Fetal Epigenetic Reprogramming: Evaluating the Role of Exposure to Maternal Dietary Metabolites and Bisphenols

by

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Dedication

To those who taught me the careful art of observation, to listen, and to always remain curious.

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Abstract

The Developmental Origins of Health and Disease (DOHaD) paradigm correlates a suboptimal intrauterine environment to increased risk of chronic disease. This association is well accepted, and recent work indicates that environmentally induced changes in cellular function and disease etiology are mediated by changes in the epigenetic profile. For the purpose of this dissertation, epigenetics is defined as the study of mitotically and/or meiotically heritable changes in gene expression that cannot be explained by changes in DNA sequence. While we are beginning to understand the outcomes associated with prenatal exposure to components like the maternal diet and bisphenol-A (BPA), few studies in humans assess the epigenetic impact of these exposures. In order to accurately discern the association between maternal exposure and epigenetic reprogramming, studies are needed that evaluate prenatal exposure to both bisphenols and maternal diet during early pregnancy. Additionally, more research is needed that establishes epigenetic signatures of exposure in peripheral tissues. We utilized the Michigan Mother-Infant Pairs (MMIP) cohort to assess (Aim 1) the association of prenatal exposure to the bisphenols BPA, BPF, and BPS and infant cord blood leukocyte DNA methylation, (Aim 2) maternal one-carbon metabolites across pregnancy and in association with DNA methylation in infant cord blood leukocytes, (Aim 3a) DNA methylation at candidate genes across three tissue types and their association with bisphenol exposure, and (Aim 3b) the association between prenatal bisphenol exposure and placental DNA methylation. Cutting edge techniques of microarray technology to profile DNA methylation at >850,000 CpG sites in both infant cord blood and

placenta, untargeted metabolomics, and pyrosequencing were combined to answer our research questions.

Aim 1 results suggest that prenatal exposure to BPA, as measured in maternal urine between 8-14 weeks of gestation, was significantly associated with differential DNA methylation at 38 CpG sites and three differentially methylated regions. Pathway analysis of BPA-associated CpG sites revealed enrichment for pathways associated with the nervous system, immune response, and neuroinflammation. Aim 2 results suggest that maternal third trimester and cord blood one-carbon metabolite S-adenosylhomocysteine (SAH) were significantly correlated with a shift in the global distribution of DNA methylation in infant cord blood leukocytes. This aim also provides evidence of patterns of one-carbon metabolites within time points and across pregnancy. For example, concentrations of maternal choline increased from first trimester to term, while betaine concentrations decreased; possibly reflecting the shift in choline dynamics during pregnancy. Aim 3a results suggest that candidate gene DNA methylation and tissue-specific associations with prenatal bisphenol exposure do significantly differ at some but not all genes tested across cord blood, cord tissue, and placenta from the same individuals. For example, mixed effect regression revealed that placental tissue DNA methylation was significantly associated with bisphenol exposure at three out of four genes, as compared to one in cord blood and none in cord tissue. These data may inform selection of surrogate tissues for environmental epigenetic studies. Lastly, Aim 3b exploratory analysis of epigenome-wide placental DNA methylation suggests prenatal exposure to bisphenols may be associated with alternations in pathways related to inflammation, vascularization, and preeclampsia.

This dissertation contributes to the burgeoning field of epigenomics and helps to establish a foundation in our understanding of maternal exposures and their influences on epigenetic

programming. Advances in epigenome-wide association studies will ultimately enable researchers, clinicians, and policymakers to target risk factors (e.g. environmental and nutritional exposures that perturb the methylome and downstream birth outcomes) and to better understand a portion of the myriad of elements underlying the developmental origins of health and disease.

Chapter 1 An Introduction to Environmental Epigenetic Studies

The intervening time between preconception and early infancy is critical in posturing an individual's lifetime health trajectory. Interruptions to typical human development, whether major, acute events or chronic exposures, can have measurable consequences as we grow and age. The Developmental Origins of Health and Disease (DOHaD) paradigm correlates a suboptimal intrauterine environment to increased risk of chronic disease. The theory of DOHaD is a foundational pillar of the work contained within this dissertation and also forms the basis of most early-life exposure research (Wadhwa et al., 2009). The field of Developmental Origins is principally attributed to David Barker; his early epidemiological work on infant and adult mortality during the late-1980's and early-1990's, and the associations he detected, has since evolved into a significantly sized field of study with a wide array of topic areas.

Barker's cardinal study investigated the geographic correlation between infant mortality in England and Wales from 1921-1925 and ischemic heart disease from 1968-1978 (Barker & Osmond, 1986). He concluded that early-life nutrition and variations therein, as related to geographic location and socioeconomic status, influenced the associated-risk and risk factors for developing heart disease later in life. This study was followed by a retrospective cohort study that evaluated men born in Hertfordshire, England between 1911-1930 and who subsequently died of an ischemic heart-related event (Barker et al., 1989). Barker and colleagues detected that those individuals born with the lowest birth weights had the highest rates of death and those with the highest birth weights had the lowest rates of death (Barker et al., 1989). Barker refined his earlier conclusions to include the notion that an early-life environment that negatively impacts

fetal and infant growth is likely followed by an adult environment with increased risk for heart disease. Barker's final work in this triptych assessed fetal undernutrition at different stages of gestation and the consequent impact on birth and adult metabolic outcomes (Barker et al., 1993). It was with this study that Barker determined that fetal undernutrition during pregnancy is correlated to a dysfunctional relationship between glucose and insulin and results in a reprogramming of key metabolic and growth factors; the perturbation of which subsequently correspond to heart disease risk during adulthood.

The conclusions generated from Barker's work are now fundamental to the way in which many scientists approach the study of developmental origins of health and disease. That an insult experienced during early life, which perturbs the prenatal environment, can be harmful to a growing fetus for its timing, duration, or nature; and then, manifest itself in shifting the risk framework for cardiometabolic and other diseases in adulthood (Lau et al., 2011). This capsule is defined as *developmental reprogramming* and it has supported the modern progression of the field to investigate an immense depth and breadth of exposures and outcomes; from air pollution and psychosocial stress to behavioral conditions and cancer. Naturally, with the discovery of these associations, comes an interest in determining the possible mechanisms of developmental reprogramming. These include, but are not limited to, alternations in nutrient sensing pathways (Brumbaugh & Friedman, 2014; Padmanabhan et al., 2016), epigenetic modifications (Guay et al., 2019), hormone and endocrine systems (Veiga-Lopez et al., 2016), and stem cell precursors (Sen et al., 2015). In other words, reprogramming can occur at the organ, cellular, and epigenetic level (Ross & Desai, 2013; Tarry-Adkins & Ozanne, 2011). That permanent effects on tissue function, cellular proportions, or gene expression might be the result of an early-life exposure has been demonstrated principally through animal models, with evidence increasing in human

cohorts. This dissertation evaluates the potential effects of two early life exposures utilizing a developmental programming framework examining epigenetic changes – specifically to DNA methylation.

