMI, education status

[b] Adjusted for infant gender, maternal age, maternal race, maternal BMI, education status, smoking status during pregnancy

* $p<0.1$; ** $p<0.05$

Figures

Figure 2-1. Analysis strategy.
840 mother-infant pairs recruited in the RICHS study had available demographic information. Neurobehavioral performance was assessed via NNNS after 24 hours of birth for 625 infants (74%), and heavy metal levels were analyzed in 192 placenta samples.

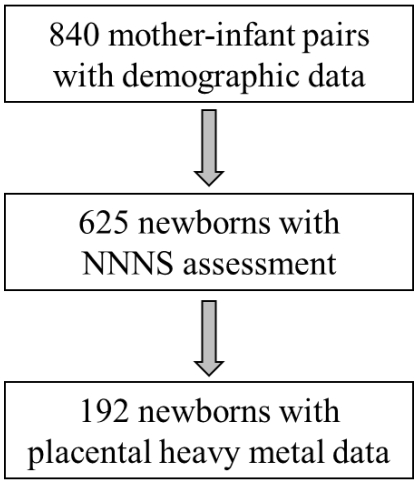


Figure 2-2. Five NNNS profiles (N=625).
NNNS summary scale z-scores across all five latent profiles as indicated via LPA among all newborns in the RICHS study population. Profile 5 (black) demonstrates atypical neurobehavior and Profile 2 (green) represents typical neurobehavioral performance.

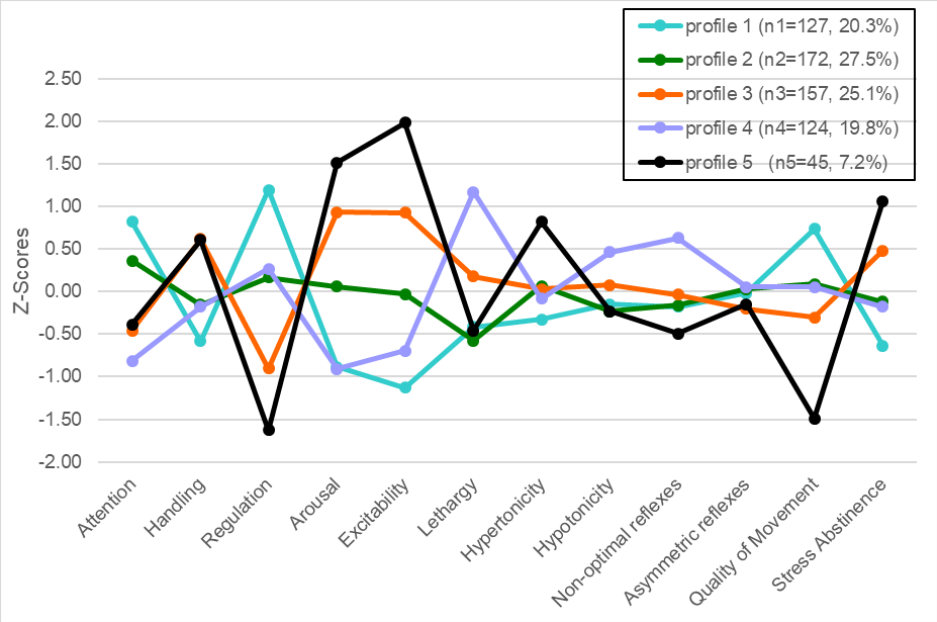
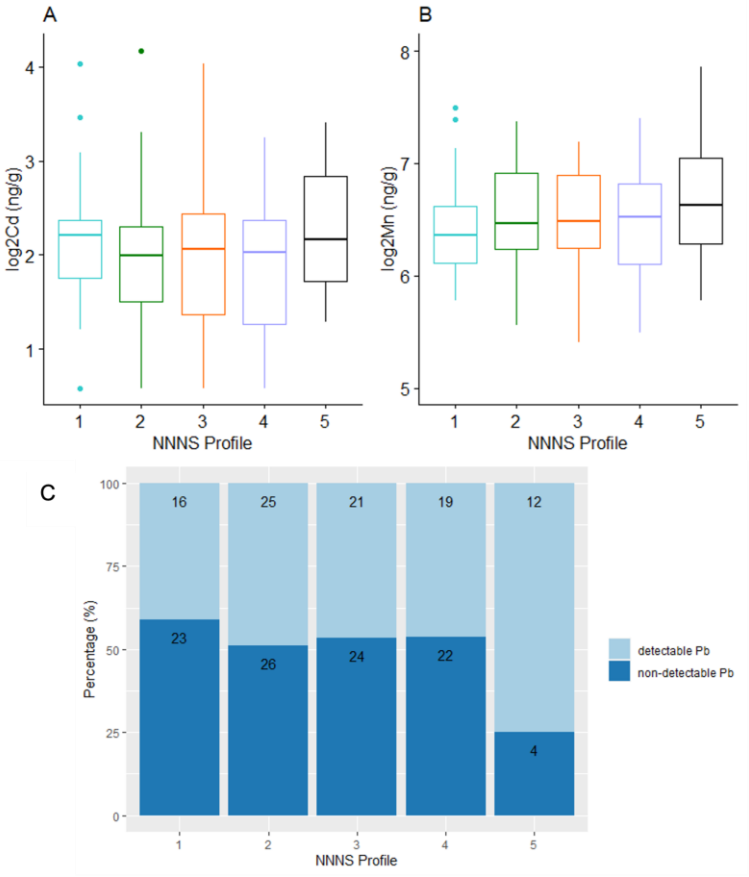


Figure 2-3. Placental heavy metal concentration and distribution across five NNNS profiles. For placental Cd (A) and Mn (B), y-axis shows log_2-transformed concentrations, while the x-axis demonstrates NNNS Profiles 1-5. For placental Pb (C), stacked bar plot represents the percentages of detectable and non-detectable distributions across the five NNNS profiles (x-axis) in the RICHS study population.



Supplemental Material

Supplemental Table 2-1. Descriptive statistics of NNNS summary scales.
Means, standard deviations, minimum and maximum values and percentiles are shown for 12 individual NNNS summary scales.

NNNS	Descriptive statistics					Percentiles						
Assessment	N	Mean	SD	Min.	Max.	5th	10th	25th	50th	75th	90th	95th
Attention	566	4.22	1.31	1.20	7.71	2.29	2.59	3.18	4.14	5.00	6.14	6.54
Handling	616	0.36	0.23	0.00	1.00	0.00	0.13	0.13	0.38	0.50	0.75	0.75
Regulation	621	4.81	0.91	2.31	7.14	3.29	3.60	4.14	4.84	5.42	6.00	6.24
Arousal	625	4.17	0.79	1.86	6.33	3.00	3.14	3.57	4.14	4.86	5.14	5.29
Excitability	625	4.47	2.84	0.00	13.00	1.00	1.00	2.00	4.00	7.00	8.00	9.80
Lethargy	625	6.28	2.50	1.00	14.00	2.20	3.00	4.00	6.00	8.00	10.00	11.00
Hypertonicity	625	0.43	0.80	0.00	5.00	0.00	0.00	0.00	0.00	1.00	1.00	2.00
Hypotonicity	625	0.53	0.76	0.00	7.00	0.00	0.00	0.00	0.00	1.00	1.00	2.00
Non-optimal reflexes	625	5.91	2.11	0.00	11.00	2.00	3.00	5.00	6.00	7.00	9.00	9.00
Asymmetrical reflexes	625	1.62	1.33	0.00	7.00	0.00	0.00	1.00	1.00	2.00	3.00	4.00
Quality of movement	624	4.16	0.66	1.80	5.67	3.00	3.33	3.83	4.17	4.60	5.00	5.17
Stress abstinence	625	0.17	0.07	0.00	0.41	0.06	0.08	0.12	0.16	0.20	0.27	0.29

Supplemental Table 2-2. Model tit statistics from latent profile analysis (LPA).
AIC: Akaike Information Criteria, BIC: Bayesian Information Criteria, BLRT: Bootstrap Likelihood Ratio Test.

Fit statistics	**2-profile**	**3-profile**	**4-profile**	**5-profile**	**6-profile**
Log-likelihood	-8542.06	-8374.50	-8210.51	-8104.95	-8016.77
AIC	17158.12	16849.00	16547.02	16361.90	16211.54
BIC	17322.32	17070.89	16826.60	16699.17	16606.50
Entropy	0.90	0.85	0.84	0.86	0.87
BLRT test	1366.58	335.13	327.98	211.12	176.36
BLRT p-value	0.00	0.00	0.00	0.00	0.00

Supplemental Table 2-3. Means and standard deviations of individual NNNS summary scales across the five NNNS profiles indicated by LPA (N=625).
NNNS: NICU network neurobehavioral scale, LPA: latent profile analysis, ANOVA: analysis of variance.

NNNS Assessment	Profile 1 (n=127, 20.3%)	Profile 2 (n=172, 27.5%)	Profile 3 (n=157, 25.1%)	Profile 4 (n=124, 19.8%)	Profile 5 (n=45, 7.2%)	ANOVA p-value
Attention	5.29 (1.04)	4.69 (1.16)	3.62 (0.97)	3.15 (0.82)	3.71 (1.28)	<.0001
Handling	0.22 (0.16)	0.32 (0.19)	0.50 (0.24)	0.31 (0.22)	0.50 (0.27)	0.000
Regulation	5.89 (0.49)	4.96 (0.44)	4.00 (0.46)	5.05 (0.56)	3.34 (0.56)	<.0001
Arousal	3.47 (0.42)	4.22 (0.44)	4.90 (0.39)	3.45 (0.46)	5.36 (0.43)	0.000
Excitability	1.25 (0.90)	4.38 (1.10)	7.10 (1.12)	2.50 (1.13)	10.11 (1.19)	<.0001
Lethargy	5.24 (1.75)	4.84 (1.78)	6.73 (2.04)	9.20 (1.80)	5.13 (2.56)	<.0001
Hypertonicity	0.17 (0.55)	0.48 (0.86)	0.45 (0.81)	0.36 (0.63)	1.09 (1.16)	0.000
Hypotonicity	0.42 (0.61)	0.36 (0.59)	0.59 (0.70)	0.88 (1.01)	0.36 (0.74)	0.000
Non-optimal reflexes	5.54 (1.69)	5.57 (1.80)	5.84 (2.29)	7.24 (1.85)	4.87 (2.67)	0.000
Asymmetric reflexes	1.60 (1.42)	1.66 (1.30)	1.59 (1.30)	1.69 (1.35)	1.42 (1.27)	0.809
Quality of Movement	4.64 (0.46)	4.22 (0.59)	3.96 (0.54)	4.19 (0.59)	3.18 (0.56)	<.0001
Stress Abstinence	0.12 (0.56)	0.16 (0.07)	0.20 (0.07)	0.16 (0.06)	0.24 (0.06)	<.0001

Supplemental Table 2-4. Placental heavy metal concentrations (ng/g) in the RICHS study population (N=192).

Metal	Mean	Standard Deviation	Minimum	Maximum
Cd	4.56	2.58	1.06	17.99
Mn	95.37	30.08	42.34	231.49
Pb [a]	4.49	3.89	1.50	32.75

[a] Among detectable Pb levels in the placenta

Supplemental Table 2-5. Odds ratio (95% CI) from multinomial regression models. (Reference group: Profiles 1~4).

	Unadjusted	**Adjusted [a]**	**Adjusted [b]**
	Profile 5	Profile 5	Profile 5
log2 Cd	1.65 (0.82, 3.32)	**2.39 (1.03, 5.52)****	1.97 (0.84, 4.58)
log2 Mn	2.72 (0.83, 8.76)	1.45 (0.39, 5.39)	1.31 (0.34, 5.00)
detectable Pb	**3.53 (1.09, 11.36)****	**3.94 (1.15, 13.46)****	**3.60 (1.02, 12.64)****

[a] Adjusted for infant gender, maternal age, maternal race, maternal BMI, education status

[b] Adjusted for infant gender, maternal age, maternal race, maternal BMI, education status, smoking status during pregnancy

* $p<0.1$; ** $p<0.05$

Chapter 3: Effects of prenatal metal mixtures on newborn neurobehavioral performances

Published in a different format as:

Tung, Pei Wen, Amber Burt, Margaret Karagas, Brian P. Jackson, Tracy Punshon, Barry Lester, and Carmen J. Marsit. 2022a. "Prenatal Exposure to Metal Mixtures and Newborn Neurobehavior in the Rhode Island Child Health Study." *Environmental Epidemiology* 6 (1): e194. https://doi.org/10.1097/EE9.0000000000000194

Abstract

Prenatal exposure to metals can affect the developing fetus and negatively impact neurobehavior. The associations between individual metals and neurodevelopment have been examined, but little work has explored the potentially detrimental neurodevelopmental outcomes associated with the combined impact of co-existing metals. The objective of this study is to evaluate prenatal metal exposure mixtures in the placenta to elucidate the link between their combined effects on newborn neurobehavior. This study included 192 infants with available placental metal and NICU Network Neurobehavioral Scale data at 24h-72h age. Eight essential and non-essential metals (cadmium, cobalt, copper, iron, manganese, molybdenum, selenium, zinc) detected in more than 80% of samples were tested for associations with atypical neurobehavior indicated by NNNS using logistic regression and in a quantile g-computation analysis to evaluate the joint association between placental metal mixture and neurobehavioral profiles. Individually, a doubling of placental cadmium concentrations was associated with an increased likelihood of being in the atypical neurobehavioral profile (OR: 2.39, 95% CI [1.05, 5.71]). In the mixture analysis, joint effects of a quartile increase in exposure to all metals was associated with 3-fold increased odds of newborns

being assigned to the atypical profile (OR: 3.23, 95% CI [0.92, 11.36]), with cadmium having the largest weight in the mixture effect. Prenatal exposure to relatively low levels of a mixture of placental metals was associated with adverse newborn neurobehavior. Examining prenatal metal exposures as a mixture is important for understanding the harmful effects of concomitant exposures in the vulnerable populations.

Introduction

Understanding the health impacts of metal exposure during the sensitive developmental periods *in utero* and early in life is imperative; neurodevelopmental processes are underway and both fetuses and newborns are sensitive to even very subtle exposures to potentially toxic metals. The placenta is a crucial organ throughout pregnancy considering its roles of transporting water, gases and nutrients between mother and fetus, regulating the progression of pregnancy, metabolism of endogenous and exogenous factors (Nugent and Bale 2015). Some studies have also documented that the placenta plays an important role in neurodevelopment, as the variety of neurotransmitters produced by the placenta throughout pregnancy stimulate normal fetal brain development (Rosenfeld 2021; Zeltser and Leibel 2011). Therefore, the placenta's part in the associations between prenatal exposure to metals and subsequent developmental outcomes in newborns has garnered attention.

Prevalent exposure to non-essential metals such as cadmium (Cd) and lead (Pb) is concerning as the population is commonly exposed to them through sources such as dietary intake or smoking (ATSDR 2012a; 2012b; K. Kim et al. 2019). Cd exposure has been frequently linked to kidney functions, and emerging evidence has shown that Cd toxicity leads to cognitive deficits in children (ATSDR 2012a; Rodríguez-Barranco et al. 2013; Sanders, Henn, and Wright 2015).

Lead (Pb) is a well-established neurodevelopmental toxicant that can result in neurodevelopmental deficits even at very low levels (Bellinger 2008; ATSDR 2020b). Arsenic (As) and mercury (Hg) are also neurotoxicants known for their negative effects on early development(Rodrigues et al. 2016; Tolins, Ruchirawat, and Landrigan 2014; Y. Kim et al. 2018; Gao et al. 2007; Llop et al. 2012). On the other hand, exposure to essential trace elements or nutrients such as manganese (Mn) and copper (Cu) can also raise concerns. Essential trace nutrients are involved in numerous biological and developmental processes and the human body require them to function properly, yet abnormal levels of such elements have been linked to adverse health outcomes in children. For instance, studies have established respiratory and neurological effects upon Mn exposure, and Mn toxicity has been linked to impaired neurodevelopment in children (ATSDR 2012b; Rodríguez-Barranco et al. 2013; Sanders, Henn, and Wright 2015; Horning et al. 2015; Yu et al. 2014). Lower Cu levels were found in children with attention deficit/hyperactivity disorder when compared to children in the control group (Kiddie et al. 2010).

An abundance of evidence on the impacts of exposure to individual metal on neurodevelopment have provided valuable insights on the importance of regulating metal exposures and protecting vulnerable populations from life-long developmental consequences. With the variety, ubiquity, and persistence of metals in our environment, it is plausible that multiple metals act concurrently, and pose threats to normal development. Metals could share common pathways to disrupt development, such as the generation of reactive oxygen species that lead to oxidative stress, effects on enzyme activities, or impacts to immunological functions, thus even at very low levels, simultaneous exposure to multiple metals can be especially detrimental (Horning et al. 2015; de Burbure et al. 2006; Farina, Aschner, and Rocha 2011; P. Chen, Miah, and Aschner 2016). However, the exact mechanisms of metals' joint effect on neurodevelopment

is unclear and the potential additive and/or protective effects of metals have yet to be thoroughly examined (Claus Henn, Coull, and Wright 2014).

Previous epidemiologic studies usually address metal exposure and neurodevelopment using single metal models or, at most, binary combination of metals, though these traditional approaches can be biased by the limited number of metals evaluated in an analysis. More importantly, as the population may never actually be exposed to only one metal at any given time, it is necessary to investigate multiple metals in one setting to better grasp the magnitude of neurodevelopmental effects upon exposure to co-existing metals. Our study objective is to examine placental metals as a mixture using a recently developed method, quantile g-computation, and evaluate the potential impact of the metal mixture on newborn neurobehavior performance indicated through NICU Network Neurobehavioral Scale (NNNS) latent profiles in the Rhode Island Child Health Study (RICHS) population. We hypothesize that a mixture of both essential and non-essential placental metals can impact neurobehavioral performance of an infant with non-essential elements most strongly contributing to poor performance.

Methods

Study population

Mother-infant pairs in the RICHS study population were recruited from the Women and Infants Hospital of Rhode Island (N=840). Briefly, the objective and design of the RICHS cohort was to understand aberrant fetal growth, thus the study population was oversampled for term infants born large for gestational age (LGA; $\geq 90^{th}$ birth weight percentile) and small for gestational age (SGA; $\leq 10^{th}$ birth weight percentile) based on the Fenton growth chart (Fenton 2003). Adequate for gestational age (AGA) infants were matched to LGA and SGA infants on sex,

maternal age (± 2 years) and gestational age (± 3 days). The study included mothers who were at least 18 years of age and did not have life-threatening medical complications. Eligible infants were born free of life-threatening medical complications or congenital or chromosomal abnormalities. Obstetric medical information was obtained from a structured medical chart review, and demographic, lifestyle and exposure histories were collected from interviewer-based questionnaires. All participants provided written informed consent approved by the Institutional Review Boards at the Women and Infants Hospital and Emory University. For this study, a series of 192 consecutive participants recruited in 2010-2011 with samples collected specifically for placental metals assessment were included.

Metals assessment

Placenta parenchyma tissue was biopsied approximately 2cm from the cord insertion site and free of maternal decidua within 2 hours of delivery. Samples were snap frozen in liquid nitrogen and stored at -80˚C until processed. Laboratory methods of assessing placental metal concentrations have been described previously (Punshon et al. 2016). Placental levels of twenty-four trace elements (aluminum (Al), arsenic (As), calcium (Ca), cadmium (Cd), cobalt (Co), chromium (Cr), copper (Cu), iron (Fe), mercury (Hg), potassium (K), magnesium (Mg), manganese (Mn), molybdenum (Mo), sodium (Na), nickel (Ni), phosphorus (P), lead (Pb), sulfur (S), antimony (Sb), selenium (Se), tin (Sn), uranium (U), vanadium (V), zinc (Zn)) were quantified in 192 samples using standardized ICP-MS protocols at the Dartmouth Trace Elements Analysis laboratory. Of this panel of metals, 14 were detectable in more than 80% of the samples (**Supplemental Table 3-1**). The current study focused on the prenatal exposure to a mixture of potentially toxic metals and micronutrients, and eight metals were included in the following

mixture analyses (Cd, Co, Cu, Fe, Mn, Mo, Se, Zn). A small number of samples showed values below the limit of detection (LOD) for three metals, Cd (9.4%), Co (12.5%) and Mo (2.6%), and these values were substituted with LOD/$\sqrt{2}$. The LOD for placental Cd, Co and Mo were 2.12 ng/g, 2.12 ng/g, and 4.24 ng/g, respectively.

Neurobehavior assessment

NICU Network Neurobehavioral Scales (NNNS) is an assessment initially designed to examine a variety of neurobehavioral performances in drug-exposed and high-risk infants (Lester and Tronick 2004; Lester, Tronick, and Brazelton 2004). A standardized and comprehensive examination of both behavioral and neurologic functioning, NNNS was commonly used for at-risk and preterm infants, and later extended its application to low-risk and term infants in the general population (Fink et al. 2012; Sucharew et al. 2012). Researchers have also established the predictive characteristics of NNNS for developmental outcomes later in life. In a generally healthy, low-risk population like ours, Sucharew et al. (2012) found that NNNS profiles were associated with early neurodevelopmental outcomes indicated through lower motor performance (Sucharew et al. 2012). In the Maternal Lifestyle study, infants from the profile with the least optimal NNNS scores showed several adverse developmental and behavioral outcomes, including lower mental development index scores at ages 1 and 2, more behavioral problems assessed through The Child Behavior Checklist at age 3, and lower IQ at 4.5 years of age (J. Liu et al. 2010).

In the RICHS study population, NNNS was administered by certified psychometrists in 625 newborns (74%) after 24 hours of birth and before discharge (Appleton et al. 2016). We utilized latent profile analysis (LPA) to further categorize the study population into mutually exclusive neurobehavior profiles (J. Liu et al. 2010; Tung et al. 2022b). Based on the NNNS score

patterns, profiles produced through the LPA method had minimized heterogeneity within a profile and maximized heterogeneity across different profiles. There are 13 summary scales in NNNS, but the habituation construct was not assessed for 54.9% of the newborns in RICHS as they would need to be in the required sleep state for assessment, so the LPA method included the remaining 12 summary scores from NNNS and generated five discrete profiles for the RICHS newborns. **Supplemental Figure 3-1** shows the NNNS summary score patterns and descriptions for the five profiles in the RICHS study population. Newborns categorized into Profile 5 demonstrated the most atypical neurobehavior, as the summary scale patterns showed more extreme neurobehavioral performances, such as highest excitability, arousal, hypertonicity and stress signs, along with exhibiting lowest regulation and quality of movement compared to the other profiles (Tung et al. 2022b).