Epigenetics, development reprogramming, and DOHaD

Discussions of epigenetics often begin with Conrad H. Waddington and his utilization of the term in his studies of embryology and genetics beginning in the 1930s (Nicoglou & Merlin, 2017). Waddington was deeply interested in development and in understanding how the elements of an embryo and genes might interact to produce differentiated cells, structures, and the varieties of human form. After many years of study, he came to define epigenetics as an integrated network of interactions with the genotype and the environment, the result of which established an organism (Nicoglou & Merlin, 2017). Waddington's work and definition were foundational and later built upon by Barbara McClintock, François Jacob and Jacques Monod; Britten and Davidson, and Nanney to provide a deeper understanding of cellular differentiation during development and the regulation of gene expression. For the work presented in this dissertation we will use a definition of epigenetics as influenced by the works of Holliday (Holliday, 1994), Russo, Martienssen, & Riggs (Robertson, 2007); Skinner (Skinner, 2011), and Dolinoy and Jirtle (Dolinoy, Weidman, et al., 2007a). Wherein "epigenetics is the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence" (Dolinoy, Weidman, et al., 2007b; Holliday, 1994; Robertson, 2007). For the purposes of this dissertation we will evaluate the epigenetic modification of DNA methylation. DNA methylation is the process by which methyl groups ($-CH_3$) are enzymatically transferred from S-adenosylmethionine (SAM) to the carbon-5 position of the cytosine ring

(Dolinoy, Weidman, et al., 2007b; Mahmoud & Ali, 2019). Cytosine methylation typically occurs on those contained within CpG dinucleotides, and the 5-methylcytosine (5mC) created is functionally important in cellular processes—most notably by interfering with transcription machinery. The non-random distribution of CpG dinucleotides throughout the genome manifest as discrete regions with specific concentrations of CpGs; often referred to as CpG islands, shores, or shelfs (Dolinoy, Weidman, et al., 2007b). CpG islands, for example, contain at least 50% CpG content, are often located near promoter regions or first exons, and are often principally unmethylated (logically so as to allow for constitutive access to and transcription of a gene) (Dolinoy, Weidman, et al., 2007b). Alternatively, CpG sites located in the promoter or regulatory regions of transposable elements are typically highly methylated so as to suppress their activity. The study of DNA methylation in the context of DOHaD is relevant and critical as mechanism through which to characterize of an epigenetic link between the prenatal environment and offspring outcomes (McMillen & Robinson, 2005). Evidence suggests that these epigenetic mechanisms are susceptible to environmental influence, particularly during development.

Periods during which the epigenome experiences extensive reprogramming are gametogenesis and pre-implantation (Reik et al., 2001). Research has shown that this exceptionally dynamic time of epigenetic activity is defined, in part, by the asymmetric epigenomic landscape of the maternal and paternal genomes during embryogenesis (Cantone & Fisher, 2013). Proper methylation/demethylation and histone/chromatin interactions during these two sensitive time periods (the length of this stage is not inconsequential although it is quite short) can affect development later by interrupting sequential activation of the maternal/paternal genomes (Cantone & Fisher, 2013). During embryonic development, the epigenome is comprehensively reprogrammed at two critical time periods. Primordial germ cells and somatic

embryonic stem cells undergo genome-wide demethylation and then remethylation (Stein & Lee Davis, 2012). Preimplantation embryos also experience methylation reprogramming; wherein the paternal genome is actively demethylated and the maternal genome is passively demethylated (Messerschmidt et al., 2014; Reik et al., 2001). The maternal and paternal genomes are remethylated around the time of implantation. Environmental exposure during this crucial time has the potential to alter the normal epigenomic operating landscape via perturbation of activation kinetics of parental alleles (Gu et al., 2011). Interruptions in or incomplete epigenetic reprogramming of the fetal genome at either phase could lead to a *de novo* establishment of 'primary epimutations' (Hitchins, 2015). Alterations in DNA methylation in somatic embryonic stem cells and primordial germ cells could result in changes that could be propagated to subsequent cells and possibly influence development and disease later in life. (Marsit, 2015)

Exposures of particular interest when experienced during pregnancy, because of growing evidence of their association with changes in fetal epigenetic reprograming, are the group of toxicants called bisphenols and maternal levels of nutrients from the one-carbon metabolism pathway that supply methyl groups and are key components of DNA methylation reactions.

Exposure to Bisphenols and their role in fetal epigenetic reprogramming

Bisphenol-A (BPA) is a chemical commonly used in receipts, plastics, and food packaging with striking evidence demonstrating its role as an endocrine disruptor (Acconcia et al., 2015). Human exposure to this toxicant is considered to be 'ubiquitous,' principally because of its wide-spread use as components in every-day products. Exposure to BPA and two of its commonly-used replacement analogues, bisphenol-F (BPF) and bisphenol-S (BPS), are readily detectable in U.S. populations (Calafat et al., 2005, 2008; Lehmler et al., 2018; Thayer et al.,

2016). Recent reports indicate that BPA is detectable in over 95% of individuals (Acconcia et al., 2015). BPF and BPS have been increasingly utilized in place of BPA as a result of consumer and scientific-based advocacy efforts. This pressure effectively elicited the Food and Drug Administration's (FDA) ban of BPA in infant-related plastics and products (Siracusa et al., 2018). While it was and is significant that companies are removing BPA from their products, remarkably less is known about the toxicity or biological effects of exposure to BPF and BPS. New evidence suggests that these replacement chemicals, which have close structural similarities to BPA, may have comparable or increased levels of potency as endocrine disruptors and may also negatively impact the reproductive system (Eladak et al., 2015; Rochester & Bolden, 2015a; Siracusa et al., 2018). A recent systematic review compared the endocrine and physiological effects of BPA, BPF, and BPS and demonstrated that BPF and BPS have similar *in vitro* metabolism, potencies, and mechanisms of action to that of BPA and additional toxicity in separate hormonal actions (Rochester & Bolden, 2015b). It is unclear to what extent humans are exposed to BPF/S particularly as, in the United States, BPF and BPS were only recently added to the list of chemicals measured in the National Health and Nutrition Examination Survey (NHANES), appearing for the first time from 2013-2014 (Lehmler et al., 2018). Rates of detection for BPF and BPS in the total population were 66.5% and 89.4% respectively. Despite the changes that have been made in 'reducing' utilization of BPA, it is evident that exposure to this environmental toxicant is still commonplace and there are additional concerns about the chemicals used in its stead. It is important to underscore that exposure in general is of concern not simply because it is common, but also because of the amount of evidence that links bisphenol exposure to serious health effects.