Covariates

Based on previous RICHS studies and literature review, covariates considered for analyses include infant sex, maternal age, maternal race, pre-pregnancy BMI, educational status and smoking status during pregnancy. Infant sex was obtained from medical records. Maternal age and pre-pregnancy BMI were considered as continuous variables. Self-reported maternal race information was dichotomized into *white* or *other,* given the small number of individuals in any of the non-white race/ethnicity groups. Highest educational attainment was also self-reported and was recoded into two groups, *more than high school* or *high school or less*. We also conducted sensitivity analysis to test whether regression results were robust when smoking status during pregnancy was included. Women reported their smoking status during pregnancy and was defined as *smoked at any point during pregnancy* or *no smoking during pregnancy*.

Statistical analysis

Descriptive information on demographic and gestational characteristics were compared between the sub-cohort with available placental metal data (N=192) and those without (N=433) using chi-square tests and t-tests. For the eight metals included in the mixture, the mean, standard deviation, minimum and maximum values, and quartile ranges were assessed.

As described in our previous work, and similar to other research groups, we utilized LPA to generate NNNS profiles with model fit criteria used to determine the ultimate number of profiles (J. Liu et al. 2010; Berlin, Williams, and Parra 2014). Based on these criteria, the 5-profile model showed the best fit.

The association between individual metals and NNNS profiles were assessed with multivariable logistic regression models, controlled for covariates previously mentioned. Based on descriptive analyses and histograms, metals were $\log_2$-transformed for normal distribution in the single metal models. NNNS profiles were further dichotomized into two groups, Profile 5 vs. all the other profiles. Based on the score patterns (**Supplemental Figure 3-1**), Profile 5 showed most of the extreme NNNS summary scores, thus newborns categorized in this profile were considered as having the most atypical neurobehavioral performance in the RICHS study population (Tung et al. 2022b).

As concurrent exposure to metals is likely the norm in the study population, we aimed to further evaluate potential impact of metals as a mixture on newborn neurobehavior. Therefore, the quantile g-computation approach was used to understand the joint association between metal mixture and NNNS profile assignment (atypical Profile 5 vs. all other profiles) in this study.

Previously described in detail in Keil et al. (2020), quantile g-computation is based on the concept of generalized linear model to estimate the impact on the outcome when simultaneously increasing all exposures in the mixture by one quantile (Keil et al. 2020). Adapted from weighted quantile sum regression (WQS), one of the main differences is the assumption of directional homogeneity (Carrico et al. 2015). Unlike WQS, quantile g-computation does not require directional homogeneity, and different exposures within the mixture may contribute oppositely (positively or negatively) to the mixture's impact on the outcome (Keil et al. 2020). With this approach, exposures will first be categorized into quartiles, and then fitted into regression models. Each exposure will be given a positive or negative weight, and weights from all components of the mixture will sum to 1. In the event that directional homogeneity does not apply, both positive and negative weights will sum to 1, and an individual exposure's weight can then be interpreted as the proportion of the positive (or negative) partial effect on the outcome due to the specific component of the mixture (Niehoff et al. 2020). For this study, we report the conditional odds ratio, and also estimate the joint effect of metal mixture on neurobehavior profile assignment with the inclusion of previously mentioned covariates through adjusted quantile g-computation models.

LPA analysis for NNNS profile membership was performed with Mplus version 8.4. All other statistical analyses were conducted using R version 3.5.1.

Results

Demographic and gestational characteristics are shown in **Table 3-1**. Among 192 newborns with available NNNS assessment scores and placental metal data in the RICHS cohort, 91 (47.4%) were female and 73.4% of the mothers were white, with 75.5% reported obtaining some post-high school education. Average gestational age in the sub-cohort was 39.31 weeks, and

the average maternal age was 29.79 years old. Demographic characteristics were similar between the included participants and those without available placental metal information (N=433), although newborns included in this study were heavier averaging 3644 grams.

Box plots for the eight metals (Cd, Co, Cu, Fe, Mn, Mo, Se, Zn) across five NNNS profiles are presented in **Figure 3-1**. Correlations between the metals are shown in **Supplemental Table 3-2**, and the strongest correlation was between Mn and Se (r=0.45).

Apart from Cu and Se, in single metal models, unadjusted logistic regression showed increased odds of belonging to the atypical Profile 5 as placental metal level increases, though the confidence intervals included the null (**Figure 3-2**). In adjusted models, every doubling of placental Cd was associated with increased odds of the newborn belonging to the atypical Profile 5 (OR: 2.39, 95% CI [1.05, 5.71]). On the other hand, with every doubling of placental Cu, we observed decreased odds of newborns belonging to the atypical profile 5 (OR: 0.42, 95% CI [0.05, 2.23]).

Quartile ranges of each metal are presented to provide additional information on the distribution of the metals included in the mixture used in the following quantile g-computation analyses (**Table 3-2**). In the g-computation mixtures analyses (**Table 3-3**), as all metals in the mixture increase by one quartile, we observed increased odds of newborns belonging to the atypical Profile 5 (OR: 3.23, 95% CI [0.92, 11.36]). This approach also demonstrated Cd as the driving factor for the overall positive association between increased levels of all metals as a mixture and atypical neurobehavior, as this metal was assigned the largest positive weight, with Zn, Mn, and Fe following with smaller positive weights. Alternatively, Cu showed the largest negative weight among the metals (**Figure 3-3**). The RICHS study population had a low

percentage of women who smoked during pregnancy, resulting in a very limited number of smokers in each profile (among infants categorized into the atypical Profile 5, only one mother reported smoking during pregnancy). Accordingly, smoking status during pregnancy was included in a sensitivity analysis, and results showed attenuated effects of the overall mixture effect on neurobehavior (OR: 2.14, 95% CI [0.71, 7.61]).

Discussion

Exposure to combinations of metals in our environment is inevitable, hence understanding the joint effects of co-existing metals is critical. Although studies have since elucidated metal-associated health effects in the general population, the potential impacts of multiple placental metals on newborn neurobehavioral performance remain unclear. Additionally, possible protective effects of some essential metals are even less studied. In this study, we observed simultaneously increased levels of eight placental metals as a mixture were associated with increased odds of impaired neurobehavior, which was indicated via membership in an atypical NNNS profile. The "atypical" profile was characterized by infants showing the most signs of arousal, stress/abstinence, excitability and hypertonicity, along with poorer regulation and quality of movement. With the quantile g-computational method, we further identified Cd as the primary metal associated with the "atypical" neurobehavior profile.

Placental levels of the eight metals included in our mixture analyses were generally comparable to what was reported in other study populations. In particular, Cd levels in RICHS would be amongst some of the lowest reported of ranges around the world (average: 4 ng/g), although it should be noted that the studies reported were from 1977-2011, and exhibit a decreasing trend over time (Esteban-Vasallo et al. 2012). While the mean (4.56 ng/g) and interquartile range

(IQR; 2.80-5.38 ng/g) of placental Cd measured in our study was relatively similar to that measured in the INMA Project (mean: 4.45 ng/g; IQR: 2.79-6.49 ng/g), RICHS Mn levels demonstrated a higher mean of 95.37 ng/g (SD ±30.08) and wider range of levels (IQR: 73.66-115.91 ng/g) compared to the INMA Project (mean: 70 ng/g; IQR: 52.50-82.24 ng/g) (Freire et al. 2019; 2018). RICHS placental Cu levels (mean: 0.97 ug/g) were comparable to another Spanish study (mean: 0.97 ug/g), though slightly higher than that measured in the New Hampshire Birth Cohort Study (mean: 0.88 ug/g) (Cerrillos et al. 2019; Kennedy et al. 2020).

Common sources of Cd in the American population are dietary intake and tobacco smoke exposure (ATSDR 2012a; K. Kim et al. 2019). Our study population had a relatively low percentage of self-reported smoking during pregnancy, and we did not obtain dietary information throughout pregnancy from the participants, which represents a limitation of the study. Despite the relatively low concentrations of Cd and low-to-moderate correlations between Cd and other metals included in the mixture, we interpret our results with caution and note that Cd exposure, individually and concurrently with other metals, during the sensitive developmental period *in utero* may lead to adverse effects on neurobehavior.

In agreement with our single-metal findings, several previously published studies also found prenatal Cd exposure to be associated with adverse impacts on neurodevelopment later in life. A Japanese birth cohort study found that maternal blood Cd concentrations negatively affect the postural-motor area of neurodevelopment in 2-year-old boys (Ma et al. 2021). Cd measured in maternal urine was found to be inversely associated with cognitive score in the Spanish INMA Project and children's IQ (verbal, performance and full scale) measured in 5-year-olds in rural Bangladesh (Forns et al. 2014; Kippler et al. 2012). Cord blood Cd also negatively associate with performance IQ in children at 5 years of age in a South Korean study (Jeong et al. 2015). However,

there remains some inconsistencies in the association between prenatal Cd exposure and developmental or behavioral outcomes in newborns or young children across different study populations. The INMA Project did not find a significant association between placental Cd and general cognitive score in preschool children, and no association was observed between maternal blood Cd and behavioral outcomes in children between the ages of 1-8 years old in the HOME study (Freire et al. 2018; W. Yang et al. 2020). Aside from Cd, we observed potential adverse effects of Mn on neurobehavior, which is consistent with some prior studies (Rodríguez-Barranco et al. 2013; Lin et al. 2013). However, we also note the limitation in our assumption of linearity in the mixture analysis, which does not address a U-shaped association between Mn and behavioral outcomes reported in some prior work (Claus Henn et al. 2010; Bhang et al. 2013). Similarly, the potential adverse effect of iron on neurobehavior observed in our study is consistent with several reports, though the exact impact of iron dysregulation on behavioral outcomes warrants further investigation (Tamura et al. 2002; Vaughn, Brown, and Carter 1986; Iglesias, Canals, and Arija 2018).

Difference seen across these studies could be attributable in part to difference in sample type used in exposure assessment. Some metals that readily pass the placental tissue (i.e., Pb and Mn) and would more likely be detected in the fetuses' blood or other tissues, would not be well represented and thus there impact on neurobehavior will be missed in this analysis, while others, such as Cd may be more likely retained in the placenta where they can exert toxic effects (Goyer 1990; Gundacker and Hengstschläger 2012; Wier et al. 1990). Elements like Zn, Cu, Fe and Se that are essential for fetal growth and development are tightly regulated but their trans-placental efficiency is less well understood. Researchers have identified metal-transporters that transport them across the placenta into the fetal system, and found the processes may be affected by

competition of toxic metals like Cd (McArdle et al. 2008; C. Cao and Fleming 2016; Z. Chen et al. 2014; Iyengar and Rapp 2001; Zalups and Ahmad 2003). Using the placenta as a biomarker has its challenges, yet the reflected exposure window and its ability of regulating passage of these elements throughout the gestational period could provide valuable insight on prenatal exposure characteristics.

With the myriad of metals present in the environment, it is challenging to capture all combinations of metals. Researchers have since focused on several trace metals, both toxic and non-toxic, that represent common exposures in the general population and/or are more well-known for their effects on human health. Kim et al. suggested an interaction between blood Cd and Pb with findings of an inverse association with Pb for both mental development index (MDI) and psychomotor development index (PDI) scores among 6-month-old infants with above-median Cd levels (Y. Kim et al. 2013). In the MOCEH study, researchers also explored the association between combined metal exposure (Cd, Hg and Pb) and children's neurodevelopment using the Bayley Scales of Infant Development (BSID). In a Bayesian kernel machine regression (BKMR) analysis, they found a joint effect from late pregnancy Pb and Hg exposures on MDI and PDI scores at 6 months (Shah-Kulkarni et al. 2020). Using the same BKMR approach for mixtures, Valeri et al. observed a negative effect on cognitive scores from joint exposure to As, Mn and Pb (Valeri et al. 2017). Alternatively, Kordas et al. (2015) did not observe an association between metals (blood Pb, hair Cd, Mn and As) and MDI or PDI scores in young children (Kordas et al. 2015).

Our observed overall mixture effect also highlighted Cu and Se to be inversely related to the risk of atypical neurodevelopment. Cu and Se are considered as essential for proper organ functioning and metabolic processes, though abnormal levels can be detrimental to health.

Adequate Cu intake is crucial in forming red blood cells and maintaining normal immune functions (Uriu-Adams et al. 2010). The main source of Cu in the general population is through diet, including vegetables, fruits, cereal, and nuts (Prohaska 2012). Though very uncommon, Cu-associated toxicity can impair numerous biological processes. Studies over the decades have largely focused on Cu deficiency and the associated effects on brain activities, such as Menkes disease, of which infants are subject to developmental disability (Uriu-Adams et al. 2010; Hordyjewska, Popiołek, and Kocot 2014). It is also suggested that Cu-deficient infants are at risk to psychomotor impacts and hypotonia, among many other vessel, bone and skin abnormalities (Uriu-Adams et al. 2010; Kaler 2013). In relation to neuropsychological outcomes, however, there remain discrepancies on the epidemiologic association between prenatal Cu concentrations and neurodevelopment or behavior early in life. For instance, a study demonstrated maternal Cu level adversely impacting the BSID mental scale accessed at 12 months, while in a Polish study, no association was found between prenatal Cu exposure and psychomotor development at 1-2 years of age (Amorós et al. 2019; Polanska et al. 2017). In our single-metal analysis, Cu was the only metal with an OR<1, indicating higher placental Cu level may lower odds of newborns belonging to the atypical NNNS profile. This association was likewise observed in our mixture analysis.

Se is generally regarded as a protective trace element to human health, with studies documenting positive effects on cognitive function and the cardiovascular and immune systems (ATSDR 2003; Pieczyńska and Grajeta 2015; Skröder et al. 2017). Se is also a crucial component of selenoproteins which oversee antioxidant defense mechanisms and protect the neuronal system (Solovyev 2015; Schofield 2017). One of the proposed mechanisms of toxic metals eliciting adverse health impacts is by generating oxidative stress and targeting normal dopamine pathway functions (Domingo-Relloso et al. 2019; Lee et al. 2006). It is possible that as part of the metal

mixture, Se could display antagonistic and anti-oxidant properties, which in turn mitigated neurodevelopmental defects resulted from co-exposure to neurotoxic metals such as Cd and Mn (Kiełczykowska et al. 2018; Schofield 2017). A Chinese study investigating two-way metal interactions found that higher Se levels may be protective towards Mn-induced toxic effects on neurodevelopment (X. Yang et al. 2014). However, further studies of placental metal mixtures that include Se as a component are needed.

By considering the real-life circumstances of concurrent exposures to multiple metals, evaluating metals as a mixture in the sensitive prenatal period indicated potential adverse impacts on newborn neurobehavior. A reasonable motivation in investigating prenatal metal exposure to metal mixtures was to reflect the reality of exposure patterns in the study population. The application of quantile g-computation enabled us to assess exposure mixture-response association, and this method also helps to identify the "bad actor" among the variety of metals present in the environment. With this information, future interventions can be designed to first target and eliminate exposure to the "bad actor" to effectively decrease negative impacts to newborn neurodevelopment. In addition, newborns that are most affected by exposures are identified early and appropriate medical follow-ups and interventions can be implemented to mitigate long term adverse developmental outcomes.

Quantiling of exposure levels when generating quantile g-computation results makes this method insensitive to exposure outliers and in turn reduces outliers' influences on model coefficients. Another advantage of this analysis method was that we were able to assess both directions of associations of metal exposures and neurobehavior. Metals like Cd are toxic and non-essential, while others such as Cu and Se, are essential to normal biological functions, and so the quantile g-computation served as an informative approach to addressing this question. A limitation,

though, is the assumption of linearity. Larger studies which can assess a broader range of exposures would be needed to better examine non-linear associations between metals and these outcomes.

Among the 24 metals accessed in the RICHS placenta samples, only 14 metals were detectable in more than 80% of the samples. Several simultaneously occurring toxic trace elements that were well-known to impact neurodevelopment, such as As, Pb and Hg, were excluded due to high percentages of <LOD. Another factor that may have affected the evaluation of placental metal mixture-neurobehavior association in our study is the relatively low to modest level for all eight metals in the RICHS study population. Although the sub-cohort with available placental metal data is representable to the full RICHS cohort, the small sample size may also affect the precision of model estimates. Coupled with our generally healthy, thus smaller proportion of atypical neurobehavior newborns, it is likely that we lack sufficient power to robustly detect an association between metal mixture and NNNS profiles. Therefore, it is also important for future research to include larger sample sizes, especially if the inherent metal exposure levels are low in the targeted population, to establish any potential neurodevelopmental impacts upon concurrent metal exposures.

Conclusion

In summary, we observed a significant association between placental Cd levels and atypical neurobehavior. As multiple placental metals were jointly investigated as a mixture, we also found the overall mixture effect to demonstrate an increased odds of newborns being assigned to the atypical NNNS profile, with Cd regarded as the driving factor of the mixture's adverse effect on neurobehavioral performance. Investigating prenatal metal exposure as a mixture provided additional insight on the adverse neurobehavior effects elicited from combined metal exposure.

Future analyses are warranted to provide and verify more robust associations between concomitant metal exposures and newborn neurobehavioral outcomes that may have persistent effects later in life.

Tables

Table 3-1. Demographic and gestational characteristics.

Characteristic	*Newborns with NNNS and placental metal data (N=192)*	*Newborns with NNNS and without placental metal data (N=433)*
	n (%)	
Infant Gender		
Female	91 (47.4%)	230 (53.1%)
Male	101 (52.6%)	203 (46.9%)
Birth Weight Category[a]		
SGA	29 (15.1%)	94 (21.7%)
AGA	93 (48.4%)	263 (60.7%)
LGA	70 (36.5%)	76 (17.6%)
Maternal Race		
White	141 (73.4%)	300 (69.3%)
Other	47 (24.5%)	120 (27.7%)
Unknown	4 (2.1%)	13 (3.0%)
Infant Race		
White	123 (64.1%)	285 (65.8%)
Other	64 (33.3%)	137 (31.6%)
Unknown	5 (2.6%)	11 (2.5%)
Maternal Education Status		
No more than high school	47 (24.5%)	119 (27.5%)
Some post-high school[b]	145 (75.5%)	314 (72.5%)
Maternal Smoking Status		
Yes	26 (13.5%)	63 (14.5%)
No	165 (85.9%)	365 (84.3%)
Unknown	1 (0.5%)	5 (1.2%)
	Mean ± SD	
Birth weight (grams)	3644 ± 680.37	3404 ± 644.89
Gestational age (weeks)	39.31 ± 0.95	39.35 ± 0.97
Maternal age (years)	29.79 ± 5.63	29.34 ± 5.43
Maternal BMI (kg/m^2)	27.48 ± 7.14	26.37 ± 6.87

[a] SGA: small for gestational weight; AGA: adequate for gestational weight; LGA: large for gestational weight

[b] Post-high school education included junior college, college or any post graduate schooling education

Table 3-2. Mean levels and quartile ranges for metals included in the mixture (ng/g).

Metal	Mean	Quartile 1	Quartile 2	Quartile 3	Quartile 4
Cd	4.56	1.06 – 2.80	>2.80 – 4.19	>4.19 – 5.38	>5.38 – 17.99
Co	3.65	1.18 – 2.56	>2.56 – 3.36	>3.36 – 4.32	>4.32 – 11.95
Cu	971.72	623.10 – 799.98	>799.98 – 878.90	>878.90 – 1042.90	>1042.90 – 2643.50
Fe (ug/g)	84.93	33.67 – 65.49	>65.49 –81.05	>81.05 – 99.30	>99.30 – 194.03
Mn	95.40	42.34 – 73.66	>73.66 – 89.12	>89.12 – 115.91	>115.91 – 231.49
Mo	6.76	3.57 – 5.85	>5.85 – 6.58	>6.58 – 7.42	>7.42 – 13.04
Se	270.68	174.44 – 247.17	>247.17 – 271.79	>271.79 – 291.22	>291.22 – 384.91
Zn (ug/g)	10.11	5.94 – 8.98	>8.98 – 9.96	>9.96 – 10.92	>10.92 – 23.13

Cd: cadmium; Co: cobalt; Cu: copper; Fe: iron; Mn: manganese; Mo: molybdenum; Se: selenium; Zn: zinc

Table 3-3. Quantile g-computation estimates (odds ratio and 95% CI) for being placed in the atypical Profile 5 for a quartile increase in all metals.

	Unadjusted	**Adjusted** [+]
mixture [a]	2.47 (0.82, 7.40)	3.23 (0.92, 11.36)

[+] Adjusted for infant sex, maternal age, race, BMI, education status
[a] 8 metals: Cd, Co, Cu, Fe, Mn, Mo, Se, Zn

Figures

Figure 3-1. Metal distribution by NNNS profiles.
Levels of the eight placental metals (y-axis) included in the mixture analysis are shown across NNNS Profiles 1-5 (x-axis) in the RICHS study population.

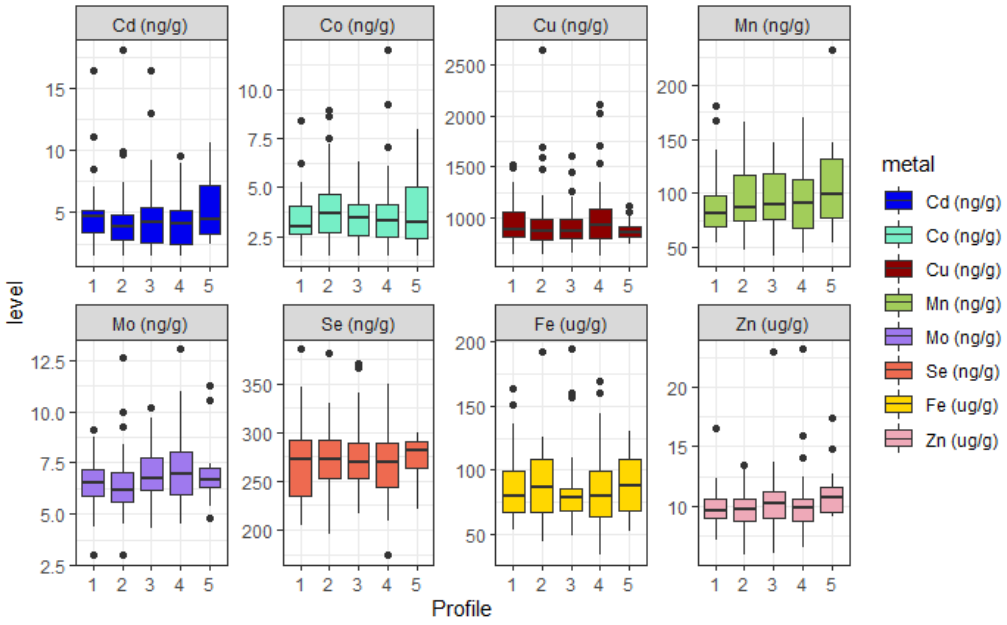


Figure 3-2. Associations between individual metals and neurobehavioral performance indicated through NNNS profiles.
Log_2-transformed levels of eight metals were individually assessed in logistic regression models. Odds ratio and 95% CI indicated the odds of newborns belonging to the atypical Profile 5 with every doubling of placental metal concentration.

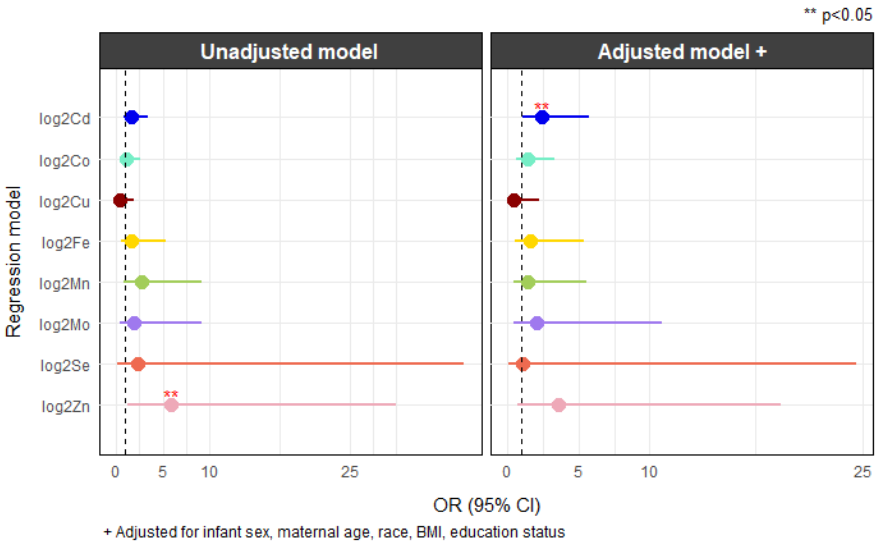
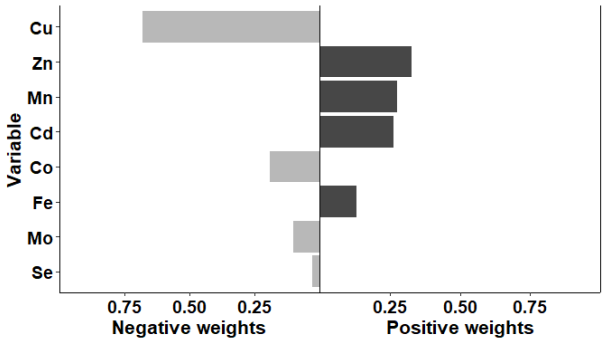
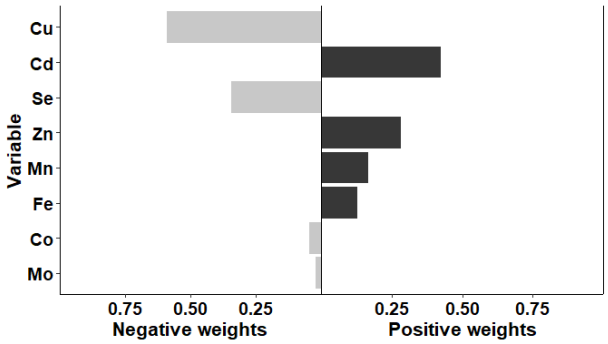


Figure 3-3. Weights for each metal in the quantile g-computation model.
Weights represent the proportion of the positive or negative partial effect for each component (metal) in the mixture on newborn neurobehavior. Shadings of the bars correspond to the overall effect size – the darker colored bars are shown in the positive direction as the overall mixture effect is positive.

Model	OR (95% CI)	Weight distribution
Unadjusted	2.47 (0.82, 7.40)	
Adjusted [+]	3.23 (0..92, 11.36)	

[+] Adjusted for infant sex, maternal age, race, BMI, education status

Supplemental Material

Supplemental Table 3-1. Panel of 24 placental metals analyzed in the RICHS study population (N=192).
14 metals had >80% of samples with >LOD levels. Bolded metals (8) are included in the mixture analysis.

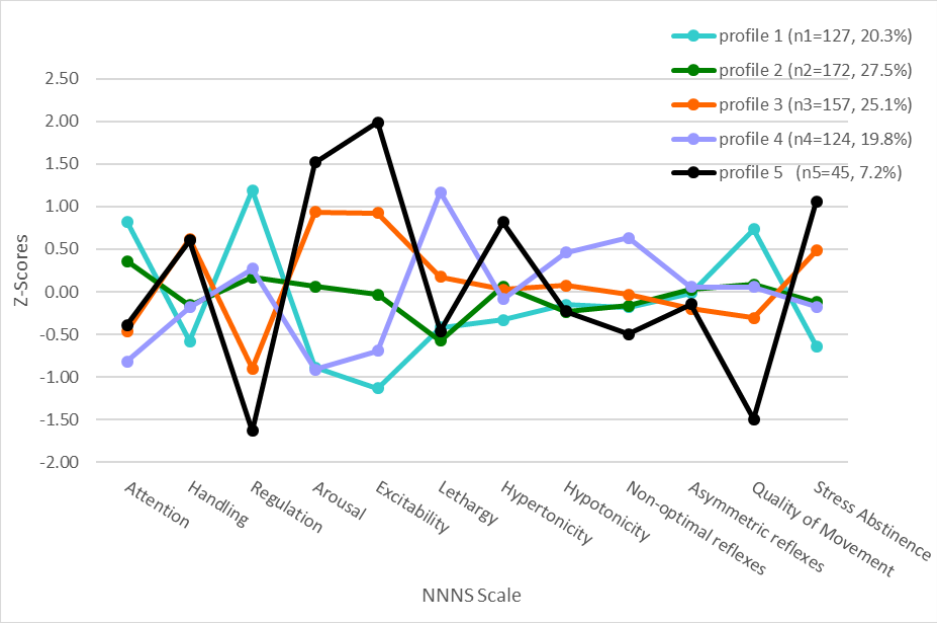
	detection limit (ng/g)	**< LOD**	**(%)**	**> LOD**	**(%)**
Mn	10.61	0	0.00	192	100.00
Na	2.12 (ug/g)	0	0.00	192	100.00
Mg	2.12 (ug/g)	0	0.00	192	100.00
P	21.22 (ug/g)	0	0.00	192	100.00
S	106.09 (ug/g)	0	0.00	192	100.00
K	2.12 (ug/g)	0	0.00	192	100.00
Ca	21.22 (ug/g)	0	0.00	192	100.00
Fe	2.12 (ug/g)	0	0.00	192	100.00
Cu	31.80	0	0.00	192	100.00
Zn	0.42 (ug/g)	0	0.00	192	100.00
Se	4.24	0	0.00	192	100.00
Mo	4.24	5	2.60	187	97.40
Cd	2.12	18	9.38	174	90.63
Co	2.12	24	12.50	168	87.50
Pb	2.12	99	51.56	93	48.44
Sb	2.12	147	76.56	45	23.44
Cr	31.83	155	80.73	37	19.27
As	2.12	175	91.15	17	8.85
Al	424.37	176	91.67	16	8.33
Sn	36.72	183	95.31	9	4.69
Hg	10.61	189	98.44	3	1.56
Ni	31.83	191	99.48	1	0.52
V	21.21	192	100.00	0	0.00
U	2.121	192	100.00	0	0.00

Mn (manganese); Na (sodium); Mg(manganese); P (phosphorus); S (sulfur); K (potassium); Ca (calcium); Fe (iron); Cu (copper); Zn (zinc); Se (selenium); Mo (molybdenum); Cd (cadmium); Co (cobalt); Pb (lead); Sb (antimony); Cr (chromium); As (arsenic); Al (aluminum); Sn (tin); Hg (mercury); Ni (nickel); U (uranium); V (vanadium)

Supplemental Table 3-2. Correlation between placental metals.

	Cd	Co	Cu	Mn	Mo	Se	Fe	Zn
Cd	1.00							
Co	0.23	1.00						
Cu	0.18	0.03	1.00					
Mn	0.10	0.30	0.27	1.00				
Mo	0.20	-0.10	0.02	0.24	1.00			
Se	0.18	0.20	0.35	0.45	0.20	1.00		
Fe	0.09	-0.13	0.12	-0.19	-0.10	0.08	1.00	
Zn	0.22	-0.09	0.01	0.21	0.43	0.36	0.05	1.00

Supplemental Figure 3-1. Five NNNS Profiles (N=625).
All five latent profiles as indicated via latent profile analysis (LPA) among all newborns in the RICHS study population. Profile 5 (black) demonstrates atypical neurobehavior and Profile 2 (green) represents typical neurobehavioral performance. The atypical Profile 5 is characterized by most extreme regulation, arousal, excitability, hypertonicity scores, more non-optimal reflexes, lowest quality of movement and highest stress abstinence signs. The typical Profile 2 is the largest profile, demonstrating overall average behavioral performances, with the exception of scoring lowest in the lethargy scale. Profile 1 newborns showed highest attention, quality of movement and regulation, along with lower stress abstinence signs, less handling, excitability and hypertonicity. Other than requiring more handling, newborns in Profile 3 showed average performance for most of the scales. Profile 4 newborns demonstrated more signs of lethargy, hypotonicity, non-optimal reflexes, and lowest attention and arousal.



Chapter 4: Association of prenatal lead exposure with placental DNA methylation and hydroxymethylation

Adapted version from an original manuscript currently under review:

Prenatal lead (Pb) exposure is associated with differential placental DNA methylation and hydroxymethylation in a human population

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Abstract

Prenatal exposure to lead (Pb) is associated with adverse developmental outcomes and has been linked to epigenetic alterations such as DNA methylation and hydroxymethylation in animal models and in newborn blood. Given the importance of the placenta in fetal development and programing, we sought to examine how prenatal Pb exposure was associated with differential placental DNA methylation and hydroxymethylation and to identify affected biological pathways potentially linked to developmental outcomes. Maternal (n=167) and infant (n=172) toenail and placenta (n=115) samples for prenatal Pb exposure were obtained from participants in a US birth cohort, and methylation and hydroxymethylation data were quantified using the Illumina Infinium MethylationEPIC BeadChip. An epigenome-wide association study was applied to identify differential methylation and hydroxymethylation associated with Pb exposure. Biological functions of the Pb-associated genes were determined by overrepresentation analysis via

ConsensusPathDB. Prenatal Pb quantified from maternal toenail, infant toenail and placenta was associated with 480, 27 and 2 differentially methylated sites ($q<0.05$), respectively. Alternatively, we identified 2, 1 and 14 differentially hydroxymethylated site(s) associated with maternal toenail, infant toenail, and placental Pb, respectively. Significantly overrepresented pathways amongst genes associated with differential methylation and hydroxymetylation ($q<0.10$) included mechanisms pertaining to nervous system and organ development, calcium transport and regulation, and signaling activities. Our results suggest that both methylation and hydroxymethylation in the placenta can be variable based on Pb exposure and that the pathways impacted could affect placental function. More work is needed to better understand the mechanisms underlying these epigenetic effects and their health impacts.

Introduction

Lead (Pb) is an environmental toxicant known to adversely affect human health. With regulatory guidelines in place, the once major sources of Pb, leaded gasoline and lead-based paint, are now lesser concerns as exposure sources. Yet even at low levels, exposure through consumer products, contaminated soil or drinking water through corroding pipes and fixtures, can still pose threats to the population (ATSDR 2020b; Dignam et al. 2019; Nriagu 1990; Lanphear et al. 2005). In addition, exposure to Pb in the sensitive pregnancy window has been shown to lead to numerous adverse health outcomes, including fetal growth restriction, low birth weight, neurodevelopmental deficits, behavior issues, and cognitive and intellectual impairment (Llanos and Ronco 2009; Jelliffe-Pawlowski et al. 2006; Bellinger 2008; Claus Henn et al. 2012; Kordas et al. 2015; Tung et al. 2022b; Canfield et al. 2003; Wasserman et al. 2000). During gestation, the placenta plays a vital role in regulating not only nutrients, but also toxicant transport between the mother and infant.

It also undertakes a wide variety of molecular processes in promoting normal growth and development. A potential mechanism for Pb exposure to affect development and contribute to diseases later in life may be through impacts on placental function in addition to known impacts directly to the fetus.

Epigenetic modifications are heritable changes in the control of gene expression that occur independently from changes in the original DNA sequences. DNA methylation, the addition of a methyl group at the C5 position of the cytosine (5mC), is one of the most assessed epigenetic markers (Weinhold 2006; Relton, Hartwig, and Davey Smith 2015). Emerging studies have suggested altered methylation patterns upon Pb exposure (Dou et al. 2019; S. Wu et al. 2017; Zeng et al. 2019). In a zebrafish model, Pb exposure results in altered DNA methylation and impaired activity of DNA methyltransferases (DNMTs), the enzymes that catalyze the methylation of DNA (Sanchez et al. 2017). Bone Pb level was negatively associated with DNA methylation within LINE-1 elements in umbilical cord blood in human studies (Wright et al. 2010; Pilsner et al. 2009). Pb was also associated with altered DNA methylation status of genes involved in brain development (Senut et al. 2014). With evidence from previous studies, Pb exposure is implied to impact DNA methylation during the sensitive gestational period, and given the critical role of placenta in fetal growth and development, Pb-induced methylation changes may partially facilitate the long-term effects of gestational Pb exposure.

Hydroxymethylation, a more recently identified type of epigenetic modification, is considered as an intermediate of the active demethylation process. With enzymes from the ten-eleven translocation (TET) family, a hydrogen atom at the C5 position of the cytosine is replaced by a hydroxymethyl group, promoting oxidation of 5mC into 5hmC (Dao et al. 2014; Ito et al. 2011; Tahiliani et al. 2009). It is suggested that hydroxymethylation (5hmC) can also be linked to

gene expression regulation as 5hmC is observed in enhancer and gene bodies in embryonic stem cells (Szulwach, Li, Li, Song, Han, et al. 2011; Bachman et al. 2014). Researchers also found that 5hmC is most abundant in the brain and is likely a key factor involved in brain development (Kinney et al. 2011; W. Li and Liu 2011; Santiago et al. 2014). Some studies have considered the highly tissue-specific 5hmC as a stable epigenetic mark, and emerging studies have shown that early mercury exposure was linked to cord blood 5hmC changes, while Pb exposure altered peripheral blood 5hmC profile in candidate genes (Bachman et al. 2014; Cardenas et al. 2017; Rygiel et al. 2021). With its' distinct features from 5mC, identifying epigenome-wide 5hmC pattern changes in the placental in response to Pb toxicity may provide addition mechanistic understandings on developmental effects resulted from early Pb exposure.

The placental epigenome may be subject to epigenetic modifications elicited by metal exposures, such as Pb, and result in disruption in placental functions and adverse birth and developmental outcomes. Given the research gaps in investigating human 5mC and 5hmC profiling in tissues aside from blood, and the well described role of the placenta in fetal development and programing, our study utilized epigenome profiling in the placenta to help elucidate Pb-induced epigenetic alterations during the prenatal window. We hypothesized that prenatal Pb, quantified through maternal and infant toenails thus reflecting prenatal exposure levels, results in differential placenta DNA methylation and hydroxymethylation, and that these Pb-associated epigenetic changes would be associated with biological mechanisms and functions in the placenta that are most relevant to fetal development.

Materials and methods

Study population

This study included mother-infant pairs enrolled in the Rhode Island Child Health Study (RICHS). Mothers at least 18 years of age, without any life-threatening medical conditions, and delivered term (≥37 weeks gestation) singletons that were free of congenital or chromosomal abnormalities were recruited from the Women & Infants Hospital in Providence, Rhode Island. RICHS oversampled for small for gestational age (SGA; lowest 10^{th} percentile) and large for gestational age (LGA; highest 10^{th} percentile) infants, and adequate for gestational age (AGA) infants were matched on sex, maternal age (± 2 years), and gestational age (± 3 days). Anthropometric and clinical data was obtained from structured medical record review, and exposure histories, demographic, and lifestyle information were collected through interviewer-based questionnaires. All participants provided written informed consent under appropriate protocols approved by the Institutional Review Boards at Women and Infants' Hospital and Emory University.

Pb exposure assessment

Following hospital discharge, first toenail clippings from all toes were requested from both mother and infant, and were mailed back to the laboratory in provided envelopes. Pb levels at microgram per gram of toenail were analyzed in the Dartmouth Trace Element Analysis laboratory using established inductively couple plasma mass spectrometry (ICP-MS) protocols as previously described (Punshon et al. 2016).

Placenta samples were collected from all RICHS participants for molecular epigenetic studies, and for a subset of consecutive participants, samples were collected to allow for the assessment of trace metals. For molecular epigenetic studies, placental parenchyma biopsies were collected with 2 hours of delivery, excised at 1-2cm from the umbilical cord insertion site and free

of maternal decidua. Samples were then placed immediately in RNAlater (Life Technology) and stored at 4ºC. After 72 hours, samples were removed from RNAlater and stored at -80ºC until DNA was extracted for further examination. For trace metals analysis, placenta parenchyma samples were collected, flash frozen in liquid nitrogen, and stored at -80ºC in trace element free tubes until analysis. The measurement of placental lead using ICP-MS has been described previously (Punshon et al. 2016). The limit of detection (LOD) for placental Pb was 2.12 ng/g.

DNA methylation and hydroxymethylation

DNA was extracted and quantified from placental samples, and methylation and hydroxymethylation analysis were conducted using standard bisulfite (BS) and oxidative bisulfite (oxBS) modification preparations, respectively, with DNeasy Blood and Tissue Kit (Qiagen, MD, USA), Qubit Fluorometer (Thermo Fisher Scientific Life Sciences) and TrueMethyl oxBS Module (NuGen, CA, USA) following manufacturer's protocols. Normalization and background correction of the BS and oxBS-converted samples were performed with the FunNorm function in R package *minfi*.

Epigenome-wide methylation and hydroxymethylation of CpG sites was assessed in paired samples using the Illumina Infinium® MethylationEPIC BeadChip (Illumina, CA, USA) at Emory University (N=230). To minimize batch effects, samples were randomized as pairs, across multiple batches.

To accurately estimate 5mC and 5hmC in the placental epigenome, we utilized the algorithm as described in detail in Green et al (2016). In brief, maximum likelihood was used to fit the data-generating model that outputs parameters indicating the unmethylated proportion (π_1), 5mC proportion (π_2), and 5hmC proportion (π_3) (Green et al. 2016). The novel approach of this

model is the constraint that disallows negative proportions for the proportion parameters. This method is publicly available from the Comprehensive R Archive Network repository as the OxyBS package (http://cran.r-project.org).

Covariates

A structured, interviewer administered questionnaire was used to collect self-reported variables, while a structured medical abstraction was used to obtain clinical information on infant and mother from the hospital record. Self-reported highest obtained education status was dichotomized into *more than high school* or *high school or* less, and self-reported maternal race was coded as either *white* or *other*, given our predominantly white study population. Maternal age was treated as a continuous variable. From medical records, height and weight were used to calculate mother's pre-pregnancy BMI, and infant sex and birth weight data were also obtained from medical chart abstraction. Additionally, infants with standardized birth weight percentiles (calculated via the Fenton growth chart) below the 10^{th} percentile were categorized into SGA; those between the 10^{th} and 90^{th} percentile were classified as AGA; and those above the 90^{th} percentile were classified as LGA (Fenton 2003).

Statistical analysis

Demographic characteristics of participants with available Pb exposure biomarkers (maternal toenail, infant toenail, placenta) were compared using chi-square and ANOVA tests. Characteristics for the sub-cohort of participants with available EPIC array data (N=230) were also assessed. The mean, standard deviation, minimum and maximum values of Pb were examined across biomarkers.

Epigenome-wide association study (EWAS) methodology was performed to identify differential methylation and hydroxymethylation associated with Pb exposure biomarkers. With R package *limma*, we performed robust linear regressions for each CpG site, regressing the methylation beta-values on $\log_2$-transformed Pb exposure measured through the three biomarkers. Models were adjusted for cell type proportions, utilizing the *planet* package in R, and included are trophoblasts, stromal cells, hofbauer cells, endothelial cells, nucleated red blood cells and syncytiotrophoblast cells, along with previously established covariates including infant sex, maternal age, maternal race to account for potential population stratification, maternal BMI and education status. To account for multiple testing, a false discovery rate (FDR) of 5% was considered as the statistically significant cut point for differentially methylated and hydroxymethylated site(s). Manhattan plots were utilized to demonstrate positions of the statistically significant CpG sites across different biomarkers from EWAS results. Analysis of epigenomic data were performed through R version 3.5.1.

To further ascertain biological functions of the differentially methylated and hydroxymethylated CpG sites, ConsensusPathDB (CPDB) was used for pathway over-representation of the top 250 sites derived from EWAS. CPDB is an online database system that utilizes 12 different source databases to understand different types of human molecular functional interactions. The web interface at http://cpdb.molgen.mpg.de demonstrates resources including protein interactions, gene regulations and signaling and genetic interactions to provide a less biased integration of functional networks and biological pathways. The system calculates an enrichment p-value from the hypergeometric distribution of genes in the user-specified candidate list and the predefined gene set (Kamburov et al. 2009). FDR was used to correct for multiple testing, and a q-value <0.10 was considered as a significantly enriched pathway or gene ontology set.

Results

The analysis strategy of this study is demonstrated in **Figure 4-1**. 230 participants had available paired EPIC array data, and the demographic and gestational characteristics of these participants that also had available Pb exposure data from the three biomarkers, placenta, maternal toenail, and infant toenail are show in **Table 4-1**. Infant gender (female to male ratio is around 1:1) and maternal race (predominantly white) distributions were similar across three subsets. Participants with maternal toenail and infant toenail samples showed similar percentages of AGA infants (57.0% vs. 56.3%), post-high school education (89%), average birth weight (3553g vs. 3552g), and average gestational age (39.46 weeks). Notable difference between the three biomarker subsets was observed in maternal age, with mothers with placental Pb data slightly younger (mean= 30.1 yrs), compared to those with maternal or infant toenail data (mean= 31.8 yrs; $p = 0.003$).

The mean Pb level measured in maternal toenail, infant toenail and placental samples were 0.31 ug/g (interquartile range, IQR: 0.23), 0.97 ug/g (IQR: 0.66), and 3.20 ng/g (IQR: 1.8), respectively. Spearman correlation results between three Pb biomarkers showed the strongest correlation was observed between maternal and infant toenail Pb ($r=0.41$), while correlations between placental Pb and maternal toenail and infant toenail Pb were -0.02 and 0.18, respectively. Histograms showed that Pb levels were right skewed in all three types of biomarkers, and $\log_2$-transformed values were used for the following epigenetics analysis to improve interpretation.