Considerable work has been done evaluating BPA exposure *in vitro* and these studies provide foundational evidence for the mechanisms of action through which BPA may alter or impact health. For example, BPA interrupts the activity of endogenous estrogens and estrogen nuclear hormone receptors; it affects androgen systems, thyroid hormone function, development, differentiation, and function of the central nervous system; the immune system; and intracellular signal transduction pathways (Wetherill et al., 2007). And while there are far fewer *in vivo* studies, their findings similarly identify BPA exposure to interfere with the mechanisms detected *in vitro* and to furthermore be associated with cancer, developmental problems, diabetes, obesity, metabolic syndrome, and possibly infertility or subfertility (Acconcia et al., 2015). What's more, both *in vitro* and *in vivo* studies recognize the potential for BPA to elicit epigenetic dysregulation, particularly when experienced prenatally. Studies that investigate the association of prenatal BPA exposure with DNA methylation in mice observe differential methylation in genes and pathways involved in neuronal and inflammatory signaling (Weinhouse et al., 2016); intracellular signaling (Anderson et al., 2017); and repetitive elements (Faulk et al., 2016). Few studies have investigated prenatal exposure to BPA and DNA methylation in humans (Nahar et al., 2014, 2015). Although these studies contribute to understanding the impact of prenatal BPA exposure on specific tissues, little is known of the effect in full-term healthy infants or later in childhood. As of this writing, four human pregnancy cohort studies have evaluated the epigenetic impact of prenatal exposure to BPA in infants or children (Alavian-Ghavanini et al., 2018; Junge et al., 2018; Miura et al., 2019; Montrose et al., 2018) (Figure 1-1). There currently are no published studies that evaluate prenatal exposure to BPF and BPS.

Maternal dietary exposures

'Maternal exposure' also encapsulates maternal dietary quality—the growing fetus is similarly exposed to the constitutive components and metabolites of a mother's diet as it is environmental toxicants. There is significant evidence to support the notion that in mammals, the maternal diet can impact DNA methylation and fetal epigenetic reprogramming in her offspring (Canani et al., 2011; Hogg et al., 2012; Lau et al., 2011; Lillycrop & Burdge, 2011, 2015; Mathers, 2007; Saffery & Novakovic, 2014; Szyf, 2009; Thornburg et al., 2010) and increased risk of later life disease (e.g. obesity, type 2 diabetes and cardiovascular disease) (Ainge et al., 2011; Gluckman et al., 2007; McMillen & Robinson, 2005; Volpato et al., 2012). Most of this evidence derives from animal studies, with the field beginning to establish studies and reveal findings and correlations in human cohorts.

Studies performed in the viable yellow agouti (Avy) mouse, which has the distinct advantage of coat-color change as a visual biosensor, have been used to demonstrate the long-term impacts of perinatal exposures (Dolinoy, 2008; Waterland & Jirtle, 2004). One such study demonstrated that supplementation of the maternal diet with methyl donors (e.g. a diet high in folate) or the phytoestrogen genistein, negated the DNA hypomethylating effect of BPA exposure (Dolinoy et al., 2006; Dolinoy, Huang, et al., 2007). Human populations display significant variety in dietary patterns, yet few studies have evaluated the association between dietary pattern, BPA exposure and alterations in DNA methylation in a comprehensive and integrated approach.

A notable study done in humans was performed by Heijmans et al. in 2008, which evaluated the impact of periconceptional and prenatal exposure to the Dutch Winter Famine of 1944-45 (Heijmans et al., 2008). The study demonstrated that periconceptional exposure to famine resulted in significantly less DNA methylation of the maternally imprinted gene *IGF2*

more than 60 years later in affected individuals. *IGF2* is a gene that is fundamental in human growth and development, and it is one of the best-characterized epigenetically regulated loci (F. M. Smith et al., 2006). Another excellent example is provided by Steegers-Theunisson et al., who conducted a study that investigated the impact of maternal dietary supplementation with folic acid on DNA methylation of the *IGF2* differentially methylated region (DMR) in offspring (Steegers-Theunissen et al., 2009). They demonstrated that infants whose mothers consumed a folic acid supplement prior to pregnancy had a 4.5% higher *IGF2* DMR methylation, as compared to those infants whose mothers did not consume the supplement. Of increasing interest and promise in the field is the use of metabolomics to assess dietary intake or nutritional status of an individual and their associations with health and disease. Through the profiling of small-molecule chemicals, nutrition and diet can be integrated to larger scale and more complex system analyses (e.g. in conjunction with epigenomics and genomics data) (Claus & Swann, 2013; Jones et al., 2012; Shin et al., 2014).

A critical mechanism through which maternal dietary components might alter the fetal epigenome is the one-carbon metabolism pathway. One-carbon metabolism (OCM) is an integrated system of biochemical reactions catalyzed by enzymes and coenzymes (Anderson, Sant, et al., 2012; Mahmoud & Ali, 2019). Coenzymes take the form of dietary micronutrients such as folate, vitamin B12, vitamin B6, betaine, choline, and methionine. Through this system one-carbon groups ($-CH_3$) are transferred from donor molecules to proteins or DNA. S-adenosylmethionine (SAM) is the universal methyl donor for DNA methylation and is the cofactor for the key enzyme DNA methyltransferase (DNMT). Because one-carbon metabolism is a cycle, a fundamental element of its integrity is negative feedback; levels and availability of

micronutrient cofactors influence the cycle's ability to generate, maintain, and replenish the components of the cycle, while also swiftly responding to the cell's needs.

A growing body of evidence supports the role of methyl donors during pregnancy and their role in modifying DNA methylation in the infant. Investigations have evaluated gene-specific DNA methylation (Hoyo et al., 2011; Pauwels, Ghosh, Duca, Bekaert, Freson, Huybrechts, A. S. Langie, et al., 2017; Pauwels, Ghosh, Duca, Bekaert, Freson, Huybrechts, Langie, et al., 2017) and epigenome-wide DNA methylation (Joubert et al., 2016; Knight et al., 2018) in infants in response to maternal dietary or supplemental intake of one-carbon metabolites. Ultimately, the field has only begun to understand the dynamics of maternal OCM across pregnancy and how they are associated with DNA methylation in the infant. Larger studies that evaluate a broader range of metabolites, assess the association between maternal and infant levels at multiple time points, and interrogate the infant epigenome at additional genes are needed to both validate existing studies and provide additional evidence of the importance of one-carbon dynamics during pregnancy.