EWAS analysis results at $q<0.05$ are summarized in **Table 4-2** (DNA methylation) and **Table 4-3** (DNA hydroxymethylation). After adjusting for cell type proportions and covariates, we observed 480 differentially methylated sites associated with maternal toenail Pb, with 313

(65.2%) sites revealing decreased methylation levels with increasing Pb concentrations. For infant toenail Pb, 27 sites were differentially methylated and 24 (88.9%) showed decreased methylation levels with increasing Pb. Only the top 10 most robust findings from the toenail Pb EWAS are included in **Table 4-2** (complete lists of differentially methylated CpG sites associated with toenail Pb are shown in **Supplemental Table 4-1** and **Supplemental Table 4-2**). Pb quantified from placenta was associated with decreased placental methylation at two sites. Hydroxymethylation results showed that two (one increased and one decreased) and one (increased hydroxymethylation) differentially hydroxymethylated site(s) were associated with maternal and infant toenail Pb, respectively. Placental Pb results showed 14 differentially hydroxymethylated sites at the statistically significant level, and among them two sites showed decreased hydroxymethylation (**Table 4-3**).

Manhattan plots for all three Pb biomarkers showed the position of the genes annotated to the differentially methylated (**Figure 4-2A-C**) and hydroxymethylated (**Figure 4-2D-F**) sites ($q<0.05$). We observed the most robustly differentially methylated probe in relation to infant toenail Pb, cg15445952 (estimate = -0.0203; p-value = 2.29E-10), was annotated to the *SCUBE1* gene, which was also identified among the differentially methylated sites associated with maternal toenail Pb.

Using genes annotated to the top 250 methylated and hydroxymethylated sites from EWAS in the candidate gene list for ConsensusPathDB, we observed significantly enriched pathways ($q<0.1$) across Pb quantified in maternal toenail, infant toenail and placenta samples. Among the three Pb biomarkers, more pathways and gene ontology (GO) terms were overrepresented among genes annotated from differential methylated and hydroxymethylated sites by infant toenail Pb. Summarized in **Table 4-4** for methylation and **Table 4-5** for hydroxymethylation, we found

overrepresented pathways across the three Pb biomarkers that shared similar biological and functional characteristics. Significant GO terms associated with both maternal and infant toenail Pb include were related to nervous system development (GO:0007399) and calcium ion binding (GO:0005509). Calcium transport and regulation pathways were also identified among placental Pb-associated genes. Multiple pathways involved in development of the brain (cerebellum cortex, hindbrain) and neuron were also linked to infant toenail Pb-associated genes. Signaling pathways and GO terms including Hedgehog and TGF-β superfamily-related (activin binding, SMAD binding) were also overrepresented among genes differentially methylated or hydroxymethylated upon prenatal Pb exposure. The complete list of significantly enriched pathways and GO terms is shown in **Supplemental Tables 4-3 - 4-8**.

Discussion

Our study explored epigenetic modifications associated with prenatal Pb exposure that was measured through maternal and infant toenails and placental tissue in the RICHS study population. We found that Pb measured in all three biomarkers was linked to differential methylation and hydroxymethylation in several CpG sites. Pb exposure quantified through toenail samples resulted in more differentially methylated sites, while placental Pb was linked to higher numbers of differentially hydroxymethylated sites. We also demonstrated various common themes of biological functions and pathways, including 1) nervous system and organ development, 2) calcium regulation and neuronal activity, and 3) signaling pathways such as Hedgehog and TGF-β receptor signaling, that were overrepresented by genes annotated to the differentially methylated and hydroxymethylated sites associated with Pb exposure.

Pb is a well-documented environmental toxicant for its' adverse health effects, including impacts on fetal growth and development, neurodevelopment, and neurologic functions(Bellinger 2008; Claus Henn et al. 2012; Kordas et al. 2015; Canfield et al. 2003). Particularly toxic to the vulnerable children's population due to environmental Pb exposure sources (i.e. surface dust, soil, deteriorating paint chips) and hand-to-mouth activity, this heavy metal has been regulated by government agencies, and CDC has established a reference range upper value of 5 ug/dL for children's blood lead level (BLLs) (ATSDR 2020b). However, the general population is still exposed to Pb, albeit at lower concentrations, through sources such as consumer products, drinking water and diet, as well as remaining contamination of painted surfaces and soil. Compared with other U.S. study populations, the average Pb level measured in infant toenail in this study (0.97 ug/g) was slightly higher than that measured in children's toenails in the Cincinnati Childhood Allergy and Air Pollution Study (CCAAPS) (0.66 ug/g), and the New Hampshire Birth Cohort Study (NHBCS) (0.31 ug/g) (Dantzer et al. 2020; Farzan et al. 2018). The mean placental Pb level observed in RICHS was similar to several epidemiologic studies in non-occupationally exposed settings in the U.S. and around the world, and generally on the lower end among the studies included in a review of placental toxic metals by Esteban-Vasallo et al (Esteban-Vasallo et al. 2012).

A growing body of research has investigated the association between prenatal Pb exposure and differential methylation. Findings from animal studies established a negative association between Pb exposure and nervous tissue DNA methylation levels, and demonstrated Pb interfered with DNA methyltransferase activities, albeit at exposure levels beyond what would be observed in our study (Eid et al. 2016; Schneider, Kidd, and Anderson 2013; Dou et al. 2019). EWAS results from a Project Viva study reported Pb exposure measured in maternal first trimester red blood

cells was associated with lower cord blood methylation level at one CpG site, and in the ELEMENT study group, maternal bone Pb was negatively associated with *Alu* and *LINE-1* methylation in cord blood (Bozack et al. 2021; Pilsner et al. 2009). To the best of our knowledge, this is the first human population study investigating the association between toenail and placental Pb concentration and placental differential DNA methylation and hydroxymethylation. Placental samples provide the concentration of Pb within the placenta at the time of collection and may provide evidence of prenatal Pb exposure levels, given the ability of Pb to transport across the placenta. Maternal toenails, on the other hand, likely reflect a longer-term measure of Pb exposure, while infant toenails may similarly provide a longer-term integrated measure of Pb exposure that reached the fetus during the later course of gestation.

From our hydroxymethylation (5hmC) results, we found in total, a lower number of differentially hydroxymethylated sites associated with Pb exposure, but in contrast to methylation results, placenta Pb was associated with the most differentially hydroxymethylated sites across the three biomarkers. 5hmC levels are highly tissue-specific, with the highest percentage found in brain tissue (Kinney et al. 2011; Globisch et al. 2010; Wen and Tang 2014). In RICHS, patterns of 5hmC were measured in the less well-explored placenta, compared to the comprehensive 5hmC profiling first conducted in brain tissue or embryonic stem cells (Pastor et al. 2011; Richa and Sinha 2014; Wen and Tang 2014). Our finding of a greater number of differentially hydroxymethylated sites associated with placental Pb, compared to differentially methylated sites, may suggest that while Pb readily passes through the placenta and is not as well sequestered in the tissue compared to other toxic metals (i.e., cadmium) (Iyengar and Rapp 2001), Pb toxicity could still result through effects on placental 5hmC dysregulation before the metal is transported from the placenta.

Epigenome-wide association studies on prenatal Pb exposure and placental 5hmC are scarce. In the ELEMENT cohort, Rygiel et al. (2021) found that trimester-specific blood Pb level was associated with gene-specific 5mC and 5hmC measured in peripheral blood (Rygiel et al. 2021). Among the four candidate genes, pre-selected based on their developmental- and neurological-related features, gestational Pb was significantly associated with 5mC in *HCN2* and 5hmC in *NINJ2* across all three trimesters. Similar to the direction of their finding, our study also found a positive association between maternal toenail Pb and placental 5mC in *HCN2*. From our placental 5hmC profiling, cg19692784, annotated to gene *NINJ2*, was not significantly associated with any of the Pb biomarkers. Rygiel et al. also assayed 5mC and 5hmC at *RAB5A* from the RAS oncogene family, but no significant associations with blood Pb were observed in the ELEMENT cohort. We observed a CpG site within the body of a different gene from this oncogene family, *RAB34*, having a negative association between maternal toenail Pb and 5mC (estimate = -0.005; FDR = 0.011).

Due to the different methods for not only delineating Pb exposure, but also 5mC and 5hmC levels in different tissue types, our study results may not be directly comparable to Rygiel et al. (2021), but certainly broadened our understandings on diverse mechanisms of Pb toxicity on epigenetic modifications in the human genome. In RICHS, we did not find significant associations between Pb exposure and the candidate genes explored in the ELEMENT study, however, we identified several Pb-associated genes that were involved in developmental- and neurologic-related pathways and functions (i.e., nervous system development; neuronal differentiation; synapse structure and organization). This expands and adds to the evidence that Pb-elicited differential epigenetic changes during the sensitive gestational period may perturb placental

pathways and functions, in some cases paralleling effects seen in other tissues, and that those impacts on placental function could consequently alter fetal development.

Development of the nervous system and major organs (i.e., brain, cerebellar cortex, and hindbrain) was one of the common themes of overrepresented pathways associated with toenail Pb. As a well-established neurotoxicant that mainly targets the CNS, Pb is known to adversely impact neurodevelopment, behavior, motor activities and cognitive functions in newborns and children, with longitudinal studies establishing persistent effects into early adolescent (Bellinger 2008; Hong et al. 2015; Jianghong Liu et al. 2014; Lanphear et al. 2005; Ris et al. 2004; Mason, Harp, and Han 2014). Given the critical role of the placenta in fetal development and programming, and the activity of a number of pathways also present in the brain, studies have suggested that differentially methylated genes in the placenta act as biomarkers for adverse fetal developmental outcomes (Koukoura, Sifakis, and Spandidos 2012; Vlahos et al. 2019). In this study, the identified developmental pathways significantly enriched for Pb-associated genes provided additional understanding on biological functions affected by placental DNA methylation and may suggest an additional way in which Pb can lead to developmental and behavioral outcomes and phenotypes later in life.

Multiple pathways in relation to calcium homeostasis and transport (calcium ion binding, regulation and release of sequestered calcium ion) and neuronal activities (neuron differentiation and neurogenesis) were also significantly enriched for Pb-associated genes. Pb is known to mimic and compete with calcium, a critical and essential metal required for maintaining regular neuron signaling and activity (Bridges and Zalups 2005; Brini et al. 2014; Kawamoto, Vivar, and Camandola 2012). A study by Lafond et al. showed that maternal blood Pb concentration was significantly linked to a decrease in calcium uptake by placental syncytiotrophoblast cells. Their

results suggest that, in the placenta, Pb exposure has the potential to decrease fetal calcium supply (Lafond et al. 2004). During gestation, high demand of calcium during fetal development can lead to maternal skeletal calcium mobilization, which could also permit the release of Pb sequestered in maternal bone into the circulating system and thus elevate exposures to the placenta and fetus (Goyer 1990; Kovacs and Kronenberg 1997; Téllez-Rojo et al. 2004). Pb-elicited disruptions to fetal calcium homeostasis can lead to alterations in synapse structure and regulation and disruption in neurotransmitter release, which then may result in lasting adverse outcomes later in life such as memory and learning impairments (Kawamoto, Vivar, and Camandola 2012; Neal and Guilarte 2010; Cory-Slechta 1995; Alkondon et al. 1990). Interfering with calcium status in placental cells can also lead to generation of reactive oxygen species (ROS), subsequently reflected by the overrepresentation of programmed cell death and apoptosis pathways related to prenatal Pb exposure in our findings (Ercal, Gurer-Orhan, and Aykin-Burns 2001; Görlach et al. 2015).

Our study also identified overrepresented Hedgehog signaling pathways associated with prenatal Pb exposure. Hedgehog signaling is involved in cell signaling, proliferation and differentiation, and the pathway is also in charge of regulating organ development and patterning during embryogenesis (Sasaki et al. 1999; Briscoe and Thérond 2013; Ingham, Nakano, and Seger 2011). There is limited research on how Pb exposure impacts Hedgehog signaling, however, previous research has linked hedgehog regulators from the GLI family zinc-finger genes, *GLI2* and *GLI3*, to arsenic exposure (Ruiz i Altaba 1997; Fei et al. 2010; J. Kim et al. 2010). *GLI3* gene expression in human placenta was also found to be negatively associated with arsenic exposure in NHBCS (Winterbottom et al. 2015). From our EWAS results, we found prenatal Pb exposure was associated with differential methylation of several Hedgehog pathway associated genes, such as

GLI2, *GLI3*, and *HHAT* (hedgehog acyltransferase), which given the consistency with findings from arsenic exposures could indicate that this pathway is impacted by a number of toxic metals.

We observed the TGF-β signaling pathway was also enriched for Pb-associated gene methylation variation. Transforming growth factor-β (TGF-β) is a major player in central regulator of growth, proliferation, differentiation, and apoptosis (Morikawa, Derynck, and Miyazono 2016; Massagué, Blain, and Lo 2000; David and Massagué 2018). TGF-β superfamily members are also critical regulators in placental development and functions for supporting healthy pregnancies and fetal development.(R. L. Jones et al. 2006) Dysregulated TGF-β members have been implicated in aberrant placental angiogenesis related to fetal growth restriction and preeclamptic pregnancies (Gunatillake et al. 2016; Caniggia et al. 1999; Goumans, Liu, and ten Dijke 2009). A potential mechanism of aberrant TGF-β signaling may be attributed to oxidative stress induced by Pb exposure (Beier et al. 2015; Krstić et al. 2015; R.-M. Liu and Desai 2015; Zuscik et al. 2007). From our EWAS results, genes identified in these pathways (i.e., *SMAD3*, *SMAD9*, *TGFBR2*, *TGFBR3*) were not significantly associated with Pb exposure after FDR adjustment. This may be due to our generally low-to-moderate levels of Pb-biomarkers measured in the study population. Further investigation is needed to better understand the plausible mechanism of dysregulated TGF-β signaling as a consequence of Pb-induced epigenetic modifications on target genes of the pathway.

Although we observed some differential hydroxymethylated sites associated with prenatal Pb exposure, the associations were not as robust as those findings of 5mC. This may be partially explained by the generally low toenail Pb levels, along with low abundance of placental 5hmC and individual variability in 5hmC across our study participants. Alternatively, we took note of the challenges of understanding the stability and role of 5hmC within different tissues and cell types.

As pointed out by a preliminary study looking at 5hmC landscape in RICHS placenta, we are also aware that while 5hmC acts as an intermediate in the demethylation process, it is possible that the measurable levels are stable 5hmC modifications, whereas some degree of 5hmC during the rapid differentiation or reprogramming periods may not be properly detected (Green et al. 2016). Regulation of TET enzymes that catalyze the demethylation process may be indirectly affected by metal exposures such as Pb. It is suggested that Pb-associated oxidative stress increased TET activity through the accumulation of TET enzyme cofactor alpha ketoglutarate (α-KG), at least partially contributes to increased 5hmC upon Pb exposure (Dao et al. 2014; Chia et al. 2011; Tretter and Adam-Vizi 1999). This aligned with our finding that among the differentially hydroxymethylated sites associated with placental Pb, 12 out of 14 sites showed increased 5hmC levels. Future studies are required to investigate more about epigenome-wide 5hmC pattern changes in response to Pb and other toxicant exposures, and to elucidate how such changes may affect biological mechanisms, mediate regulatory responses in genes, and contribute to disease progression.

A strength of this study is utilizing data prepared with the algorithm that calculated and prevented any of the 5mC, 5hmC or unmethylated proportions to be below 0, refining our 5hmC profiling in the RICHS placental epigenome, as previous approaches to 5hmC estimation may result in negative numbers due to the subtraction method (Green et al. 2016). Another advantage of this study is leveraging 5mC and 5hmC data quantified through placental tissue in a human population, which provided a more analogous interpretation on prenatal Pb toxicity and epigenetic dysregulation in the sensitive developmental window. Existing literature on how toxicant exposure alters 5hmC levels mainly focused on animal models, with few studies exploring 5hmC levels in human tissue such as stem cells or blood (Cardenas et al. 2017; Sen et al. 2015). Although brain

tissue is of particular interest when researchers assess Pb toxicity, utilizing noninvasive and surrogate tissues like the placenta captures information unique to the pregnancy period. Given the placenta serves as an interface for toxicant regulation between the mother and infant during the gestation period, we suggest that perturbation to the placental epigenome by prenatal Pb exposure likely led to impaired cellular functions and resulted in adverse developmental outcomes.

Conclusion

In summary, our study shows that prenatal exposure to Pb, a known developmental toxicant, is associated with differential placental DNA methylation and hydroxymethylation, and demonstrates Pb-induced epigenetic changes are related to multiple biological pathways and functions, including developmental, calcium transport and regulation, and signaling activities. With our placental epigenetic profiling, we provide evidence that not only DNA methylation but also hydroxymethylation, may serve as potential response markers to environmental toxicant exposures during the gestation period.

Tables

Table 4-1. Demographic and gestational characteristics for subsets with different Pb biomarkers in the RICHS study population.

Characteristic	*With available Pb exposure data in*		
	Maternal toenail (N=172)	***Infant toenail (N=167)***	***Placenta (N=115)***
	n (%)		
Infant Gender			
Female	87 (50.6%)	84 (50.3%)	54 (47.0%)
Male	85 (49.4%)	83 (49.7%)	61 (53.0%)
Birth Weight Category[a]			
SGA	28 (16.3%)	28 (16.8%)	19 (16.5%)
AGA	98 (57.0%)	94 (56.3%)	53 (46.1%)
LGA	46 (26.7%)	45 (26.9%)	43 (37.4%)
Maternal Race			
White	134 (77.9%)	132 (79.0%)	83 (72.2%)
Other	32 (18.6%)	29 (17.4%)	31 (27.0%)
N/A	6 (3.5%)	6 (3.6%)	1 (0.9%)
Infant Race			
White	128 (74.4%)	126 (75.4%)	74 (64.3%)
Other	38 (22.1%)	35 (21.0%)	39 (33.9%)
N/A	6 (3.5%)	6 (3.6%)	2 (1.8%)
Maternal Education Status			
No more than high school	18 (10.5%)	18 (10.8%)	23 (20.0%)
Some post-high school	154 (89.5%)	149 (89.2%)	92 (80.0%)
		Mean ± SD	
Birth weight (grams)	3553 ± 643.47	3552 ± 648.09	3610 ± 703.41
Gestational age (weeks)	39.46 ± 0.93	39.46 ± 0.94	39.26 ± 0.97
Maternal age (years)	31.75 ± 4.16	31.78 ± 4.12	30.10 ± 5.46
Maternal BMI (kg/m^2)	26.58 ± 6.42	26.61 ± 6.35	26.48 ± 6.48

[a] SGA: small for gestational weight; AGA: adequate for gestational weight; LGA: large for gestational weight

Table 4-2. Epigenome-wide association study results for differentially methylated sites (q < 0.05) associated with Pb quantified in maternal toenail, infant toenail and placenta samples.

Probe	Position	Gene	Estimate	SE	p-value	q-value
Maternal toenail[+]						
cg16285217	chr2:20095489		-0.0022	0.0696	7.57E-09	0.0053
cg11374425	chr3:62359677	*FEZF2*	0.0033	0.1025	7.20E-08	0.0101
cg10012394	chr5:63461371	*RNF180*	0.0114	0.3406	1.95E-08	0.0063
cg06821993	chr8:114449011	*CSMD3*	0.0322	1.0060	4.70E-08	0.0082
cg19902005	chr11:57545678	*TMX2-CTNND1; CTNND1*	0.0089	0.2929	1.81E-07	0.0114
cg06090833	chr14:32288033	*NUBPL*	-0.0047	0.1652	1.89E-07	0.0114
cg00049033	chr16:49317080	*CBLN1*	0.0251	0.8204	1.33E-07	0.0114
cg10018294	chr17:911609	*ABR*	-0.0080	0.2470	2.69E-08	0.0063
cg04295815	chr17:27045188	*RAB34*	-0.0054	0.1752	1.04E-07	0.0114
cg21667047	chr20:9495726	*LAMP5-AS1; LAMP5*	0.0207	0.7004	1.99E-07	0.0114
Infant toenail[+]						
cg10814131	chr1:203009651	*PPFIA4*	-0.0087	0.3359	1.23E-07	0.0108
cg11365072	chr5:154392274	*KIF4B*	-0.0031	0.1162	3.53E-08	0.0058
cg10299585	chr12:54321717		0.0147	0.5599	4.95E-08	0.0058
cg07136023	chr16:86537316		0.0137	0.5140	4.70E-08	0.0058
cg09422806	chr19:3364015	*NFIC*	-0.0149	0.5936	2.12E-07	0.0148
cg04640975	chr19:3464991		-0.0129	0.4600	9.39E-09	0.0033
cg02859421	chr19:3465071		-0.0091	0.3503	7.02E-08	0.0070
cg13822446	chr19:46235676	*BHMG1*	-0.0042	0.1673	1.74E-07	0.0135
cg03359161	chr20:39191619		-0.0020	0.0730	4.46E-08	0.0058
cg15445952	chr22:43653574	*SCUBE1*	-0.0203	0.6330	2.29E-10	0.0002
Placenta						
cg04773990	chr6:32820410	*TAP1*	-0.0039	0.0553	2.78E-11	<0.0001
cg09465791	chr20:19633301	*SLC24A3*	-0.0106	0.1867	4.55E-08	0.0159

[+] Top 10 sites are shown.

Table 4-3. Epigenome-wide association study results for differentially hydroxymethylated sites (q < 0.05) associated with Pb quantified in maternal toenail, infant toenail and placenta samples.

Probe	Position	Gene	Estimate	SE	p-value	q-value
Maternal toenail						
cg15320238	chr16:51091402		-0.0027	0.0797	1.14E-08	0.0079
cg15591645	chr19:46147397	*EML2*	0.0052	0.1737	1.21E-07	0.0417
Infant toenail						
cg20800997	chr21:47018175		0.0036	0.1269	7.16E-09	0.0049
Placenta						
cg09869950	chr1:59783505	*FGGY*	0.0060	0.1274	8.40E-07	0.0441
cg11036421	chr1:61510287		0.0100	0.1869	4.94E-07	0.0401
cg25805115	chr1:81964207		-0.0128	0.2356	1.29E-07	0.0295
cg19519828	chr1:206664973	*IKBKE*	0.0027	0.0457	1.38E-08	0.0062
cg25195477	chr3:148847134	*HPS3*	0.0005	0.0093	6.79E-07	0.0401
cg25687585	chr5:42756950	*CCDC152*	0.0075	0.1468	2.48E-07	0.0401
cg13694662	chr9:140214551	*EXD3*	-0.0107	0.2136	3.76E-07	0.0401
cg26252498	chr10:34865917	*PARD3*	0.0063	0.1237	5.57E-07	0.0401
cg16119628	chr10:88282035	*WAPAL*	0.0005	0.0106	8.98E-07	0.0441
cg09847717	chr11:126318677	*KIRREL3*	0.0049	0.0891	7.01E-07	0.0401
cg15574100	chr12:27168121	*TM7SF3*	0.0048	0.0947	4.69E-07	0.0401
cg19704558	chr17:35296732	*LHX1*	0.0069	0.1187	1.81E-08	0.0062
cg05279901	chr19:42498662	*ATP1A3*	0.0024	0.0466	6.34E-07	0.0401
cg24118151	chr20:42354839	*GTSF1L*	0.0048	0.0856	3.36E-07	0.0401

Table 4-4. Significant pathways and gene ontology (GO) terms (q < 0.10) with an overrepresentation of differentially methylated genes associated with Pb.
Summary of significantly enriched pathways and GO terms involved in neuronal, developmental, and cellular signaling processes. Candidate gene set comprised of genes annotated to the top 250 differentially methylated CpGs by Pb quantified in maternal toenail, infant toenail and placenta.