Approaches in environmental epigenomic studies

Environmental epigenomic studies that evaluate a prenatal exposure in humans, whether dietary or toxicant, often examine its association with DNA methylation in infant umbilical cord blood leukocytes (Herzog et al., 2020). A common topic of debate is whether cord blood serves as an accurate surrogate tissue in the measurement of these associations. Infant cord blood and whole blood in general is commonly utilized to measure epigenome-wide DNA in response to environmental exposure, because it is readily available, acceptable to collect (particularly in human birth cohorts), and the DNA methylation profile in whole blood is associated with a

variety of health conditions or exposures (Houseman et al., 2015). Recent work has started to integrate the use of algorithms that control for cell-composition effects of whole blood—a method that, when combined with epigenomic analysis in whole-blood DNA, offers the potential to assess many components of exposure response (Bakulski et al., 2016). However, increasing evidence also points to the potential for differential methylation across the epigenome to be tissue-and cell type-specific. For example, Herzog et al. detected tissue-specific differentially methylated regions (tDMRs) associated with human umbilical vein endothelial cells and with placenta (Herzog et al., 2020). Similar studies by Lin et al. and Wu et al. also detected tissue-specific results when comparing epigenome-wide DNA methylation between cord blood and cord tissue (Lin et al., 2017; Y. Wu et al., 2019). These findings are important to acknowledge, because with the advances in technology to survey epigenome-wide DNA methylation, comes the additional responsibility to select appropriate tissues for the exposure and outcomes of interest. Studies are needed that not only evaluate tissue-specific DNA methylation in multiple tissues, but also evaluate such differences in the context of response to exposure and offer this perspective in a paired fashion.

Currently, the correlation between exposure-induced epigenetic alterations in target and in surrogate tissues is unclear. Furthermore, this association may depend on the established epigenetic landscape of the tissues, the timing, route, and dose of exposure, among other components. Determining the utility of surrogate tissues in epigenomic analyses will enable more effective use of population-based studies to make connections between exposure, epigenetic changes, and the development of disease. Furthermore, it remains to be elucidated if cessation or elimination of pertinent exposure that may result in reversal of phenotype is associated with changes in the epigenome. This dissertation involves investigating first trimester

exposures—a highly relevant time in the context of epigenetic reprogramming. As a result, peripheral tissues like cord blood or cord tissue could be more appropriate for our research questions than they traditionally are, because of the propagation of early gestational epigenetic changes across tissues in the growing fetus.

A healthy placenta is essential for a successful pregnancy and proper fetal development

The placenta is a crucial mediator between mother and fetus; regulating nutrient supply and waste exchange. The placenta possesses an immense ability to adapt to the maternal environment, particularly during suboptimal conditions, ultimately, however, compromised placental development impacts fetal development (Myatt, 2006). Altered maternal environment, such as underfeeding, as primarily examined in animal models, has demonstrated to alter placental area (McCrabb et al., 1991), reduce placental vascularization and circulation (Reynolds et al., 2005), reduce placental nutrient transfer capacity (Maiiendran et al., 1993), and alter placental endocrine function (Sibley et al., 2004). DNA methylation and other epigenetic modifications are required for normal placental form and function (Tarrade et al., 2015), with evidence starting to indicate a role for altered placental DNA methylation in the etiology of adult or later-life disease. Furthermore, exposure to specific toxicants or maternal dietary components has been shown to alter DNA methylation of specific candidate genes or the whole DNA methylome (S.-A. Lee & Ding, 2012; Marsit, 2015). Studies are needed that examine altered maternal environment, placental epigenomics and offspring outcomes from a wider array of human exposures.

Human pregnancy cohort

The samples to be used as part of this dissertation are derived from the Michigan Mother-Infant Pairs study (MMIP; PI-Vasantha Padmanabhan), which initiated in 2012. This pregnancy cohort is valuable in its recruitment of women during their first trimester and emphasis on collecting a variety of maternal and infant biological samples (e.g., maternal blood and urine). Establishing a formal link between a given exposure and increased disease risk via epigenetic reprogramming, however, requires studies that examine the relationship from many different angles. These include: demonstrating epigenetic variability in early life in response to specific environmental exposures, establishing a link between epigenetic change and disease prior to onset, and determining the functional relevance of specific epigenetic changes (Saffery, 2014). Studies that utilize longitudinal models that begin prior to birth and follow offspring after birth have the greatest potential to elucidate the role of epigenetic reprogramming in the wide variety of human disease. Currently, only a few such studies exist that evaluate the prenatal environment and its consequent impact on the fetal and later-life epigenome in this type of longitudinal approach.

Aims

There is a critical need to evaluate early prenatal exposure to bisphenols and maternal diet in association with epigenetic outcomes in the offspring. Given the expansion of capabilities and tools in environmental epigenomics, it is vital that studies include additional tissues to assist in our understanding of the utility of surrogate tissues and in the diversity of fetal tissue response to prenatal exposure. Approaches that consider overall dietary quality and its impact on the fetal epigenome are a necessary part of facilitating risk evaluation; particularly as the necessary element of this work being that we should not only identify the consequences of prenatal toxicant

exposure, but as public health scholars we should also be able to provide actionable remedies to help individuals and populations live healthfully. The aims and goals of this dissertation are as follows.

The Michigan Mother-Infant Pair (MMIP) pregnancy cohort will be used to investigate the **hypothesis that prenatal exposure to bisphenols or maternal dietary components leads to the alteration of the offspring epigenome.** Ultimately, these changes may contribute to differences in infant birth outcomes and disease risk later in life**.** Maternal biological specimens (e.g. blood and urine) collected at a first trimester visit and at delivery in conjunction with infant cord blood and tissue collected at delivery allow for evaluation of the relationship between maternal exposure (BPA, BPF, and BPS and maternal one-carbon metabolites) and developmental epigenetic reprogramming. Placental and umbilical cord tissue collected at delivery from a subset of families, in conjunction with umbilical cord blood, allows for the evaluation of the epigenetic differences in tissue response to exposure. Few studies combine the innovative technologies of epigenetics and metabolomics to consider an integrative and longitudinal view of maternal exposure and offspring outcomes. This proposal will address the following three aims:

Aim 1: To identify changes in infant cord blood DNA methylation associated with maternal exposures to the bisphenols BPA, BPF, and BPS. Maternal first-trimester urine samples were evaluated for bisphenol concentration in partnership with NSF International (Ann Arbor, MI). Quantification of DNA methylation at >850,000 CpG sites in N=69 infant umbilical cord blood samples was performed with the use of the Infinium Methylation EPIC Beadchip (Illumina Platform). Multivariate regression models were used to investigate adjusted associations between

maternal bisphenols exposure and mean infant cord blood DNA methylation to evaluate the impact of maternal exposure on the infant epigenome. *We hypothesize that maternal bisphenol levels will be associated with differentially methylated CpG sites (DMS) in infant cord blood.*