Pathway ID / GO Term	Pathway description / Term name
Maternal toenail	
GO:0005509	calcium ion binding
GO:0043169	cation binding
GO:0046872	metal ion binding
GO:0007399	nervous system development
Infant toenail	
R-HSA-1266738[a]	Developmental Biology
R-HSA-5610787[a]	Hedgehog, off, state
R-HSA-5358351[a]	Signaling by Hedgehog
R-HSA-5635851[a]	GLI proteins bind promoters of Hh responsive genes to promote transcription
path:hsa04340[b]	Hedgehog signaling pathway - Homo sapiens (human)
GO:0005509	calcium ion binding
GO:0007399	nervous system development
GO:0048856	anatomical structure development
GO:0021695	cerebellar cortex development
GO:0048731	system development
GO:0021549	cerebellum development
GO:0030902	hindbrain development
GO:0030182	neuron differentiation
GO:0048666	neuron development
GO:0022008	neurogenesis
GO:0007507	heart development
GO:0048568	embryonic organ development
Placenta	
GO:0051279	regulation of release of sequestered calcium ion into cytosol
GO:0008528	G protein-coupled peptide receptor activity
GO:0070410	co-SMAD binding
GO:0010522	regulation of calcium ion transport into cytosol
GO:0051282	regulation of sequestering of calcium ion

[a] Pathway source: Reactome; [b] Pathway source: KEGG

Table 4-5. Significant pathways and gene ontology (GO) terms (q < 0.10) with an overrepresentation of differentially hydroxymethylated genes associated with Pb.
Summary of significantly enriched pathways and GO terms involved in neuronal, developmental, and cellular signaling processes. Candidate gene set comprised of genes annotated to the top 250 differentially hydroxymethylated CpGs by Pb quantified in maternal toenail, infant toenail and placenta.

Pathway ID / GO Term	Pathway description / Term name
Maternal toenail	
GO:0050807	regulation of synapse organization
GO:0050803	regulation of synapse structure or activity
Infant toenail	
WP4816[a]	TGF-beta receptor signaling in skeletal dysplasias
path:hsa05220[b]	Chronic myeloid leukemia - Homo sapiens (human)
WP560[a]	TGF-beta Receptor Signaling
GO:0004674	protein serine/threonine kinase activity
GO:0034673	inhibin-betaglycan-ActRII complex
GO:0048185	activin binding
GO:0004675	transmembrane receptor protein serine/threonine kinase activity
GO:0046332	SMAD binding
Placenta	
GO:0035904	aorta development

[a] Pathway source: Wikipathways; [b] Pathway source: KEGG

Figures

Figure 4-1. Analysis strategy.
RICHS study population comprised of 840 mother-infant pairs with demographic information, and 230 participants had available EPIC array data. Participants with Pb exposure data quantified from different biomarkers are included in this study: maternal toenail (N=172), infant toenail (N=167), and placenta (N=115).

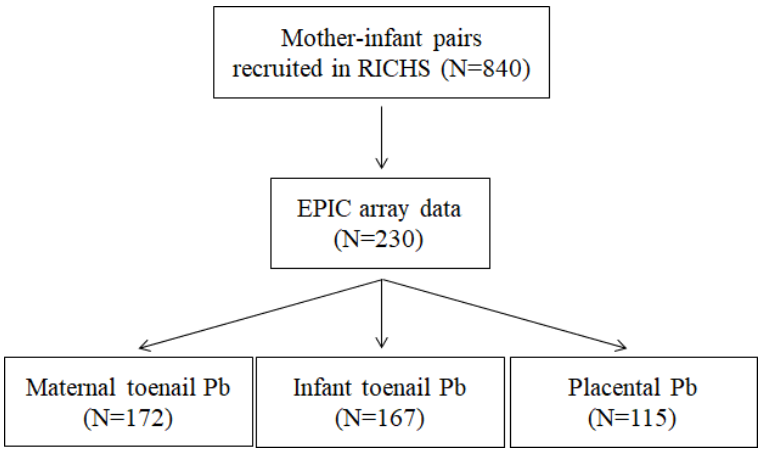
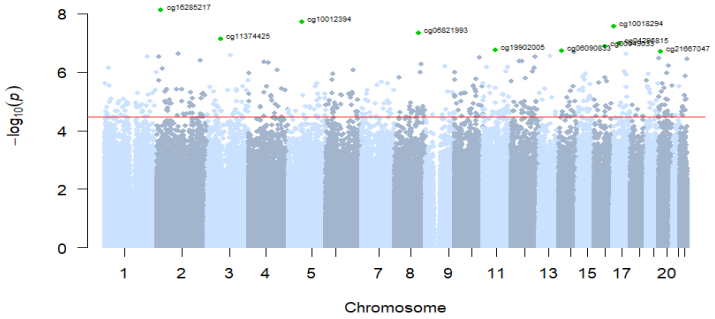
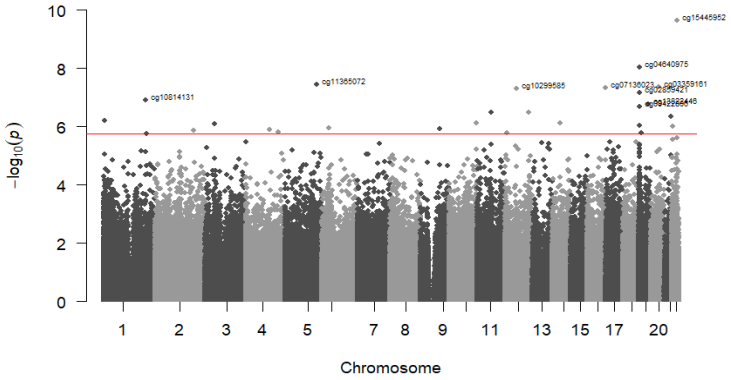


Figure 4-2. Manhattan plots showing the positions of the differentially methylated and hydroxymethylated CpG sites (q < 0.05).
X-axis represents the genomic location of the probes and the y-axis represents -$\log_{10}$(p-value) for the CpG sites associated with Pb biomarkers: maternal toenail [(A)$^+$ and (D)], infant toenail [(B)$^+$ and (E)], and placenta [(C) and (F)]. FDR cutoff is indicated as the horizontal red line. $^+$ Top 10 sites are labeled.

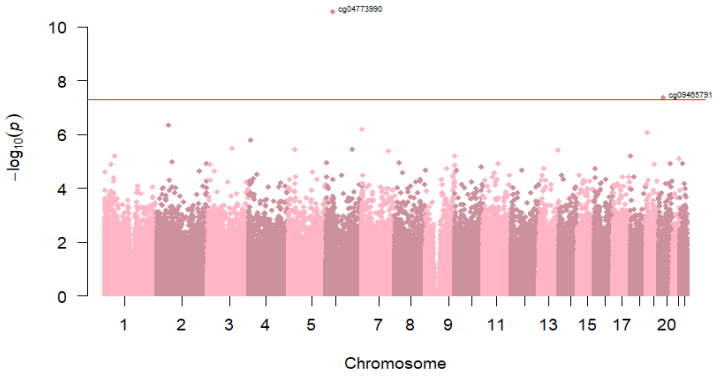
(A)$^+$



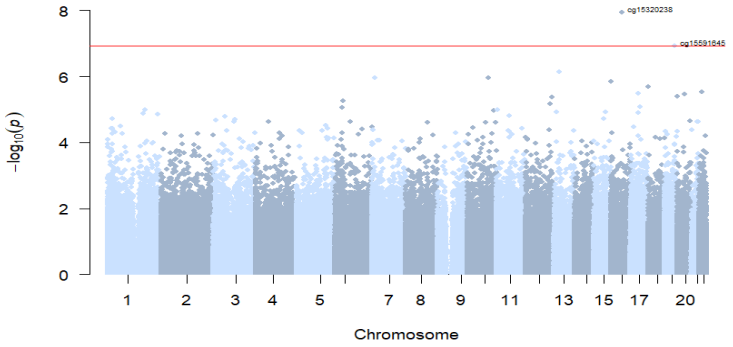
(B)$^+$



(C)



(D)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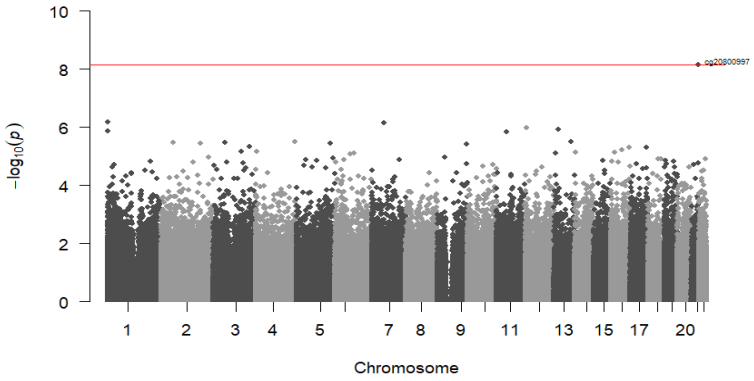
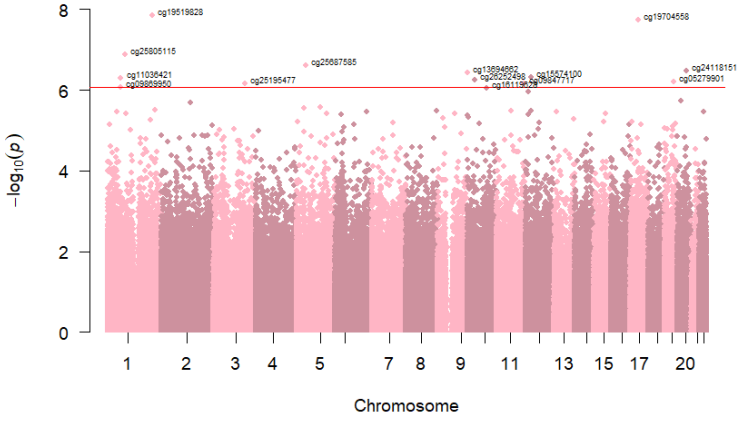
cg15591645
8
6
4
2
0
−log10(p)
1
2
3
4
5
7
8
9
11
13
15
17
20
Chromosome

(E)


cg20800997
10
8
6
4
2
0
−log10(p)
1
2
3
4
5
7
8
9
11
13
15
17
20
Chromosome

(F)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cg19704558
cg25805115
cg25687585
cg11036421
cg09869950
cg25195477
cg15574100
cg24118151
cg05279901
8
6
4
2
0
−log10(p)
1
2
3
4
5
7
8
9
11
13
15
17
20
Chromosome

Supplemental Material

Supplemental Table 4-1. Differentially methylated sites (q < 0.05) associated with maternal toenail Pb.

Probe	Position	Gene	Estimate	SE	p-value	q-value
cg16285217	chr2:20095489		-0.0022	0.0696	7.57E-09	0.0053
cg10012394	chr5:63461371	RNF180	0.0114	0.3406	1.95E-08	0.0063
cg10018294	chr17:911609	ABR	-0.0080	0.2470	2.69E-08	0.0063
cg06821993	chr8:114449011	CSMD3	0.0322	1.0060	4.70E-08	0.0082
cg11374425	chr3:62359677	FEZF2	0.0033	0.1025	7.20E-08	0.0101
cg04295815	chr17:27045188	RAB34	-0.0054	0.1752	1.04E-07	0.0114
cg00049033	chr16:49317080	CBLN1	0.0251	0.8204	1.33E-07	0.0114
cg19902005	chr11:57545678	TMX2-CTNND1; CTNND1	0.0089	0.2929	1.81E-07	0.0114
cg06090833	chr14:32288033	NUBPL	-0.0047	0.1652	1.89E-07	0.0114
cg21667047	chr20:9495726	LAMP5-AS1; LAMP5	0.0207	0.7004	1.99E-07	0.0114
cg16943230	chr14:91932755	PPP4R3A	-0.0035	0.1329	2.16E-07	0.0114
cg18923673	chr17:59724605		-0.0055	0.2055	2.32E-07	0.0114
cg13723230	chr2:103465190		0.0035	0.1072	2.43E-07	0.0114
cg22146131	chr3:109215086		-0.0089	0.3258	2.66E-07	0.0114
cg08362982	chr13:74535677	KLF12	-0.0024	0.0832	2.83E-07	0.0114
cg01331275	chr2:9908576		-0.0052	0.1760	3.03E-07	0.0114
cg03461704	chr1:205818484	PM20D1	-0.0080	0.2745	3.05E-07	0.0114
cg02705918	chr12:114087236		0.0050	0.1665	3.07E-07	0.0114
cg16496322	chr10:116729482	TRUB1	-0.0032	0.1197	3.10E-07	0.0114
cg24596259	chr20:1511125		-0.0166	0.5821	3.36E-07	0.0118
cg09357255	chr22:50739893	PLXNB2	-0.0054	0.1819	3.66E-07	0.0122
cg10663482	chr2:208124866		-0.0040	0.1373	3.96E-07	0.0125
cg21396441	chr12:53491214	IGFBP6	0.0007	0.0228	4.20E-07	0.0125
cg10618461	chr12:39535355		-0.0033	0.1212	4.28E-07	0.0125
cg11209631	chr4:72204651	SLC4A4	-0.0112	0.3995	4.47E-07	0.0125
cg06216505	chr4:95321549		-0.0119	0.4415	4.67E-07	0.0126
cg07454113	chr8:125498222	RNF139	-0.0015	0.0513	5.34E-07	0.0139
cg04146241	chr20:44884711	CDH22	-0.0086	0.3001	6.02E-07	0.0151
cg11893539	chr9:95187249	OMD; CENPP	-0.0048	0.1824	6.42E-07	0.0155
cg06720945	chr11:16834902	PLEKHA7	-0.0037	0.1315	6.81E-07	0.0155
cg24590524	chr11:118023353	SCN4B	0.0042	0.1417	6.91E-07	0.0155
cg27189424	chr1:17914734	ARHGEF10L	0.0041	0.1403	7.25E-07	0.0155
cg09625338	chr12:111022506	PPTC7	0.0100	0.3530	7.31E-07	0.0155
cg22313606	chr2:26943931	KCNK3	-0.0039	0.1447	7.75E-07	0.0160
cg14942437	chr4:139177576		-0.0007	0.0276	8.44E-07	0.0168

cg18884295	chr6:131234267	EPB41L2	-0.0055	0.1970	8.61E-07	0.0168
cg15641657	chr12:103344564		0.0130	0.4723	9.44E-07	0.0168
cg01584174	chr17:54916198	DGKE	-0.0073	0.2773	9.57E-07	0.0168
cg19814100	chr11:111322009		-0.0074	0.2636	9.66E-07	0.0168
cg23458989	chr10:433254	DIP2C	-0.0042	0.1473	9.97E-07	0.0168
cg21260512	chr8:121549533	SNTB1	-0.0010	0.0388	1.01E-06	0.0168
cg12466022	chr1:244777505	C1orf101	-0.0011	0.0390	1.01E-06	0.0168
cg09113613	chr20:37101903	RALGAPB	0.0010	0.0351	1.04E-06	0.0170
cg14565059	chr4:3355126	RGS12	-0.0008	0.0304	1.08E-06	0.0172
cg09497409	chr19:8275083	LASS4	-0.0037	0.1350	1.12E-06	0.0172
cg18797229	chr6:33267523	TAPBP; RGL2	0.0014	0.0484	1.13E-06	0.0172
cg02972064	chr15:57544255	TCF12	-0.0045	0.1803	1.16E-06	0.0173
cg26924979	chr17:63175961	RGS9	-0.0053	0.1944	1.22E-06	0.0178
cg17246765	chr22:43619731	SCUBE1	-0.0229	0.8118	1.38E-06	0.0197
cg05110632	chr15:44673032	CASC4	-0.0020	0.0778	1.49E-06	0.0208
cg00317355	chr16:49308483		0.0213	0.7539	1.53E-06	0.0209
cg20815371	chr8:22311197	PPP3CC	-0.0028	0.1031	1.56E-06	0.0209
cg01078378	chr12:69634480	CPSF6	0.0018	0.0687	1.58E-06	0.0209
cg04476927	chr10:102489929		0.0078	0.2752	1.73E-06	0.0222
cg16012719	chr10:102985022		0.0041	0.1530	1.77E-06	0.0222
cg04825276	chr10:529387	DIP2C	-0.0052	0.1913	1.84E-06	0.0222
cg20070464	chr15:55666568	MIR628; CCPG1	-0.0012	0.0472	1.85E-06	0.0222
cg10443518	chr6:26999326		-0.0013	0.0494	1.85E-06	0.0222
cg25807199	chr3:193345871	OPA1-AS1; OPA1	-0.0026	0.1087	1.90E-06	0.0222
cg04605388	chr18:77748761	TXNL4A	0.0009	0.0304	1.90E-06	0.0222
cg04850254	chr2:8722072		-0.0022	0.0786	1.94E-06	0.0223
cg04557383	chr16:56703568	MT1H	0.0060	0.2162	2.01E-06	0.0227
cg27201750	chr12:2781468	CACNA1C-AS2; CACNA1C	-0.0035	0.1267	2.06E-06	0.0229
cg19643442	chr7:95705636	DYNC1I1	0.0011	0.0455	2.13E-06	0.0233
cg06579338	chr16:20814739	ERI2	-0.0016	0.0655	2.30E-06	0.0242
cg08351203	chr19:53141793	ZNF83	0.0007	0.0244	2.32E-06	0.0242
cg24057514	chr11:2293181	ASCL2	0.0088	0.3286	2.38E-06	0.0242
cg16022081	chr1:2564329	MMEL1	-0.0108	0.3893	2.41E-06	0.0242
cg26818820	chr5:135468000	SMAD5	-0.0126	0.4559	2.44E-06	0.0242
cg27582912	chr20:18326596		-0.0036	0.1386	2.45E-06	0.0242
cg10330371	chr7:122527499	CADPS2	0.0009	0.0335	2.46E-06	0.0242
cg26853340	chr15:81409649		-0.0063	0.2410	2.64E-06	0.0257
cg21828319	chr7:73645966	RFC2	-0.0012	0.0448	2.73E-06	0.0260
cg21954572	chr1:20511920	UBXN10; UBXN10-AS1	-0.0050	0.1905	2.87E-06	0.0260
cg12757181	chr10:88025053	GRID1; MIR346	-0.0008	0.0303	2.94E-06	0.0260
cg08589418	chr20:61993913	CHRNA4	-0.0042	0.1606	3.00E-06	0.0260

cg23114881	chr14:88851550	SPATA7	0.0014	0.0541	3.01E-06	0.0260
cg25983553	chr10:130339134		0.0140	0.5235	3.05E-06	0.0260
cg03139244	chr3:168762673		-0.0027	0.1073	3.06E-06	0.0260
cg26741373	chr21:44585506		-0.0063	0.2292	3.08E-06	0.0260
cg08117431	chr19:5455497	ZNRF4	-0.0008	0.0288	3.08E-06	0.0260
cg08568504	chr5:126172293	LMNB1	-0.0009	0.0350	3.11E-06	0.0260
cg25932097	chr10:89381759		-0.0016	0.0603	3.16E-06	0.0260
cg02874994	chr4:87581763	PTPN13	-0.0095	0.4023	3.17E-06	0.0260
cg15436954	chr11:31325916	DCDC1	-0.0157	0.6226	3.18E-06	0.0260
cg02099676	chr3:12490694		0.0064	0.2340	3.19E-06	0.0260
cg05879129	chr17:61515854	CYB561	-0.0045	0.1644	3.35E-06	0.0270
cg04222582	chr15:45405103	DUOX2	0.0227	0.8356	3.41E-06	0.0272
cg10704729	chr11:60722107		-0.0037	0.1349	3.49E-06	0.0274
cg12624481	chr13:47326847	LRCH1	-0.0007	0.0277	3.51E-06	0.0274
cg09979523	chr20:25071315		-0.0075	0.2907	3.56E-06	0.0274
cg16700392	chr4:156680207	GUCY1B3	0.0355	1.2894	3.60E-06	0.0275
cg02976668	chr3:141741702	TFDP2	-0.0052	0.2084	3.80E-06	0.0276
cg12081194	chr2:95653444		-0.0083	0.3101	3.80E-06	0.0276
cg05403744	chr2:121746753	GLI2	-0.0041	0.1520	3.81E-06	0.0276
cg12591302	chr10:99258515	UBTD1; MMS19	0.0014	0.0525	3.85E-06	0.0276
cg22928999	chr2:48046329	FBXO11	-0.0020	0.0799	3.93E-06	0.0276
cg24360651	chr19:14586317	PTGER1	0.0020	0.0776	3.94E-06	0.0276
cg06855877	chr15:30248561	TJP1	-0.0067	0.2831	4.03E-06	0.0276
cg13396060	chr1:243670224	AKT3	-0.0024	0.0928	4.05E-06	0.0276
cg19968631	chr4:175750792	GLRA3	0.0011	0.0414	4.07E-06	0.0276
cg16273664	chr3:185430333	IGF2BP2; C3orf65	-0.0077	0.2949	4.08E-06	0.0276
cg04362407	chr3:98189197	OR5K1	-0.0028	0.1088	4.10E-06	0.0276
cg17003465	chr7:154795674	PAXIP1; PAXIP1-AS1	0.0009	0.0341	4.10E-06	0.0276
cg18361162	chr7:25255671		-0.0015	0.0571	4.21E-06	0.0281
cg21396877	chr14:74963393		-0.0008	0.0300	4.28E-06	0.0283
cg16609941	chr3:139108945	COPB2	0.0013	0.0496	4.44E-06	0.0290
cg20049927	chr15:45777254	SLC30A4	-0.0022	0.0926	4.49E-06	0.0290
cg09373765	chr2:39320345	SOS1	-0.0014	0.0564	4.56E-06	0.0290
cg12365907	chr3:28373304	AZI2	-0.0018	0.0733	4.58E-06	0.0290
cg27338102	chr9:107769113		-0.0009	0.0336	4.59E-06	0.0290
cg20580833	chr10:99628950	CRTAC1	-0.0051	0.1943	4.72E-06	0.0294
cg03304641	chr20:13227047	ISM1	-0.0014	0.0504	4.75E-06	0.0294
cg15347676	chr4:73176816	ADAMTS3	-0.0014	0.0569	4.78E-06	0.0294
cg04227973	chr9:129327236		0.0048	0.1789	4.93E-06	0.0296
cg26713963	chr2:162308719		-0.0025	0.1011	4.95E-06	0.0296
cg05163650	chr6:125502705	TPD52L1	-0.0022	0.0843	5.06E-06	0.0296
cg09362115	chr2:220463095	STK11IP	0.0007	0.0285	5.12E-06	0.0296