Aim 2: To identify genome-wide changes in infant cord blood DNA methylation associated with exposure to one-carbon metabolites. Untargeted metabolomic analysis via LC-MS/MS was performed on maternal first-trimester, third trimester, and umbilical cord plasma for N=89 maternal-infant pairs. From these data, we extracted one-carbon cycle metabolites betaine, choline, methionine, SAM, and SAH for further analysis. Multivariate regression models were used to investigate adjusted associations between these one-carbon metabolites and the cumulative distribution of infant cord blood DNA methylation across all CpG sites to evaluate the impact of maternal exposure on the infant epigenome and relative patterns of association between the timepoints. *We hypothesize that one-carbon metabolites will be associated with global shifts in the DNA methylation profile in infant cord blood.*

Aim 3: To compare DNA methylation response to bisphenols between matched umbilical cord blood, umbilical cord tissue, and placental samples. Using N=23 tissue triads, four candidate genes identified as associated with BPA exposure in Aim 1 in cord blood were assessed via pyrosequencing to investigate the following: 1) How does DNA methylation at candidate genes *FN1, SNAP25, HOXA-AS3, and PRSS22* compare in cord blood, cord tissue, and placenta? 2) How does environmental response to bisphenols differ across these tissues? 3) What is the placental epigenome-wide response to prenatal bisphenol exposure? Mixed effects regression was used to assess the response of these tissues to maternal exposure to bisphenols.

We hypothesize that DNA methylation at the candidate genes will be significantly different across tissue type, that prenatal bisphenol exposure will be differentially significantly associated by tissue, and that placental epigenome-wide DNA methylation will be associated with bisphenol exposure.

Innovation

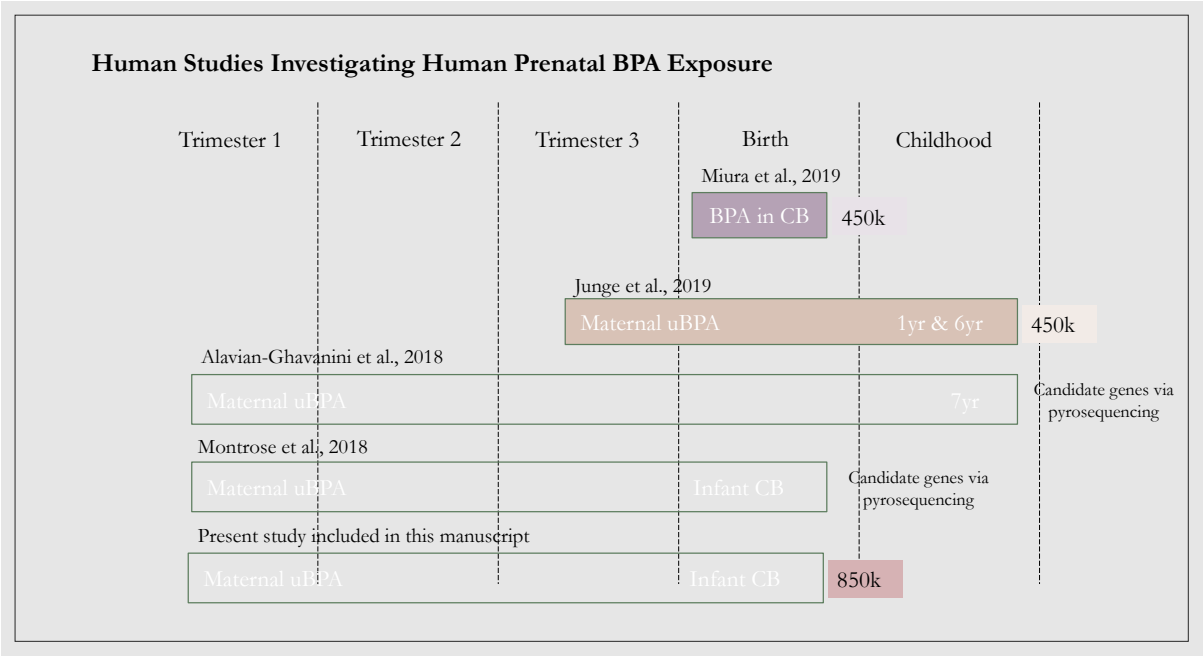
Successful completion of the proposed research will contribute fundamental knowledge to the field and understanding of epigenetic reprogramming in response to maternal exposure. Figure 1-1 displays currently published human studies that evaluate maternal bisphenol exposure and infant DNA methylation. These studies, however, are not uniform across their approach in three key elements: (1) the time point and sample type in which bisphenol exposure was measured (e.g., urinalysis during pregnancy or cord blood)], (2) the type of DNA methylation profiling (e.g., in candidate genes or epigenome-wide), and (3) the timepoint at which DNA methylation was analyzed in offspring. Establishing a formal link between a given exposure and increased disease risk via epigenetic reprogramming requires studies that examine the relationship from many different angles. These include: demonstrating epigenetic variability in early life in response to specific environmental exposures, demonstrating genetic and sex-specific effects, establishing a link between epigenetic change and disease prior to onset, and determining the functional relevance of specific epigenetic changes (Saffery, 2014). Studies that utilize longitudinal models that begin prior to birth and follow offspring after birth have the greatest potential to elucidate the role of epigenetic reprogramming in the wide variety of human disease. This study is of the few to evaluate prenatal exposure to bisphenols or metabolites during the first trimester and its epigenome-wide association with DNA methylation infant cord

blood. As of this writing, this is the first study to not only include cord blood, umbilical cord, and placenta in DNA methylation analyses, but also to evaluate the differences between each tissue and prenatal bisphenol exposure. Furthermore, this is also the first study to perform epigenome-wide analysis in placenta evaluating its association with prenatal bisphenol exposure.

Public health significance

With the understanding that early-life exposures like BPA or maternal diet can have consequences for later-life disease, there is a genuine need to develop better tools for (1) assessing risk of exposure, (2) understanding the variation in consequences for an infant's long-term health, and (3) the potential for nutrient co-exposures with toxicant exposures to harm or to help mitigate risk. Improved insight into whole dietary patterns by newer dietary evaluation methods and the association with fetal epigenetic reprogramming will facilitate risk evaluation and development of better maternal nutrition counseling. Advances in epigenome-wide association studies will ultimately enable researchers, clinicians, and policymakers to target risk factors (e.g. environmental and nutritional exposures that perturb the methylome and downstream birth outcomes) and to better understand a portion of the myriad of elements underlying the developmental origins of health and disease.

Figure 1-1: A comparison of currently published human pregnancy cohort studies and the work proposed in this dissertation that investigate human prenatal bisphenol exposure and its association with offspring DNA methylation.



Chapter 2 Maternal Environmental Exposure to Bisphenols and Epigenome-Wide DNA Methylation in Infant Cord Blood

Introduction

Bisphenol-A (BPA), a chemical commonly used in receipts, plastics and food packaging, is considered to be a 'ubiquitous exposure,' principally because of its wide-spread usage and high rate (over 95%) of detection in human urine (Calafat et al., 2008). Exposure to BPA and two of its commonly-used replacement analogues, bisphenol-F (BPF) and bisphenol-S (BPS), are readily detectable in U.S. populations (Calafat et al., 2005, 2008; Lehmler et al., 2018; Thayer et al., 2016). BPF and BPS are now increasingly utilized in place of BPA particularly as a result of consumer and scientific-based advocacy efforts. This pressure effectively elicited the U.S. Food and Drug Administration's ban of BPA in infant-related plastics and products (Siracusa et al., 2018). However, significantly less is known about BPF and BPS, with new evidence suggesting that these replacement chemicals with close structural similarities to BPA may have comparable or increased levels of potency as endocrine disruptors and may also negatively impact the reproductive system (Eladak et al., 2015; Rochester & Bolden, 2015a; Siracusa et al., 2018). In the U.S., BPF and BPS were only recently added to the list of chemicals measured in the National Health and Nutrition Examination Survey (NHANES), appearing for the first time from 2013-2014 (Lehmler et al., 2018).

While the proportion of adults and children with detectable levels of these bisphenols is concerning, the exposure patterns experienced by pregnant and lactating mothers introduce an additional layer of consideration. Specifically, when the potential impact on fetal development

and lifetime health trajectory are evaluated. Pregnant women in the U.S. and internationally are typically exposed to or have biological concentrations of urinary bisphenols at similar levels to non-pregnant women (Arbuckle et al., 2015; Callan et al., 2013; Gerona et al., 2016; Woodruff et al., 2011). Furthermore, BPA, BPF and BPS have the potential to cross the placenta at differing rates and with inter-individual variation (Grandin et al., 2018, 2019; J. Lee et al., 2018).

Environmental research establishes the framework of time around conception, gestation, and birth as one of the most developmentally susceptible times of life. This aligns with the Developmental Origins of Health and Disease (DOHaD) hypothesis, which recognizes the connection between maternal exposure during pregnancy and the risks posed to her offspring's health and later-life disease (Dolinoy, Weidman, et al., 2007a). An increasing number of studies have investigated the impact of prenatal exposure to bisphenols on phenotypic outcomes in infants and children. Maternal and prenatal exposure to bisphenols in humans is associated with pregnancy duration and birth weight (Ferguson et al., 2016; Veiga-Lopez et al., 2015; Wan et al., 2018; Weinberger et al., 2014), increased risk of preeclampsia (Cantonwine et al., 2016), early childhood behavior (Braun et al., 2009; Evans et al., 2014; Perera et al., 2012), childhood body mass index (BMI) (Harley et al., 2013), and peripubertal metabolic homeostasis (Ashley-Martin et al., 2014; Watkins et al., 2016). Studies in mice have demonstrated that prenatal or early-life exposure to bisphenols is associated with altered brain development and behavior (Jašarević et al., 2013) as well as disruptions in metabolic homeostasis (Alonso-Magdalena et al., 2010; Anderson et al., 2013; J. Liu et al., 2013; Van Esterik et al., 2014), glucose metabolism (García-Arévalo et al., 2016; J. Li et al., 2016), neuroendocrine function (Franssen et al., 2016; Witchey et al., 2019), and immune function (Fischer et al., 2016; Weinhouse et al., 2014). Despite these developments in understanding of the association between prenatal bisphenol exposure and

phenotypic outcomes in offspring, less is known of the possible mechanism through which bisphenols elicit these outcomes.

Recent work indicates that environmentally induced disease etiology may be mediated by changes in the epigenetic profile (Cardenas et al., 2019; Ladd-Acosta & Fallin, 2019; Witt et al., 2018). For the purposes of this chapter, we define the epigenome as consisting of chemical modifications (e.g. DNA methylation and histone modification) that are mitotically heritable and regulate gene expression but are not the result of a change in the DNA sequence (Dolinoy, Weidman, et al., 2007a). Currently, very few studies exist that evaluate prenatal bisphenol exposure and its consequent longitudinal impact on the fetal and later-life epigenome (Kundakovic & Champagne, 2011; McCabe et al., 2017). Most studies were completed in mice; with evidence suggesting that prenatal exposure to bisphenols is associated with changes in DNA methylation in genes regulating hepatic function (Strakovsky et al., 2015), metabolism (Anderson et al., 2016a; Anderson, Nahar, et al., 2012), neuronal (Kundakovic et al., 2013) and inflammatory pathways (Weinhouse et al., 2016), and other regulatory epigenetic machinery (Senyildiz et al., 2017). Four human studies in pregnancy cohorts have evaluated the epigenetic impact of prenatal exposure to bisphenols (Alavian-Ghavanini et al., 2018; Junge et al., 2018; Miura et al., 2019; Montrose et al., 2018). From these collective investigations comes significant insight into elements of the association between prenatal BPA exposure and DNA methylation in offspring growth and neurological function in addition to its sexually dimorphic nature. However, these studies are not uniform across their approach in three key elements: (1) the time point and sample type in which bisphenol exposure was measured (e.g., urinalysis during pregnancy or cord blood)], (2) the type of DNA methylation profiling (e.g., in candidate genes or epigenome-wide), and (3) the timepoint at which DNA methylation was analyzed in offspring.

With advances in exposure science and DNA methylation technology, it is critical to evaluate exposure to multiple bisphenols from the first trimester, a time during which the epigenome is highly susceptible to reprogramming; and measure outcomes at birth, utilizing methods that generate data at all genes.

This study discussed in this chapter aimed to test the association between maternal exposure to the bisphenol BPA or its substitute chemicals, BPF and BPS, and cord blood leukocyte DNA methylation at >800,000 loci in a longitudinal pregnancy cohort. *We hypothesized that maternal bisphenol levels would be associated with differentially methylated CpG sites (DMS) in infant cord blood.* This study is of the few to evaluate prenatal exposure to bisphenols during the first trimester and its epigenome-wide association with DNA methylation infant cord blood. Importantly, we are the first to use this method to also investigate the replacement phenols BPF and BPS.