cg05533552	chr11:34074141	CAPRIN1	0.0016	0.0611	5.13E-06	0.0296
cg25308531	chr10:51575388	NCOA4; TIMM23B	-0.0040	0.1547	5.20E-06	0.0296
cg00756178	chr13:21996653	ZDHHC20	-0.0048	0.2011	5.20E-06	0.0296
cg27519395	chr1:231004784	C1orf198	0.0010	0.0398	5.25E-06	0.0296
cg13923648	chr6:27173910		0.0079	0.2918	5.32E-06	0.0296
cg27463491	chr10:43626431		-0.0060	0.2207	5.34E-06	0.0296
cg23207876	chr19:15162821	CASP14	0.0150	0.5675	5.34E-06	0.0296
cg05822949	chr1:159802791	SLAMF8	-0.0034	0.1314	5.38E-06	0.0296
cg18512628	chr6:124811541	NKAIN2	0.0070	0.2944	5.45E-06	0.0296
cg22287711	chr5:139282860	NRG2	-0.0101	0.3760	5.45E-06	0.0296
cg15105011	chr4:940614	TMEM175	-0.0049	0.1842	5.52E-06	0.0296
cg08345465	chr6:108901318	FOXO3	-0.0021	0.0818	5.60E-06	0.0296
cg15827508	chr2:218679705	TNS1	-0.0035	0.1354	5.61E-06	0.0296
cg00558019	chr7:23205338	KLHL7	-0.0011	0.0462	5.65E-06	0.0296
cg18365757	chr5:176830143	F12	0.0117	0.4543	5.68E-06	0.0296
cg14622897	chr10:30328751	KIAA1462	-0.0040	0.1534	5.68E-06	0.0296
cg14613491	chr11:98892283	CNTN5	0.0217	0.8144	5.73E-06	0.0296
cg03583857	chr1:208085022	CD34	-0.0068	0.2549	5.75E-06	0.0296
cg18259092	chr11:94960077	SESN3	-0.0042	0.1814	5.99E-06	0.0306
cg00524818	chr6:71607590	B3GAT2	-0.0111	0.4537	6.03E-06	0.0306
cg18058524	chr2:228498185	C2orf83	-0.0028	0.1152	6.11E-06	0.0308
cg00866946	chr5:68687729	RAD17	-0.0017	0.0719	6.17E-06	0.0308
cg01543767	chr4:109540778	RPL34	-0.0010	0.0370	6.20E-06	0.0308
cg24901743	chr15:81293243	MESDC1	0.0004	0.0167	6.27E-06	0.0308
cg26110605	chr10:1981558		0.0209	0.8047	6.29E-06	0.0308
cg24691024	chr6:76501887	MYO6	-0.0037	0.1622	6.35E-06	0.0309
cg11946336	chr22:37816333	ELFN2	0.0022	0.0860	6.40E-06	0.0309
cg19719208	chr10:102976430		0.0061	0.2311	6.64E-06	0.0319
cg13308122	chr4:105641126		-0.0076	0.3200	6.85E-06	0.0327
cg16629616	chr17:67363884		-0.0034	0.1416	6.97E-06	0.0327
cg25006249	chr20:259898	C20orf96	0.0253	0.9661	7.10E-06	0.0327
cg25197886	chr11:2413949	CD81	-0.0049	0.1932	7.16E-06	0.0327
cg06191454	chr11:122850545	BSX	0.0012	0.0453	7.16E-06	0.0327
cg12470036	chr10:649019	DIP2C	-0.0015	0.0577	7.17E-06	0.0327
cg16081957	chr14:57671658	EXOC5	-0.0021	0.0922	7.23E-06	0.0327
cg15682717	chr15:75772834	PTPN9	-0.0047	0.1949	7.24E-06	0.0327
cg23715904	chr13:82067535		0.0288	1.1216	7.26E-06	0.0327
cg13533456	chr6:116818106	BET3L	-0.0034	0.1426	7.28E-06	0.0327
cg17286256	chr2:169322797	CERS6	0.0111	0.4433	7.70E-06	0.0344
cg06663310	chr5:176153056		0.0069	0.2601	7.82E-06	0.0345
cg11901248	chr5:149866502		0.0011	0.0406	7.83E-06	0.0345
cg10595984	chr15:86299156	LOC101929679	-0.0015	0.0613	7.92E-06	0.0347
cg00928989	chr2:214093892		-0.0016	0.0649	8.08E-06	0.0352

cg11459705	chr1:90534674		-0.0029	0.1209	8.25E-06	0.0356
cg23936463	chr22:48884885	FAM19A5	0.0100	0.3889	8.29E-06	0.0356
cg18244708	chr20:36152923	BLCAP	-0.0059	0.2235	8.33E-06	0.0356
cg17879376	chr1:32212024	BAI2	-0.0033	0.1283	8.42E-06	0.0358
cg04260507	chr16:123601	RHBDF1	-0.0030	0.1181	8.75E-06	0.0365
cg17403653	chr20:42198669	SGK2	-0.0013	0.0509	8.81E-06	0.0365
cg23628297	chr13:32892854	BRCA2	-0.0048	0.1889	8.85E-06	0.0365
cg26882009	chr5:140346106	PCDHAC2; PCDHA7; PCDHA12; PCDHA6; PCDHA10; PCDHA4; PCDHA11; PCDHA8; PCDHA1; PCDHA9; PCDHA13; PCDHA5; PCDHA3	0.0244	0.9690	8.86E-06	0.0365
cg20067765	chr7:16524513		-0.0012	0.0498	8.96E-06	0.0365
cg25340361	chr2:62131978	COMMD1	-0.0025	0.0983	9.00E-06	0.0365
cg20425597	chr6:149004588		-0.0016	0.0655	9.03E-06	0.0365
cg01320000	chr22:43619727	SCUBE1	-0.0155	0.6042	9.07E-06	0.0365
cg15890574	chr15:65739067	DPP8	-0.0026	0.1139	9.13E-06	0.0365
cg24458300	chr11:1302168	TOLLIP	-0.0014	0.0544	9.24E-06	0.0365
cg04369018	chr4:113626366		0.0021	0.0803	9.29E-06	0.0365
cg00695244	chr7:123134038	IQUB	-0.0052	0.2031	9.31E-06	0.0365
cg22236250	chr19:46580441		0.0228	0.8759	9.33E-06	0.0365
cg21332500	chr7:27233480		-0.0096	0.3584	9.37E-06	0.0365
cg12669892	chr3:37727660	ITGA9	-0.0059	0.2321	9.48E-06	0.0365
cg21883261	chr5:135170208	LOC153328	0.0025	0.0959	9.53E-06	0.0365
cg10797690	chr2:41370132		-0.0008	0.0305	9.64E-06	0.0365
cg21543067	chr6:16744551	ATXN1	-0.0079	0.3237	9.64E-06	0.0365
cg07561192	chr7:29203827	CHN2	-0.0060	0.2506	9.72E-06	0.0365
cg21414048	chr12:118766325	TAOK3	-0.0010	0.0445	9.76E-06	0.0365
cg19851029	chr3:138669137	C3orf72	0.0025	0.0991	9.81E-06	0.0365
cg19621877	chr4:1393418		0.0175	0.6836	9.81E-06	0.0365
cg18548381	chr1:215571083		0.0144	0.5542	9.93E-06	0.0365
cg07792422	chr15:50639978	GABPB1	-0.0006	0.0233	9.95E-06	0.0365
cg14729709	chr14:97297779	VRK1	-0.0013	0.0542	9.99E-06	0.0365
cg14582298	chr10:31610093	ZEB1	0.0015	0.0581	1.01E-05	0.0365
cg19918758	chr1:108508113	VAV3	0.0007	0.0270	1.01E-05	0.0365
cg05697394	chr12:65701642	MSRB3	-0.0032	0.1408	1.01E-05	0.0365
cg13195890	chr2:120848033	EPB41L5	-0.0012	0.0530	1.01E-05	0.0366
cg26157031	chr11:102208366	BIRC3	-0.0023	0.0950	1.02E-05	0.0366
cg02641990	chr1:66999785	SGIP1	0.0040	0.1564	1.02E-05	0.0366
cg00527174	chr1:20880663	FAM43B	0.0026	0.0977	1.04E-05	0.0369
cg17020516	chr12:118797962	TAOK3	-0.0022	0.0980	1.04E-05	0.0369
cg19388017	chr19:15285100	NOTCH3	-0.0006	0.0240	1.06E-05	0.0372
cg09901748	chr12:12098691		-0.0079	0.3050	1.06E-05	0.0372

cg04921109	chr14:69952104	FLJ44817	0.0057	0.2271	1.07E-05	0.0372
cg09278646	chr3:167466262	SERPINI1	-0.0042	0.1785	1.08E-05	0.0373
cg06232130	chr2:157177008		0.0074	0.2804	1.09E-05	0.0373
cg23088511	chr6:41396106		0.0038	0.1497	1.10E-05	0.0373
cg21148188	chr2:42324450		-0.0017	0.0657	1.10E-05	0.0373
cg20590313	chr8:74705377	UBE2W	-0.0049	0.2110	1.10E-05	0.0373
cg04209173	chr1:150037321		-0.0052	0.2196	1.10E-05	0.0373
cg07103093	chr17:35297752	LHX1	0.0040	0.1563	1.11E-05	0.0374
cg02793774	chr7:130737598	LINC-PINT	-0.0036	0.1445	1.12E-05	0.0376
cg14506158	chr12:48111918	ENDOU	-0.0035	0.1372	1.14E-05	0.0378
cg26562600	chr2:61243860	PUS10; PEX13	0.0008	0.0363	1.14E-05	0.0378
cg25087851	chr11:60623918	GPR44	-0.0053	0.2127	1.14E-05	0.0378
cg05114334	chr7:65705962	TPST1	-0.0023	0.0956	1.17E-05	0.0383
cg19318403	chr7:107389546	CBLL1	-0.0027	0.1244	1.17E-05	0.0383
cg08229967	chr2:69134616		-0.0012	0.0502	1.18E-05	0.0383
cg16713168	chr16:80716710	CDYL2	-0.0046	0.1849	1.18E-05	0.0383
cg00207865	chr1:111149644	KCNA2	0.0014	0.0564	1.19E-05	0.0383
cg11223980	chr10:87863164	GRID1	-0.0088	0.3506	1.19E-05	0.0383
cg08144546	chr1:62941484	DOCK7	-0.0012	0.0527	1.20E-05	0.0385
cg27332018	chr7:129846548	TMEM209; C7orf45	-0.0036	0.1502	1.22E-05	0.0389
cg02225054	chr16:12210834	SNX29	-0.0004	0.0145	1.24E-05	0.0391
cg14142047	chr3:31052252		-0.0058	0.2285	1.24E-05	0.0391
cg20989305	chr19:30586538		-0.0082	0.3234	1.25E-05	0.0391
cg18563384	chr6:72312521		-0.0022	0.0883	1.25E-05	0.0391
cg24111276	chr1:110512547		-0.0017	0.0693	1.26E-05	0.0391
cg25757747	chr4:129083788	LARP1B	-0.0068	0.3073	1.28E-05	0.0391
cg04550363	chr6:11732512	ADTRP	-0.0052	0.2141	1.29E-05	0.0391
cg06949264	chr1:103238555		-0.0026	0.1112	1.29E-05	0.0391
cg27118178	chr20:31116012	NOL4L	-0.0021	0.0810	1.29E-05	0.0391
cg16190644	chr18:9750329	RAB31	-0.0034	0.1337	1.30E-05	0.0391
cg26939283	chr1:152731117	KPRP	-0.0124	0.4949	1.31E-05	0.0391
cg01925344	chr6:44310098	SPATS1	-0.0147	0.5893	1.32E-05	0.0391
cg11436333	chr2:169759454	G6PC2	-0.0060	0.2641	1.33E-05	0.0391
cg05576754	chr10:115904364	CCDC186	-0.0026	0.1164	1.33E-05	0.0391
cg12396622	chr6:119390418	FAM184A; MIR548B	-0.0007	0.0328	1.33E-05	0.0391
cg02984262	chr12:62048371		0.0152	0.6219	1.34E-05	0.0391
cg18240794	chr20:16332248	KIF16B	-0.0066	0.2593	1.35E-05	0.0391
cg24332770	chr1:152658287	LCE2B	-0.0102	0.4122	1.35E-05	0.0391
cg09128310	chr6:378457		0.0108	0.4352	1.35E-05	0.0391
cg19547141	chr11:118313315	MLL	-0.0007	0.0276	1.37E-05	0.0391
cg09851951	chr16:54323498		0.0165	0.6941	1.38E-05	0.0391
cg21176488	chr3:188251742	LPP	-0.0011	0.0450	1.39E-05	0.0391

cg02297173	chr5:140772023	PCDHGA8; PCDHGA1; PCDHGA2; PCDHGA3; PCDHGB1; PCDHGA4; PCDHGB2; PCDHGA5; PCDHGB3; PCDHGA6; PCDHGA7; PCDHGB4	0.0109	0.4449	1.39E-05	0.0391
cg13994321	chr20:9495723	LAMP5-AS1; LAMP5	0.0096	0.3825	1.39E-05	0.0391
cg26811372	chr5:140772182	PCDHGA4; PCDHGA6; PCDHGA1; PCDHGA5; PCDHGB1; PCDHGB4; PCDHGA3; PCDHGA8; PCDHGA2; PCDHGA7; PCDHGB2; PCDHGB3	0.0194	0.7633	1.39E-05	0.0391
cg11760363	chr12:26581154	ITPR2	-0.0014	0.0614	1.40E-05	0.0391
cg24926320	chr5:150163018	SMIM3	-0.0047	0.1864	1.40E-05	0.0391
cg01034678	chr17:59951926	INTS2	-0.0014	0.0637	1.40E-05	0.0391
cg07930539	chr2:177025975		0.0255	1.0060	1.40E-05	0.0391
cg06298772	chr10:125979200		-0.0042	0.1675	1.41E-05	0.0391
cg14980467	chr14:64762315	ESR2; MIR548AZ	-0.0045	0.1875	1.42E-05	0.0391
cg03966582	chr1:152540156	LCE3E	-0.0100	0.4252	1.42E-05	0.0391
cg20851097	chr14:65289835	SPTB	-0.0039	0.1598	1.42E-05	0.0391
cg08789530	chr3:47091820	SETD2	-0.0067	0.2987	1.43E-05	0.0391
cg03916903	chr17:30860657	MYO1D	-0.0034	0.1409	1.43E-05	0.0391
cg27167982	chr1:93175467	EVI5	-0.0012	0.0533	1.43E-05	0.0391
cg06488467	chr1:238216183		0.0211	0.8275	1.44E-05	0.0391
cg22008490	chr1:202129255	PTPN7	-0.0036	0.1413	1.44E-05	0.0391
cg09297225	chr4:56656788		0.0105	0.4129	1.46E-05	0.0395
cg00401552	chr16:2835710	PRSS33	0.0030	0.1237	1.47E-05	0.0395
cg22344419	chr4:139982108	ELF2	-0.0040	0.1731	1.47E-05	0.0395
cg00850245	chr22:31836332	EIF4ENIF1	-0.0016	0.0631	1.47E-05	0.0395
cg21614762	chr12:65674271	MSRB3	-0.0086	0.3422	1.48E-05	0.0395
cg05022087	chr14:62407567		0.0076	0.3053	1.51E-05	0.0396
cg20256494	chr22:30116439	CABP7	0.0054	0.2208	1.52E-05	0.0396
cg02919936	chr8:70982285	PRDM14	0.0068	0.2702	1.52E-05	0.0396
cg13576566	chr8:133805116	PHF20L1	-0.0037	0.1582	1.52E-05	0.0396
cg14912644	chr2:157176601		0.0152	0.6050	1.52E-05	0.0396
cg23278483	chr5:102194124		-0.0114	0.4774	1.52E-05	0.0396
cg13827350	chr5:83680045	EDIL3	0.0033	0.1306	1.53E-05	0.0396
cg07755760	chr3:196943586	DLG1	-0.0031	0.1400	1.53E-05	0.0396
cg14256814	chr1:166808417	POGK	0.0012	0.0469	1.54E-05	0.0397
cg10144104	chr3:50518896	CACNA2D2	-0.0136	0.5350	1.56E-05	0.0401
cg23455227	chr16:56703561	MT1H	0.0014	0.0553	1.58E-05	0.0404
cg01976992	chr6:36238410	PNPLA1	-0.0017	0.0688	1.58E-05	0.0404
cg20328532	chr4:55414602		-0.0063	0.2466	1.59E-05	0.0404
cg27561954	chr1:10057312	RBP7	0.0012	0.0489	1.61E-05	0.0404
cg11784305	chr20:1757812		0.0011	0.0415	1.61E-05	0.0404

cg01690182	chr15:27018874	GABRB3	0.0116	0.4621	1.62E-05	0.0404
cg20870512	chr7:1272515	UNCX	0.0010	0.0410	1.63E-05	0.0404
cg08895932	chr1:152778580	LCE1C	-0.0204	0.8193	1.63E-05	0.0404
cg03712600	chr1:87159493		-0.0058	0.2569	1.63E-05	0.0404
cg27318570	chr8:109143688		0.0119	0.4896	1.64E-05	0.0404
cg15118604	chr12:109742398	FOXN4	-0.0080	0.3245	1.65E-05	0.0404
cg03046801	chr12:80621582	OTOGL	-0.0011	0.0504	1.65E-05	0.0404
cg20349411	chr11:111252661		-0.0059	0.2443	1.65E-05	0.0404
cg04193415	chr22:30643063	LIF	-0.0086	0.3348	1.66E-05	0.0404
cg09312135	chr6:53519753	KLHL31	-0.0041	0.1702	1.66E-05	0.0404
cg20655220	chr5:102246737	PAM	-0.0033	0.1352	1.68E-05	0.0404
cg05503219	chr12:80987677	PTPRQ	-0.0076	0.3402	1.68E-05	0.0404
cg12890561	chr12:80853517	PTPRQ	-0.0065	0.2959	1.68E-05	0.0404
cg08363415	chr2:54345792	ACYP2	-0.0062	0.2520	1.68E-05	0.0404
cg10476288	chr17:49101055	SPAG9	-0.0044	0.1952	1.70E-05	0.0407
cg00270036	chr15:32928807	ARHGAP11A	-0.0016	0.0694	1.71E-05	0.0407
cg04434491	chr19:16058640	OR10H4	0.0215	0.8743	1.72E-05	0.0410
cg12109743	chr11:3400108	ZNF195	0.0008	0.0318	1.74E-05	0.0411
cg00503017	chr10:79151564	KCNMA1	-0.0060	0.2485	1.77E-05	0.0415
cg15811446	chr16:69820935	WWP2	-0.0013	0.0541	1.77E-05	0.0415
cg05717082	chr12:96251548	SNRPF	-0.0040	0.1685	1.77E-05	0.0415
cg07071464	chr7:102388091	FAM185A	0.0104	0.4335	1.79E-05	0.0418
cg03040622	chr7:5536937	MIR589; FBXL18	0.0059	0.2386	1.80E-05	0.0418
cg23640002	chr6:33084933	HLA-DPB2	0.0090	0.3642	1.81E-05	0.0418
cg21611830	chr15:68113461	SKOR1	0.0023	0.0965	1.82E-05	0.0418
cg13368367	chr5:124549915	LOC101927421	-0.0015	0.0658	1.83E-05	0.0418
cg00973677	chr7:136553595	CHRM2	0.0011	0.0454	1.83E-05	0.0418
cg14840561	chr12:121418741	HNF1A	-0.0009	0.0376	1.83E-05	0.0418
cg20738192	chr10:71333365	NEUROG3	-0.0017	0.0695	1.83E-05	0.0418
cg14409559	chr8:72756341	MSC	0.0065	0.2630	1.85E-05	0.0418
cg22458427	chr3:136648752	NCK1	-0.0050	0.2219	1.85E-05	0.0418
cg07944907	chr3:184098758	THPO; CHRD	0.0120	0.4890	1.85E-05	0.0418
cg05280133	chr15:45670068	GATM; LOC145663	0.0170	0.6879	1.87E-05	0.0420
cg16887334	chr20:3052151	OXT	-0.0018	0.0771	1.87E-05	0.0420
cg16239482	chr3:181437145	SOX2OT	0.0148	0.5891	1.87E-05	0.0420
cg25279318	chr15:81488917	IL16	-0.0110	0.4561	1.89E-05	0.0422
cg05403282	chr9:96930797		0.0022	0.0949	1.91E-05	0.0424
cg00455988	chr11:8554936	STK33	-0.0029	0.1173	1.92E-05	0.0424
cg12466737	chr18:35146589	BRUNOL4	0.0170	0.6732	1.93E-05	0.0424
cg24751561	chr5:179207393		-0.0009	0.0377	1.93E-05	0.0424
cg05723219	chr4:187605157	FAT1	-0.0035	0.1460	1.93E-05	0.0424
cg15284545	chr12:123757474	CDK2AP1	-0.0025	0.1026	1.94E-05	0.0424