Methods

Study Population

The samples used in this study were derived from the Michigan Mother-Infant Pairs pregnancy cohort (MMIP), which initiated in 2011. Briefly, women providing informed, written consent were enrolled during their first prenatal visit to the University of Michigan Women's Hospital clinic. At this visit, maternal first trimester blood and urine were collected. Women also completed a questionnaire that gathered socio-demographic factors, health behaviors, food consumption and personal care product use, among other measures. Exclusion criteria included: age <18 years, prior infertility treatment, pregnancy with multiple fetuses, and pregnancy <8 weeks or >14 weeks gestation. Women were provided study materials between weeks 34-38 of

gestation for blood and urine collection upon admission into labor. Maternal blood and urine were collected when admitted and umbilical cord blood samples were collected at delivery. At the time of writing, 331 mothers have enrolled in MMIP, and 200 have been followed-up through labor and delivery. For the analysis described here, a subset of MMIP families enrolled between 2011 and 2017 with first trimester exposure assessment of three urinary bisphenols and DNA methylation analysis via the Infinium EPIC were included (n=69). The University of Michigan Medical School Institutional Review Board approved all study procedures (HUM00017941).

Epigenome-Wide DNA Methylation Analysis of Infant Umbilical Cord Blood

Infant cord blood samples (N=69) were collected into PaxGene Blood DNA tubes (PreAnalytix) with the use of butterfly needles at the time of birth and stored at -80°C until processing. Total DNA was extracted with the PaxGene Blood DNA kit. DNA quality and concentration were assessed via Qubit at the University of Michigan Advanced Genomics Core. DNA was bisulfite converted using the EZ-96 DNA Methylation Kit (Zymo), wherein approximately 500ng of input DNA was used. The kit utilized sodium bisulfite to convert un-methylated cytosines to uracil and ultimately thymine, while methylated cytosines were protected (Grunau et al., 2001).

Following bisulfite treatment, DNA methylation at >850,000 CpG sites was evaluated using the Illumina Infinium MethylationEPIC BeadChip (‘EPIC’) at the University of Michigan Advanced Genomics Core according to standard protocols. Cord blood samples were run on three separate days, and these experimental batches are considered in statistical models.

Processing and Quality Control of Infinium MethylationEPIC Data

Arrays were assessed for quality of samples and probes using a standard pipeline. Briefly, the pipeline utilized the minfi package (Aryee et al., 2014) (R Project for Statistical Computing) to read in raw data image files. Quality control of samples was assessed by comparing estimated sex (from methylation values on the X and Y chromosomes) with known infant sex, detection p-values of probes, and intensity signals.

Probes with poor detection (positions that failed detection in more than 10% of samples N=1475), cross-reactive probes, and probes that target polymorphic CpG sites in the Illumina HumanMethylation arrays were dropped (McCartney et al., 2016). The Functional Normalization (Fortin et al., 2014) R package was used to correct for background and perform dye-bias normalization.

Using estimateCellCounts, the relative proportion of B-cells, CD4, CD8T, granulocytes, monocytes, neutrophils, and nucleated red blood cells (nRBCs) were estimated for each cord blood sample using an established algorithm based on DNA methylation profiles of sorted major cord blood cell types (Bakulski et al., 2016). estimateCellCounts is a cell proportion estimation algorithm that estimates the relative proportions of cell types within a given sample based on DNA methylation signatures of each cell type.

These preprocessing steps resulted in 822,020 retained probes from N=69 cord blood samples that passed all quality control measures. Finally, M-values, defined as the log2 ratio of intensities of methylated probe versus unmethylated probes, were generated for each sample at these CpG sites and were used in downstream statistical analyses unless otherwise noted.

Maternal Bisphenol Measurement

Bisphenols (BPA, BPF, and BPS) were measured in spot urine samples collected from mothers during their first trimester visit (between 8-14 weeks) for this subset of MMIP participants (n=69). Samples were collected into polypropylene urine collection containers, aliquoted into glass vials, and frozen at -80°C until analysis. Total urinary BPA, BPF, and BPS were measured at NSF International (Ann Arbor, MI) using isotope dilution-liquid chromatography-tandem mass spectrometry (ID-LC-MS/MS), as reported previously. (Goodrich et al., 2019) Specific gravity (SG) was measured using a handheld digital refractometer (Atago Co., Ltd., Tokyo, Japan) at the time of sample analysis. Urinary bisphenol values below the limit of detection (LOD, 0.2 ng/mL) were replaced with LOD/√2 (0.141 ng/mL).

Statistical Analysis

All statistical analyses were performed in R version 3.6.0 (Platform: x86_64-apple-darwin15.6.0 (64-bit) & Running under: macOS Mojave 10.14.6). We first performed univariate analyses on all exposure biomarkers and potential covariates of interest. We then assessed relationships between exposures and covariates to identify potential confounders via chi-square tests, t-tests, and Spearman correlations. First-trimester urinary BPA was modeled as a continuous variable, and BPF and BPS were modeled as categorical (above or below the LOD) (Supplemental Tables 1-3). Singular Value Decomposition (SVD) analysis was performed with the ChAMP package (Tian et al., 2017). The correlation between principal components of the methylation data with biological and technical covariates was determined using linear regression (continuous variables) or Kruskal-Wallis (categorical variables). We did not identify potential confounders (i.e. covariates associated with both BPA and DNA methylation) to include in the model. However, due to their significant ($p<1x10^{-5}$) association with the DNA methylation data

in the SVD analysis, infant sex, B-cells, nRBCs, and sample-plate (batch) were selected as covariates to adjust for in final models.

Single-Site Association Analysis

Linear regression was used to identify differentially methylated CpG sites (using M-values) by each maternal urinary bisphenol exposure, adjusting for covariates described above (infant sex, B-cells, nRBCs, sample plate). An empirical Bayes method in the limma (Smyth, 2005) R package was then used to shrink probe-wise variances towards a pooled estimate and calculate a moderated t-statistic. M-values were selected for statistical analysis given their advantages which include meeting the assumption of homoscedasticity and superior performance in Detection Rate (DR) and True Positive Rate (TPR), especially for highly methylated and unmethylated sites (Du et al., 2010; Xie et al., 2019). P-value correction by the Benjamini-Hochberg false discovery rate (FDR) method was used, (Hochberg & Benjamini, 1990) and a 5% FDR (i.e. $q<0.05$) were considered significant.

Sensitivity analyses were performed. One maternal urinary BPA sample was identified as a statistical outlier ($\pm$2 standard deviations (SD) from the mean). The outlier was removed, and the single site analyses were rerun. The direction and significance of the sites identified as significant in the initial model were compared to the results from the model without the outlier. Additional analyses included examining scatterplots of the relationship between BPA and methylation at each significant site.

In order to test whether the bisphenol exposures may be influencing the same genes, we calculated the Pearson correlation between the effect estimates of all CpG sites from models for each bisphenol.