cg09844907	chr16:15489619	MPV17L	0.0009	0.0344	1.96E-05	0.0427
cg09896315	chr11:94172892	MRE11A	-0.0007	0.0304	1.99E-05	0.0427
cg18102098	chr14:78432386		0.0013	0.0551	1.99E-05	0.0427
cg20281659	chr15:30365390		-0.0023	0.0931	1.99E-05	0.0427
cg13314908	chr2:46307119	PRKCE	-0.0014	0.0565	1.99E-05	0.0427
cg13138000	chr8:125669849	MTSS1	-0.0040	0.1695	2.00E-05	0.0427
cg21489989	chr22:43616909	SCUBE1	-0.0164	0.6698	2.00E-05	0.0427
cg15716642	chr19:58071595	ZNF550	0.0024	0.1002	2.00E-05	0.0427
cg01106410	chr6:31549159	LTB	0.0045	0.1886	2.00E-05	0.0427
cg14994639	chr11:121422921	SORL1	0.0085	0.3490	2.01E-05	0.0428
cg04110478	chr5:2142052		0.0152	0.6064	2.02E-05	0.0428
cg25137918	chr12:52545323		-0.0029	0.1189	2.03E-05	0.0428
cg20728696	chr11:2158702	INS-IGF2; IGF2	0.0013	0.0504	2.03E-05	0.0428
cg13713522	chr8:141109051	TRAPPC9	-0.0056	0.2403	2.04E-05	0.0428
cg01131229	chr10:17622581		-0.0041	0.1824	2.04E-05	0.0428
cg23815646	chr8:131961143	ADCY8	0.0124	0.4898	2.05E-05	0.0429
cg18209502	chr5:34944941	DNAJC21	-0.0012	0.0526	2.07E-05	0.0430
cg17985656	chr1:97186397	PTBP2	-0.0008	0.0352	2.09E-05	0.0430
cg24945028	chr8:87552374	CPNE3	-0.0008	0.0380	2.09E-05	0.0430
cg00059015	chr1:60340545	HOOK1	-0.0015	0.0691	2.10E-05	0.0430
cg06296151	chr3:32522806	CMTM6	-0.0057	0.2504	2.10E-05	0.0430
cg23326633	chr1:185177216	SWT1	-0.0025	0.1090	2.10E-05	0.0430
cg15207742	chr20:43438809	RIMS4	0.0021	0.0801	2.10E-05	0.0430
cg18444689	chr3:185080423	MAP3K13	-0.0029	0.1212	2.12E-05	0.0430
cg19142497	chr6:149898426	GINM1	-0.0005	0.0192	2.13E-05	0.0430
cg05561386	chr17:76455102	DNAH17	-0.0006	0.0224	2.13E-05	0.0430
cg21458907	chr3:62860802	CADPS	0.0144	0.5778	2.14E-05	0.0430
cg16905280	chr3:69985202	MITF	-0.0041	0.1831	2.14E-05	0.0430
cg19610007	chr19:42449034		-0.0012	0.0490	2.14E-05	0.0430
cg25943307	chr2:51944827		-0.0049	0.2274	2.15E-05	0.0430
cg12253142	chr12:12347284	LRP6	-0.0125	0.5226	2.16E-05	0.0430
cg13897996	chr12:9202584		-0.0065	0.2667	2.16E-05	0.0430
cg06572849	chr11:45354316		0.0009	0.0354	2.17E-05	0.0430
cg23234083	chr1:53552366		-0.0041	0.1737	2.17E-05	0.0430
cg24765016	chr19:57874875	ZNF547; TRAPPC2P1	0.0006	0.0256	2.18E-05	0.0430
cg15090562	chr1:6534449	PLEKHG5	-0.0099	0.4061	2.19E-05	0.0430
cg26865109	chr14:36784004	MBIP	-0.0015	0.0687	2.19E-05	0.0430
cg09317096	chr3:196159456	UBXN7	0.0013	0.0533	2.20E-05	0.0430
cg07046546	chr7:128550967	KCP	-0.0038	0.1563	2.20E-05	0.0430
cg22570122	chr16:49315822	CBLN1	0.0027	0.1137	2.23E-05	0.0433
cg24916281	chr12:98937145	TMPO	-0.0019	0.0889	2.23E-05	0.0434
cg03230592	chr15:69552888	GLCE	-0.0052	0.2370	2.24E-05	0.0434

cg10262037	chr1:216897002	ESRRG	-0.0010	0.0435	2.26E-05	0.0435
cg07183148	chr10:71712878	COL13A1	-0.0136	0.5736	2.26E-05	0.0435
cg03206741	chr8:105235385	RIMS2	0.0016	0.0690	2.27E-05	0.0435
cg13613180	chr11:74560694	XRRA1	-0.0037	0.1506	2.27E-05	0.0435
cg15281774	chr15:73661908	HCN4	0.0127	0.5314	2.28E-05	0.0435
cg15294275	chr6:143607481	AIG1	0.0060	0.2494	2.29E-05	0.0435
cg24055030	chr22:25561051	KIAA1671	-0.0043	0.1719	2.29E-05	0.0435
cg10694152	chr13:99404506	SLC15A1	0.0112	0.4629	2.31E-05	0.0437
cg16309506	chr12:60651018		0.0045	0.2038	2.33E-05	0.0440
cg22291850	chr20:30456013	DUSP15	-0.0100	0.4137	2.36E-05	0.0444
cg07262457	chr3:128777371		-0.0067	0.2700	2.36E-05	0.0444
cg14437725	chr9:137787387		0.0179	0.7258	2.37E-05	0.0444
cg13416727	chr8:26115975		-0.0014	0.0567	2.38E-05	0.0444
cg07812805	chr15:27525898	GABRG3	0.0199	0.8302	2.38E-05	0.0444
cg04767500	chr6:38682956		0.0132	0.5462	2.41E-05	0.0447
cg19451873	chr7:40027199	CDK13	-0.0040	0.1739	2.42E-05	0.0447
cg20846447	chr1:177140305	FAM5B	0.0094	0.3950	2.42E-05	0.0447
cg01273734	chr14:92342200	FBLN5	-0.0065	0.2719	2.42E-05	0.0447
cg07290552	chr8:79578143	FAM164A	0.0006	0.0276	2.44E-05	0.0448
cg00520601	chr11:111305794		-0.0072	0.3013	2.44E-05	0.0448
cg06391839	chr8:134510900	ST3GAL1	-0.0043	0.1790	2.46E-05	0.0448
cg00651099	chr4:125599866	ANKRD50	-0.0016	0.0727	2.47E-05	0.0448
cg26703261	chr17:79171051	CEP131	-0.0056	0.2304	2.47E-05	0.0448
cg25055477	chr6:34360916	NUDT3	-0.0058	0.2440	2.47E-05	0.0448
cg24932925	chr6:31708299	MSH5	0.0009	0.0371	2.48E-05	0.0449
cg11307857	chr11:128599031	FLI1	-0.0029	0.1225	2.49E-05	0.0449
cg02621151	chr5:3092997		0.0168	0.6967	2.49E-05	0.0449
cg12989851	chr3:147074517		0.0155	0.6496	2.51E-05	0.0451
cg25767314	chr8:120806025	TAF2	-0.0046	0.2069	2.52E-05	0.0452
cg02683759	chr2:157177072		0.0196	0.8165	2.53E-05	0.0453
cg04185729	chr11:65382031	MAP3K11	0.0008	0.0325	2.54E-05	0.0453
cg17491545	chr9:139559373	EGFL7	-0.0049	0.2088	2.57E-05	0.0455
cg21377489	chr10:102985196		0.0074	0.3028	2.57E-05	0.0455
cg11816739	chr17:42926867	HIGD1B	-0.0050	0.2054	2.57E-05	0.0455
cg23157284	chr12:80120320		-0.0032	0.1464	2.58E-05	0.0455
cg09045552	chr2:169506620	LASS6	-0.0133	0.5457	2.58E-05	0.0455
cg02425372	chr12:53074023	KRT1	0.0130	0.5444	2.60E-05	0.0457
cg15784996	chr10:74894550	ECD	-0.0010	0.0458	2.61E-05	0.0458
cg13760583	chr2:143635616	KYNU	-0.0019	0.0815	2.62E-05	0.0458
cg15568408	chr1:11825928		-0.0075	0.3160	2.63E-05	0.0458
cg22881941	chr3:42642053	NKTR	0.0003	0.0145	2.65E-05	0.0460
cg08515841	chr2:208029740	KLF7	0.0150	0.6203	2.65E-05	0.0461
cg20798469	chr7:139680133	TBXAS1	-0.0006	0.0240	2.67E-05	0.0462

cg16784468	chr10:134230041	PWWP2B	-0.0022	0.0936	2.69E-05	0.0465
cg24626646	chr7:2685064	TTYH3	-0.0053	0.2227	2.71E-05	0.0465
cg22663098	chr2:128512817	WDR33	-0.0019	0.0850	2.71E-05	0.0465
cg11995282	chr7:16746042	BZW2	-0.0015	0.0662	2.72E-05	0.0466
cg00939030	chr9:116298734	RGS3	0.0038	0.1580	2.75E-05	0.0469
cg26745222	chr20:42543878	TOX2	0.0076	0.3254	2.75E-05	0.0469
cg03578022	chr17:67057578	ABCA9	-0.0056	0.2491	2.77E-05	0.0471
cg23811122	chr1:78463167		-0.0038	0.1636	2.79E-05	0.0472
cg18875629	chr22:27834439		0.0165	0.6799	2.79E-05	0.0472
cg11563860	chr3:2141937	CNTN4	0.0205	0.8578	2.80E-05	0.0472
cg26763380	chr8:109233795	EIF3E	-0.0031	0.1355	2.81E-05	0.0472
cg19561508	chr9:33140866	B4GALT1	0.0040	0.1673	2.81E-05	0.0472
cg11178281	chr14:36983734	SFTA3	0.0135	0.5696	2.81E-05	0.0472
cg22073869	chr13:98866089	FARP1	-0.0044	0.1828	2.82E-05	0.0472
cg15328302	chr11:93479482	C11orf54	-0.0028	0.1196	2.84E-05	0.0473
cg11269491	chr2:218692587	TNS1	-0.0032	0.1330	2.85E-05	0.0473
cg01272851	chr12:110092636		-0.0105	0.4335	2.85E-05	0.0473
cg22896480	chr3:122379438		0.0209	0.8878	2.86E-05	0.0473
cg07042319	chr9:81638357		0.0064	0.2642	2.86E-05	0.0473
cg22855900	chr5:72529662		0.0012	0.0496	2.90E-05	0.0478
cg19004285	chr12:95468616	NR2C1	-0.0016	0.0668	2.92E-05	0.0478
cg18809403	chr20:30504995	TTLL9	-0.0142	0.5874	2.92E-05	0.0478
cg13091717	chr11:75898195	WNT11	-0.0037	0.1535	2.93E-05	0.0478
cg13706097	chr22:30295853	MTMR3	-0.0014	0.0627	2.94E-05	0.0478
cg23521138	chr18:29026303	DSG3	-0.0055	0.2364	2.94E-05	0.0478
cg02859466	chr3:167448491	PDCD10	-0.0017	0.0767	2.94E-05	0.0478
cg11820270	chr14:56072740	KTN1	0.0042	0.2009	2.95E-05	0.0478
cg03161912	chr5:133229901		-0.0074	0.3062	2.96E-05	0.0478
cg19265606	chr6:146611484	GRM1	-0.0181	0.7565	2.96E-05	0.0478
cg10332039	chr11:1848665		-0.0043	0.1827	2.98E-05	0.0479
cg11245243	chr19:48744484	CARD8	-0.0018	0.0769	2.99E-05	0.0479
cg19678828	chr21:27945413	CYYR1	0.0223	0.8910	3.00E-05	0.0479
cg16195091	chr17:76227996	LOC283999	0.0167	0.7003	3.01E-05	0.0479
cg14928293	chr20:37591018	DHX35	0.0009	0.0384	3.01E-05	0.0479
cg25033325	chr2:98612560	TMEM131	0.0012	0.0522	3.02E-05	0.0479
cg11825681	chr10:134902460	ADGRA1	0.0103	0.4214	3.02E-05	0.0479
cg23641145	chr11:101000566	PGR	0.0012	0.0499	3.02E-05	0.0479
cg03173827	chr20:62688717	TCEA2	-0.0038	0.1594	3.05E-05	0.0482
cg07047620	chr8:140761733	TRAPPC9	-0.0019	0.0796	3.06E-05	0.0482
cg03485508	chr3:43372060	SNRK	-0.0021	0.0944	3.07E-05	0.0482
cg22733664	chr1:10338586	KIF1B	-0.0011	0.0485	3.08E-05	0.0482
cg16290431	chr5:173342997	CPEB4	-0.0029	0.1357	3.08E-05	0.0482
cg19121352	chr2:133247007	GPR39	0.0120	0.4981	3.08E-05	0.0482

cg07252680	chr14:94857224	SERPINA1	-0.0042	0.1725	3.10E-05	0.0482
cg16286080	chr18:60742636		0.0012	0.0543	3.10E-05	0.0482
cg25317631	chr11:44541905		-0.0039	0.1681	3.10E-05	0.0482
cg01023982	chr5:139891775	ANKHD1; ANKHD1-EIF4EBP3	-0.0018	0.0757	3.11E-05	0.0482
cg22054189	chr14:35344773	BAZ1A	0.0011	0.0489	3.12E-05	0.0482
cg24274117	chr20:62185401	C20orf195	0.0132	0.5541	3.13E-05	0.0483
cg15896796	chr4:74459396	RASSF6	-0.0046	0.2013	3.19E-05	0.0491
cg11248542	chr20:31864167		-0.0094	0.3892	3.20E-05	0.0491
cg24833575	chr15:44375222	FRMD5	-0.0043	0.1867	3.23E-05	0.0493
cg26963797	chr16:51189291		0.0230	0.9773	3.23E-05	0.0493
cg20140657	chr10:32832249	CCDC7	-0.0073	0.3450	3.24E-05	0.0493
cg12340301	chr1:100060046		-0.0031	0.1383	3.24E-05	0.0493
cg25880785	chr5:12277867		-0.0018	0.0780	3.24E-05	0.0493
cg06524264	chr20:259925	C20orf96	0.0261	1.0821	3.25E-05	0.0493
cg04633513	chr1:206224027	AVPR1B	0.0009	0.0372	3.26E-05	0.0493
cg06272272	chr10:112684117	SHOC2	-0.0035	0.1592	3.26E-05	0.0493
cg18765874	chr16:8620227	TMEM114	0.0180	0.7503	3.28E-05	0.0493
cg17647441	chr7:67753554		0.0213	0.8793	3.29E-05	0.0493
cg02570501	chr7:64125557	ZNF107	-0.0020	0.0939	3.30E-05	0.0493
cg09442654	chr8:53477881	FAM150A	0.0049	0.2064	3.30E-05	0.0493
cg06503521	chr14:74164171	DNAL1	-0.0022	0.0954	3.30E-05	0.0493
cg06031433	chr11:96022822	MAML2	-0.0009	0.0427	3.31E-05	0.0493
cg19208718	chr1:190233978	BRINP3	-0.0050	0.2131	3.33E-05	0.0496
cg10376133	chr14:57049860	C14orf101	-0.0052	0.2357	3.36E-05	0.0497
cg17289868	chr2:210334953	MAP2	-0.0017	0.0736	3.38E-05	0.0497
cg01641514	chr11:2013461		-0.0028	0.1165	3.38E-05	0.0497
cg23381646	chr7:44187310	GCK	-0.0061	0.2586	3.38E-05	0.0497
cg27506462	chr2:223184557		0.0011	0.0479	3.38E-05	0.0497
cg27578046	chr4:156275021	LOC102724776; MAP9	-0.0052	0.2399	3.39E-05	0.0497
cg00015373	chr9:134247288		-0.0018	0.0753	3.39E-05	0.0497
cg23231734	chr1:39620146	MACF1	-0.0025	0.1057	3.42E-05	0.0500

Supplemental Table 4-2. Differentially methylated sites (q < 0.05) associated with infant toenail Pb.

Probe	Position	Gene	Estimate	SE	p-value	q-value
cg15445952	chr22:43653574	SCUBE1	-0.0203	0.6330	2.29E-10	0.0002
cg04640975	chr19:3464991		-0.0129	0.4600	9.39E-09	0.0033
cg11365072	chr5:154392274	KIF4B	-0.0031	0.1162	3.53E-08	0.0058
cg03359161	chr20:39191619		-0.0020	0.0730	4.46E-08	0.0058
cg07136023	chr16:86537316		0.0137	0.5140	4.70E-08	0.0058
cg10299585	chr12:54321717		0.0147	0.5599	4.95E-08	0.0058
cg02859421	chr19:3465071		-0.0091	0.3503	7.02E-08	0.0070
cg10814131	chr1:203009651	PPFIA4	-0.0087	0.3359	1.23E-07	0.0108
cg13822446	chr19:46235676	BHMG1	-0.0042	0.1673	1.74E-07	0.0135
cg09422806	chr19:3364015	NFIC	-0.0149	0.5936	2.12E-07	0.0148
cg12412088	chr12:115605267		-0.0064	0.2574	3.20E-07	0.0189
cg04494051	chr11:67142562	LOC100130987; CLCF1	-0.0082	0.3258	3.23E-07	0.0189
cg23003220	chr21:43928228	SLC37A1	-0.0104	0.4257	4.62E-07	0.0249
cg15902830	chr1:3387913	ARHGEF16	-0.0049	0.1996	6.15E-07	0.0308
cg22434786	chr14:59537154		-0.0092	0.3900	7.74E-07	0.0339
cg25057461	chr10:130507759		-0.0089	0.3728	7.76E-07	0.0339
cg17378686	chr3:46925524	PTH1R	-0.0073	0.2989	8.23E-07	0.0339
cg25826463	chr19:3369820	NFIC	-0.0186	0.7904	8.93E-07	0.0348
cg26906447	chr22:22093503		-0.0122	0.5111	9.93E-07	0.0366
cg23239444	chr6:34433320	PACSIN1	-0.0055	0.2267	1.14E-06	0.0395
cg15651103	chr9:96357066	MIR548AU; PHF2	-0.0020	0.0817	1.18E-06	0.0395
cg15882726	chr4:114293805	ANK2	0.0186	0.8084	1.28E-06	0.0401
cg14613413	chr2:187244031		-0.0041	0.1839	1.32E-06	0.0401
cg00630993	chr4:158122378		-0.0158	0.6681	1.57E-06	0.0443
cg08917665	chr12:9861036		-0.0044	0.1959	1.60E-06	0.0443
cg22681218	chr19:14636831		-0.0072	0.3194	1.64E-06	0.0443
cg17029220	chr1:210709518	HHAT	-0.0079	0.3357	1.77E-06	0.0459

Supplemental Table 4-3. Gene ontology (GO) terms and pathways with a significant ($q < 0.10$) overrepresentation of differentially methylated genes associated with maternal toenail Pb.

GO term	**q-value**	**GO category**	**Term name**
GO:0007156	7.01E-24	BP	homophilic cell adhesion via plasma membrane adhesion molecules
GO:0098742	1.01E-18	BP	cell-cell adhesion via plasma-membrane adhesion molecules
GO:0098609	2.44E-12	BP	cell-cell adhesion
GO:0005509	3.11E-12	MF	calcium ion binding
GO:0007155	5.02E-09	BP	cell adhesion
GO:0044459	1.33E-07	CC	plasma membrane part
GO:0031226	1.88E-06	CC	intrinsic component of plasma membrane
GO:0005887	4.23E-06	CC	integral component of plasma membrane
GO:0043169	4.09E-05	MF	cation binding
GO:0046872	8.62E-05	MF	metal ion binding
GO:0005886	0.001	CC	plasma membrane
GO:0031224	0.001	CC	intrinsic component of membrane
GO:0071944	0.001	CC	cell periphery
GO:0016021	0.002	CC	integral component of membrane
GO:0043167	0.007	MF	ion binding
GO:0007399	0.013	BP	nervous system development
GO:0031252	0.029	CC	cell leading edge
GO:0003689	0.088	MF	DNA clamp loader activity
GO:0031256	0.088	CC	leading edge membrane
BP: biological process; CC: cellular component; MF: molecular function			
Pathway ID	**q-value**	**Pathway Source**	**Pathway description**
rac1_reg_pathway	0.094	PID	Regulation of RAC1 activity
WP4746	0.094	Wikipathways	Thyroid hormones production and their peripheral downstream signaling effects
ecadherin_stabilization_pathway	0.094	PID	Stabilization and expansion of the E-cadherin adherens junction
path:hsa04662	0.094	KEGG	B cell receptor signaling pathway - Homo sapiens (human)
R-HSA-109581	0.094	Reactome	Apoptosis
R-HSA-193648	0.094	Reactome	NRAGE signals death through JNK
R-HSA-5357801	0.094	Reactome	Programmed Cell Death
R-HSA-5218920	0.094	Reactome	VEGFR2 mediated vascular permeability
R-HSA-2871809	0.094	Reactome	FCERI mediated Ca+2 mobilization
R-HSA-8876493	0.094	Reactome	InlA-mediated entry of Listeria monocytogenes into host cells
WP185	0.094	Wikipathways	Integrin-mediated Cell Adhesion

path:hsa04922	0.094	KEGG	Glucagon signaling pathway - Homo sapiens (human)
R-HSA-114452	0.094	Reactome	Activation of BH3-only proteins
path:hsa04929	0.094	KEGG	GnRH secretion - Homo sapiens (human)
R-HSA-198693	0.094	Reactome	AKT phosphorylates targets in the nucleus
R-HSA-391908	0.094	Reactome	Prostanoid ligand receptors
R-HSA-9607240	0.098	Reactome	FLT3 Signaling
None	0.098	INOH	IGF signaling
path:hsa04924	0.098	KEGG	Renin secretion - Homo sapiens (human)
WP4239	0.098	Wikipathways	Epithelial to mesenchymal transition in colorectal cancer
R-HSA-204998	0.098	Reactome	Cell death signalling via NRAGE, NRIF and NADE
path:hsa04917	0.098	KEGG	Prolactin signaling pathway - Homo sapiens (human)
R-HSA-9614399	0.098	Reactome	Regulation of localization of FOXO transcription factors
atr_pathway	0.098	PID	ATR signaling pathway
R-HSA-9634638	0.098	Reactome	Estrogen-dependent nuclear events downstream of ESR-membrane signaling
R-HSA-416482	0.098	Reactome	G alpha (12/13) signalling events
R-HSA-5693616	0.098	Reactome	Presynaptic phase of homologous DNA pairing and strand exchange
R-HSA-157858	0.098	Reactome	Gap junction trafficking and regulation
R-HSA-1358803	0.098	Reactome	Downregulation of ERBB2:ERBB3 signaling
R-HSA-73887	0.098	Reactome	Death Receptor Signalling
R-HSA-9013149	0.098	Reactome	RAC1 GTPase cycle
R-HSA-162582	0.098	Reactome	Signal Transduction
R-HSA-9012999	0.098	Reactome	RHO GTPase cycle
R-HSA-5693579	0.098	Reactome	Homologous DNA Pairing and Strand Exchange
rhoa_reg_pathway	0.098	PID	Regulation of RhoA activity
R-HSA-391903	0.098	Reactome	Eicosanoid ligand-binding receptors
R-HSA-1250347	0.098	Reactome	SHC1 events in ERBB4 signaling
path:hsa04068	0.098	KEGG	FoxO signaling pathway - Homo sapiens (human)
WP4806	0.099	Wikipathways	EGFR Tyrosine Kinase Inhibitor Resistance
ptenpathway	0.099	BioCarta	pten dependent cell cycle arrest and apoptosis
R-HSA-111447	0.099	Reactome	Activation of BAD and translocation to mitochondria
path:hsa04210	0.099	KEGG	Apoptosis - Homo sapiens (human)
fanconi_pathway	0.099	PID	Fanconi anemia pathway
path:hsa04540	0.099	KEGG	Gap junction - Homo sapiens (human)
arf6_traffickingpathway	0.099	PID	Arf6 trafficking events
insulin_pathway	0.099	PID	Insulin Pathway
longevitypathway	0.099	BioCarta	the igf-1 receptor and longevity
R-HSA-1963640	0.099	Reactome	GRB2 events in ERBB2 signaling

Supplemental Table 4-4. Gene ontology (GO) terms and pathways with a significant ($q < 0.10$) overrepresentation of differentially methylated genes associated with infant toenail Pb.

GO term[+]	q-value	GO category	Term name
GO:0007156	1.91E-11	BP	homophilic cell adhesion via plasma membrane adhesion molecules
GO:0098742	6.98E-09	BP	cell-cell adhesion via plasma-membrane adhesion molecules
GO:0005509	3.24E-08	MF	calcium ion binding
GO:0044459	2.06E-07	CC	plasma membrane part
GO:0098609	1.36E-05	BP	cell-cell adhesion
GO:0005887	7.11E-05	CC	integral component of plasma membrane
GO:0031226	7.96E-05	CC	intrinsic component of plasma membrane
GO:0007155	3.00E-04	BP	cell adhesion
GO:0007399	4.40E-04	BP	nervous system development
GO:0048856	0.001	BP	anatomical structure development
GO:0071944	0.001	CC	cell periphery
GO:0005886	0.001	CC	plasma membrane
GO:0048468	0.002	BP	cell development
GO:0021695	0.002	BP	cerebellar cortex development
GO:0048731	0.003	BP	system development
GO:0061032	0.004	BP	visceral serous pericardium development
GO:0007275	0.004	BP	multicellular organism development
GO:0021549	0.004	BP	cerebellum development
GO:0048645	0.004	BP	animal organ formation
GO:0030902	0.004	BP	hindbrain development
GO:0022037	0.004	BP	metencephalon development
GO:0030182	0.008	BP	neuron differentiation
GO:0023061	0.009	BP	signal release
GO:0009887	0.009	BP	animal organ morphogenesis
GO:0061005	0.011	BP	cell differentiation involved in kidney development
GO:0048666	0.011	BP	neuron development
GO:0048771	0.012	BP	tissue remodeling
GO:0032989	0.017	BP	cellular component morphogenesis
GO:0021680	0.017	BP	cerebellar Purkinje cell layer development
GO:0035107	0.017	BP	appendage morphogenesis
GO:0048598	0.017	BP	embryonic morphogenesis
GO:0007442	0.017	BP	hindgut morphogenesis
GO:0007389	0.020	BP	pattern specification process
GO:0048699	0.021	BP	generation of neurons
GO:0022008	0.022	BP	neurogenesis
GO:0030073	0.022	BP	insulin secretion
GO:0035113	0.022	BP	embryonic appendage morphogenesis

GO:0007507	0.024	BP	heart development
GO:0042476	0.024	BP	odontogenesis
GO:0048562	0.024	BP	embryonic organ morphogenesis
GO:0000902	0.024	BP	cell morphogenesis
GO:0001501	0.024	BP	skeletal system development
GO:0048568	0.024	BP	embryonic organ development
GO:2000242	0.024	BP	negative regulation of reproductive process
GO:0061326	0.024	BP	renal tubule development
GO:0035108	0.026	BP	limb morphogenesis
GO:0000904	0.027	BP	cell morphogenesis involved in differentiation
GO:0051174	0.027	BP	regulation of phosphorus metabolic process
GO:0030326	0.027	BP	embryonic limb morphogenesis
GO:0046879	0.028	BP	hormone secretion
GO:0072102	0.030	BP	glomerulus morphogenesis
GO:0021696	0.030	BP	cerebellar cortex morphogenesis
GO:0098589	0.031	CC	membrane region
GO:0009914	0.033	BP	hormone transport
GO:0060573	0.033	BP	cell fate specification involved in pattern specification
GO:0035850	0.033	BP	epithelial cell differentiation involved in kidney development
GO:0042475	0.034	BP	odontogenesis of dentin-containing tooth
GO:0001773	0.041	BP	myeloid dendritic cell activation
GO:0048736	0.041	BP	appendage development
GO:0021910	0.042	BP	smoothened signaling pathway involved in ventral spinal cord patterning
GO:0030072	0.042	BP	peptide hormone secretion
GO:0009306	0.043	BP	protein secretion
GO:0035773	0.043	BP	insulin secretion involved in cellular response to glucose stimulus
GO:0021681	0.044	BP	cerebellar granular layer development
GO:0021587	0.044	BP	cerebellum morphogenesis
GO:0009790	0.044	BP	embryo development
GO:0009799	0.044	BP	specification of symmetry
GO:0030154	0.044	BP	cell differentiation
GO:0009653	0.045	BP	anatomical structure morphogenesis
GO:0003207	0.045	BP	cardiac chamber formation
GO:0021521	0.045	BP	ventral spinal cord interneuron specification
GO:0061525	0.045	BP	hindgut development
GO:0021575	0.046	BP	hindbrain morphogenesis
GO:0003211	0.047	BP	cardiac ventricle formation
GO:0009954	0.047	BP	proximal/distal pattern formation
GO:0001709	0.048	BP	cell fate determination
GO:0010817	0.049	BP	regulation of hormone levels

GO:0021532	0.050	BP	neural tube patterning
GO:0023051	0.051	BP	regulation of signaling
GO:0072006	0.051	BP	nephron development
[+] First 80 GO terms listed.			
BP: biological process; CC: cellular component; MF: molecular function			
Pathway ID	**q-value**	**Pathway Source**	**Pathway description**
R-HSA-422356	0.031	Reactome	Regulation of insulin secretion
R-HSA-1266738	0.031	Reactome	Developmental Biology
R-HSA-5610787	0.031	Reactome	Hedgehog ,off, state
R-HSA-5358351	0.031	Reactome	Signaling by Hedgehog
nos1pathway	0.031	BioCarta	nitric oxide signaling pathway
R-HSA-163685	0.043	Reactome	Integration of energy metabolism
R-HSA-373760	0.045	Reactome	L1CAM interactions
path:hsa04340	0.046	KEGG	Hedgehog signaling pathway - Homo sapiens (human)
R-HSA-5635851	0.051	Reactome	GLI proteins bind promoters of Hh responsive genes to promote transcription
R-HSA-444473	0.058	Reactome	Formyl peptide receptors bind formyl peptides and many other ligands
path:hsa04020	0.058	KEGG	Calcium signaling pathway - Homo sapiens (human)
None	0.058	INOH	Hedgehog
WP4787	0.058	Wikipathways	Osteoblast differentiation
path:hsa05200	0.058	KEGG	Pathways in cancer - Homo sapiens (human)
R-HSA-112316	0.058	Reactome	Neuronal System
R-HSA-112315	0.058	Reactome	Transmission across Chemical Synapses
R-HSA-111933	0.058	Reactome	Calmodulin induced events
R-HSA-111997	0.058	Reactome	CaM pathway
path:hsa05202	0.058	KEGG	Transcriptional misregulation in cancer - Homo sapiens (human)
R-HSA-111996	0.061	Reactome	Ca-dependent events
R-HSA-1489509	0.064	Reactome	DAG and IP3 signaling
WP306	0.064	Wikipathways	Focal Adhesion
R-HSA-442720	0.064	Reactome	CREB1 phosphorylation through the activation of Adenylate Cyclase
agpcrpathway	0.064	BioCarta	attenuation of gpcr signaling
WP4947	0.064	Wikipathways	NO metabolism in cystic fibrosis
WP4249	0.064	Wikipathways	Hedgehog Signaling Pathway
WP4823	0.064	Wikipathways	Genes controlling nephrogenesis
nfatpathway	0.066	BioCarta	nfat and hypertrophy of the heart
a6b1_a6b4_integrin_pathway	0.067	PID	a6b1 and a6b4 Integrin signaling
WP5053	0.067	Wikipathways	Development of ureteric collection system
R-HSA-422475	0.067	Reactome	Axon guidance
R-HSA-6788467	0.067	Reactome	IL-6-type cytokine receptor ligand interactions

R-HSA-112310	0.067	Reactome	Neurotransmitter release cycle
R-HSA-112043	0.067	Reactome	PLC beta mediated events
WP1991	0.067	Wikipathways	SRF and miRs in Smooth Muscle Differentiation and Proliferation
WP47	0.067	Wikipathways	Hedgehog Signaling Pathway Netpath
R-HSA-425393	0.067	Reactome	Transport of inorganic cations/anions and amino acids/oligopeptides
cd8tcrpathway	0.067	PID	TCR signaling in naïve CD8+ T cells
R-HSA-9662360	0.067	Reactome	Sensory processing of sound by inner hair cells of the cochlea
R-HSA-164378	0.067	Reactome	PKA activation in glucagon signalling
R-HSA-163615	0.067	Reactome	PKA activation
sppapathway	0.067	BioCarta	aspirin blocks signaling pathway involved in platelet activation
R-HSA-9675108	0.070	Reactome	Nervous system development
R-HSA-111931	0.070	Reactome	PKA-mediated phosphorylation of CREB
R-HSA-1181150	0.070	Reactome	Signaling by NODAL
R-HSA-112040	0.071	Reactome	G-protein mediated events
R-HSA-3000178	0.071	Reactome	ECM proteoglycans
R-HSA-9659379	0.071	Reactome	Sensory processing of sound
path:hsa04928	0.072	KEGG	Parathyroid hormone synthesis, secretion and action - Homo sapiens (human)
R-HSA-6783589	0.091	Reactome	Interleukin-6 family signaling
R-HSA-373753	0.091	Reactome	Nephrin family interactions
tcr_pathway	0.091	PID	TCR signaling in naïve CD4+ T cells
WP474	0.091	Wikipathways	Endochondral Ossification
WP4808	0.091	Wikipathways	Endochondral Ossification with Skeletal Dysplasias
R-HSA-210500	0.091	Reactome	Glutamate Neurotransmitter Release Cycle
hedgehog_2pathway	0.091	PID	Signaling events mediated by the Hedgehog family
gpcrpathway	0.094	BioCarta	signaling pathway from g-protein families
WP4172	0.094	Wikipathways	PI3K-Akt signaling pathway
R-HSA-1500931	0.094	Reactome	Cell-Cell communication

Supplemental Table 4-5. Gene ontology (GO) terms and pathways with a significant ($q < 0.10$) overrepresentation of differentially methylated genes associated with placental Pb.

GO term	q-value	GO category	Term name
GO:0004667	0.019	MF	prostaglandin-D synthase activity
GO:0015184	0.019	MF	L-cystine transmembrane transporter activity
GO:0051279	0.023	BP	regulation of release of sequestered calcium ion into cytosol
GO:0070412	0.027	MF	R-SMAD binding
GO:0000099	0.029	MF	sulfur amino acid transmembrane transporter activity
GO:0008528	0.029	MF	G protein-coupled peptide receptor activity
GO:0070410	0.029	MF	co-SMAD binding
GO:0010522	0.043	BP	regulation of calcium ion transport into cytosol
GO:0051283	0.059	BP	negative regulation of sequestering of calcium ion
GO:0051282	0.059	BP	regulation of sequestering of calcium ion
GO:0070567	0.089	MF	cytidylyltransferase activity
GO:0035257	0.093	MF	nuclear hormone receptor binding
BP: biological process; MF: molecular function			

Supplemental Table 4-6. Gene ontology (GO) terms and pathways with a significant ($q < 0.10$) overrepresentation of differentially hydroxymethylated genes associated with maternal toenail Pb.

GO term	q-value	GO category	Term name
GO:0050807	0.065	BP	regulation of synapse organization
GO:0050803	0.069	BP	regulation of synapse structure or activity
GO:0050808	0.071	BP	synapse organization
BP: biological process			

Supplemental Table 4-7. Gene ontology (GO) terms and pathways with a significant ($q < 0.10$) overrepresentation of differentially hydroxymethylated genes associated with infant toenail Pb.

GO term	**q-value**	**GO category**	**Term name**
GO:0004674	0.006	MF	protein serine/threonine kinase activity
GO:0034673	0.021	CC	inhibin-betaglycan-ActRII complex
GO:0048185	0.025	MF	activin binding
GO:0004675	0.032	MF	transmembrane receptor protein serine/threonine kinase activity
GO:0071495	0.073	BP	cellular response to endogenous stimulus
GO:0002457	0.073	BP	T cell antigen processing and presentation
GO:0004672	0.074	MF	protein kinase activity
GO:0071772	0.077	BP	response to BMP
GO:0140096	0.088	MF	catalytic activity, acting on a protein
GO:0046332	0.093	MF	SMAD binding
GO:0005524	0.093	MF	ATP binding
GO:0016772	0.093	MF	transferase activity, transferring phosphorus-containing groups
GO:0043168	0.093	MF	anion binding
GO:0015232	0.095	MF	heme transporter activity
BP: biological process; CC: cellular component; MF: molecular function			
Pathway ID	**q-value**	**Pathway Source**	**Pathway description**
WP4816	0.077	Wikipathways	TGF-beta receptor signaling in skeletal dysplasias
path:hsa05220	0.077	KEGG	Chronic myeloid leukemia - Homo sapiens (human)
R-HSA-6811440	0.077	Reactome	Retrograde transport at the Trans-Golgi-Network
None	0.077	INOH	GPCR Dopamine D1like receptor
WP560	0.077	Wikipathways	TGF-beta Receptor Signaling
rnapol3pathway	0.077	BioCarta	rna polymerase iii transcription
WP4904	0.077	Wikipathways	LDLRAD4 and what we know about it
None	0.077	INOH	BMP2 signaling TGF-beta MV
alk1pathway	0.077	PID	ALK1 signaling events

Supplemental Table 4-8. Gene ontology (GO) terms and pathways with a significant ($q < 0.10$) overrepresentation of differentially hydroxymethylated genes associated with placental Pb.

GO term	q-value	GO category	Term name
GO:0072525	0.024	BP	pyridine-containing compound biosynthetic process
GO:0035904	0.024	BP	aorta development
GO:0036449	0.059	CC	microtubule minus-end
BP: biological process; CC: cellular component			

Chapter 5: Summary and conclusions

Summary

Prenatal exposure to the ubiquitous toxic metals in our environment has been shown to result in adverse health outcomes in the vulnerable children's population. Alluding to the DOHaD hypothesis, the intrauterine environment that is critical to promoting normal development and growth can be especially susceptible to these environmental triggers. Establishing the associations between toxic metals quantified in placental tissue and atypical neurobehavior added to existing evidence the consequential effects of prenatal environmental metal influences on newborn health that may persist throughout the life course.

While there are population-based studies documenting the negative impacts of neurotoxic metals on children's neurodevelopment, exposure to metals most likely occurs concurrently and not in isolation. The need to understand the impact of multiple exposures brought about the emerging field of mixtures studies. With the variety of compounds concomitantly present in our environment, assessment of the combined impact of both non-essential and essential metals and trace elements on neurobehavior not only more accurately reflects the exposure patterns of the study population, but also highlights the fact that low levels of multiple exposures during the critical developmental period may jointly induce negative neurobehavioral outcomes early in life.

During gestation, the placenta is a diverse organ integral to the transport and metabolism of a myriad of biological compounds as well as in the production of growth and neuroactive factors critical for appropriate fetal development. It also plays a central role in fetal developmental programming by responding to the environment and driving physiologic change in the fetus. Epigenetic mechanisms, including DNA methylation and the more recently established mark, hydroxymethylation, have been postulated as the underlying factors linking prenatal perturbations

and developmental implications. However, little is known about the association between placental epigenetic modifications and metal exposures, particularly in the human population. Thus, understanding the placental epigenome through DNA methylation and hydroxymethylation may clarify the mechanisms of which developmental toxicants such as lead exert its toxic effects in the prenatal period and within a highly relevant tissue, the placenta.

This body of work applied a variety of approaches to investigate prenatal metal exposure and the potential neurobehavioral and epigenetic effects in participants from RICHS, a U.S. birth cohort study. The results demonstrated that early life environmental metal influences adversely impacted neurobehavior, and resulted in epigenetic modifications in the placental epigenome.

In Chapter 2, we utilized placental levels of metal exposures and demonstrated that placental cadmium (Cd) was associated with atypical neurobehavior, and newborns with detectable lead (Pb) was also at higher risks of showing atypical neurobehavioral performances. Our work provided additional evidence on prenatal metal exposure characteristics through the placenta as a biomarker, and may suggest a specificity for impacts of certain metals through impacts on the placenta. Additionally, RICHS NNNS profile patterns coincided with patterns from the at-risk newborn population NNNS was initially designed to examine, suggesting the generalizability and reproducibility of latent profile analysis profile classification in varying study settings. Our application of NNNS profiles adds to current knowledge that NNNS can serve as a useful assessment tool for neurobehavior within hours of life in healthy, low-risk newborns. Overall, the findings demonstrated that even at subtle levels, prenatal exposure to toxic metals Cd and Pb negatively impacted newborn neurobehavioral performance.

In Chapter 3, we applied quantile g-computation to assess the overall metal mixtures effect on newborn neurobehavior and found that placental metal mixture was associated with an

increased risk of atypical neurobehavior. In addition, placental Cd was labeled as the "bad actor" of the overall impact on neurobehavior in our findings. Through the mixtures approach, we were able to evaluate co-existing metals and trace elements for their partial positive or negative effects on neurobehavior. Notably, elements such as copper and selenium portrayed opposite effects on the outcome as the non-essential cadmium, which fit our expectations given these elements' essential features in biological and cellular processes. Taken together, the findings showed that concurrent exposure to placental metal mixtures increased the risk of atypical neurobehavior, and the identification of the driving factor of the mixture impact on neurobehavior can be useful for effective mitigation efforts to ensure better newborn health. They also further support a role for the placenta in mediating the impacts of toxic trace metal exposures on newborn neurodevelopment.

In Chapter 4, we explored the association between Pb exposure and placental epigenetic changes. We observed prenatal Pb exposure quantified from three distinct biomarkers was significantly associated with differential methylation and hydroxymethylation in various numbers of CpG sites. Comparing across the EWAS results, maternal toenail Pb was associated with the highest number of significantly differentially methylated sites, while the highest number of significantly differentially hydroxymethylated sites was associated with placental Pb. Moreover, overrepresentation analysis showed that biological functions and pathways involving major organ and nervous system development, calcium transport and regulation and cell signaling activities were significantly enriched for Pb-associated genes. This work is the first to link prenatal Pb exposure to epigenome-wide DNA methylation in human placenta, and this is also one of the few studies that investigated hydroxymethylation as an epigenetic mark in relation to environmental metal exposures. In summary, our findings suggested placental DNA methylation and

hydroxymethylation may act as response markers for prenatal Pb exposure and shed insight on the critical placental functions consequently affected upon exposure.

Limitations and future directions

Our study design demonstrated the utilization of placental tissue as a metal biomarker and established the pertinent application of placental tissue in understanding prenatal environmental exposure characteristics. The presented study findings showed robust associations between placental toxic metals (Cd and Pb) and atypical neurobehavior, and further determined exposure to a mixture of placental metals also increased the risk of atypical neurobehavior in newborns.

The placenta is a multifaceted organ important to the prenatal period, but there are limited studies that addressed children's environmental health research from the placental perspective. While our work underlined the tissue's features in prenatal metal exposure and adverse newborn neurobehavior, larger sample sizes would be needed for verification of these findings and for providing more robust associations on prenatal metal exposures and adverse neurodevelopmental outcomes. Health effects elicited from concomitant metal exposures may differ from those resulted from individual metal exposure, hence, particularly in setting environments where overall toxicant exposure levels are low, larger sample sizes will have the improved ability to pinpoint the underlying impacts between multiple metal exposures and outcome. Furthermore, such studies may be able to properly distinguish linear and non-linear associations and detect potential sex-specific differences in the target population.

Our mixtures study design enabled us to identify the driving factor (placental Cd) of the combined metal mixtures effect on newborn neurobehavior. This identification can be especially useful for agencies to establish guidelines and interventions to effectively eliminate the specific

metal's adverse impact on human health. Future mixtures studies motivated by the need for exposure reduction strategies in the vulnerable children's population may similarly apply this methodology. Moreover, future research may improve the models by also incorporating non-chemical stressors, including acculturation, stress, neighborhood, and nutritional factors, as well as additional chemical stressors, in the exposure mixture for their potential influences on children's health.

Our findings observed that prenatal exposure to Pb, quantified through both placental tissues and toenails, induced epigenetic modifications through placental DNA methylation and hydroxymethylation. Placental epigenetic dysregulation was further shown to result in altered biological functions and pathways that are involved in developmental processes.

While it is evident that prenatal exposure to metals adversely impacts children's health, further research on characterizing the intrauterine environment and distinguishing the role of placental epigenome changes upon exposure is needed. In the human population, DNA methylation patterns are relatively well profiled in various tissues, yet the hydroxymethylation landscape in tissues other than stem cells remain to be determined. Our work explored not only DNA methylation, but also the newly emerged epigenetic mark, hydroxymethylation, in the human placental epigenome and was able to discern the links between prenatal Pb and differential epigenetic modifications, along with associations to disrupted placental functions. If this study design can be extended to examine other developmental toxicants such as arsenic and mercury, and the associated placental epigenetic modifications, findings could add to current understandings of epigenetics as the underlying mechanism for metals' impacts on intrauterine health and the consequential offspring growth and development processes. In addition, different types of epigenetic mechanism, including histone modification and microRNAs, should also be

incorporated in the study of prenatal toxic metal exposures' effect on the placental epigenome to fully substantiate the role of epigenetic regulations in the predisposition of potential long-term health outcomes.

Referring to the DOHaD concept, more work is needed to extend our understandings on prenatal Pb-induced, epigenetic-dysregulated placental pathways and potential neurodevelopmental phenotypes later in early childhood or adult life. Neurodevelopmental and behavioral traits may continue to develop and change throughout childhood and into early adolescence. Standardized assessments appropriate for older children, such as the Child Behavioral Checklist or The Bayley Scales of Infant and Toddler Development, may help characterize their motor activities and cognitive functions later in life. Likewise, physician-diagnosed neurodevelopmental disorders such as autism spectrum disorder, may also be an associated endpoint with origins traced back to prenatal and placental toxicant exposure. Therefore, designing longitudinal cohorts with the incorporation of later-life neurophysiological assessment data under this study framework is warranted in future studies.

Conclusion

In conclusion, this work underscored the associations between environmental metal exposure, both individually and as a mixture, and adverse neurobehavioral performances. Furthermore, our unique approach of understanding human placental epigenome changes upon prenatal metal exposure added to the crosstalk between exogenous stressors and intrauterine environment disruptions during the critical developmental period. Collectively, our work emphasized the placenta's role as a mediator in the complex interrelations among exposure

characteristics, epigenetic mechanisms, and the implied health outcomes later in the life course, and demonstrated a pivotal study avenue in the children's environmental health field.

Reference

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