Lastly, we compared results of previously published epigenome-wide studies focused on BPA exposure with our results.(Junge et al., 2018; Miura et al., 2019) Pearson correlation was run between the effect estimates for sites reported by Miura et al. as significant at $p \leq 0.0001$ for all infants (Miura et al., 2019) and the corresponding results in our BPA model. Results from Junge et al. and Alavian-Ghavanini et al. were compared to our results for replication of the direction of the effect of BPA.

Differentially Methylated Regions (DMRs)

We utilized dmrcate (Peters et al., 2015) to test for differentially methylated regions by maternal first trimester urinary phenols exposure. A differentially methylated region had to consist of at least 2 consecutive probes. Probes that were two nucleotides or closer to a single-nucleotide polymorphism (SNP) that had minor allele frequency greater than 0.05 were filtered out first. The model was adjusted for cell type (Bcell and nRBC), infant sex, and batch. GenomicRanges (Lawrence et al., 2013) was used to graph an annotated representation of the DMRs. GenomicRanges requires the use of beta values (e.g., proportion of DNA methylation at CpG sites), and data are displayed as averaged across quartiles of BPA. Quartile cut-offs are as follows: Q1 [<LOD, 0.348], Q2 [0.349, 0.897], Q3 [0.898,1.90], Q4 [1.91,6.76] in ng/mL BPA. DMRcate analysis was also repeated without the BPA outlying subject.

Pathway Analysis

LRpath (J. H. Kim et al., 2012) was utilized to perform gene-set enrichment across all probes annotated to genes (using Entrez Gene IDs) using concepts (also known as gene-sets) from KEGG and GO (Biological Process, Molecular Function, and Cellular Component). LRpath

uses raw p-values, fold changes, and Entrez gene IDs for each probe mapping to a known gene from the single-site linear model for the association between each bisphenol and DNA methylation. LRPath utilizes logistic regression in determining gene set membership status (dependent variable) by the statistical significance of genes' differential methylation (independent variable, raw p-value). Concepts from both Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases were selected from LRPath's internal annotation database of gene-sets (concepts) as those onto which our data should be mapped, and only gene-sets with a minimum of 10 and a maximum of 250 genes were used; a directional test was included based on the direction of association between BPA and DNA methylation at each site. LRPath tests the odds that the genes in a concept have higher significance values (e.g., lower p-values from the differential methylation analysis) than expected at random, and FDR of 5% was considered a statistically significant enriched gene-set.

Results:

Study Population Characteristics

Table 2-1 contains the demographic data of the maternal-infant pairs included in this study. The mean maternal age was 32, and on average, the number of weeks to delivery was 39.5 weeks. After adjusting for specific gravity, mean maternal, first-trimester urinary BPA concentration was 1.19 ng/mL (range <LOD - 6.78 ng/mL) (Figure 2-1). The highest maternal BPA exposure was determined to be an outlier (e.g., greater than two SD away from the mean). However, her exposure levels were biologically plausible, given its fitting within the distribution of measured samples in the most recent NHANES report from 2014-2015. Therefore, this sample was retained. Fifty-nine of 69 (85.5%) maternal samples had urinary BPA levels above the LOD.

Mean maternal first-trimester urinary BPA concentration was 1.27 ng/mL (range <LOD – 19.97 ng/mL) (Figure 2-1). Thirty-nine of 69 (56.5%) maternal samples had urinary BPF levels above the LOD, and two outliers were detected. Mean maternal first-trimester urinary BPS concentration was 0.37 ng/mL (range <LOD – 4.50 ng/mL) (Figure 2-1). Forty of 69 (57.9%) maternal samples had urinary BPS levels above the LOD.

When we assessed relationships between maternal first trimester urinary BPA exposure and covariates of interest, including maternal characteristics and estimated cord blood cell type proportions (Appendix Table 1) using Spearman correlations or t-tests, none were statistically significant. Similarly, t-tests and chi-square tests for covariates of interest with maternal first trimester urinary BPF and BPS (modeled as categorical variables) were not statistically significant except for pre-pregnancy BMI by BPF (detected vs. <LOD) (Appendix Table 2 & 3).

Single-Site DNA methylation

Single-Site Association Analysis revealed maternal first-trimester urinary BPA exposure was associated with 38 differentially methylated sites (DMS) in infant cord blood at $q<0.05$. The genomic inflation factor (lambda) for the analysis was 0.823. Increasing BPA concentrations were associated with lower DNA methylation at 87% of significant sites (Table 2-2). The five most significantly differentially methylated sites ($q<0.003$) were within the genes *SLC2A1-AS1, KIF21B, CRYL1, HSPBAP1,* and *FN1*. For interpretability, Table 2-2 also shows effect estimates from a model of the beta values. To more clearly demonstrate percent difference in methylation at each site, M-values were replaced with beta values in the single-site analysis. For example, for every 1ng/mL increase in BPA, DNA methylation at *SLC2A1-AS1* decreased by 10%, while DNA methylation at *KIF21B* increased by 2.7%.

Appendix Table 4 provides results for the 38 DMS associated with prenatal BPA exposure when the model is run without the BPA outlying subject. When the outlier was removed, only two sites remained significantly associated with BPA at $p \leq 0.0001$, lambda=0.948 (in *SLC2A1-AS1* and *RAD52*). The remaining CpG sites may be false positives or may only be perturbed at higher levels of exposure; this should be tested further in future studies.

BPF exposure, dichotomized as below or above the LOD, was not associated with DMS at FDR of $q<0.05$, but was associated with 19 DMS at $p \leq 0.0001$, lambda=0.788. BPS exposure, also dichotomized as below or above the LOD, was not associated with DMS at the FDR of $q<0.05$ but was associated with one differentially methylated site at *VPS53* at $p \leq 0.0001$, lambda=0.674.

The effect estimates from the BPA, BPF, and BPS models were significantly correlated. BPA and BPF (cor=0.194), BPA and BPS (cor=0.116), and BPF and BPS (cor=0.179) were each significantly, positively correlated at $p<2.2e^{-16}$.

Differentially Methylated Regions

Three differentially methylated regions (DMRs) were detected in association with maternal first trimester urinary BPA exposure, wherein each region possessed at least seven CpG sites. These genes were *HOXA-AS3*, *PRSS22*, and *ZSCAN12P1*. Two of the three regions (*HOXA-AS3* and *PRSS22*) displayed an increase in DNA methylation with increasing BPA (Table 2-3). Figure 2-2 includes the 18 *HOXA-AS3* CpG sites contained within the DMR. Similarly, for *PRSS22*, across its 13 CpG sites higher maternal exposure first-trimester BPA exposure was associated with increased percent DNA methylation, and this association remained after exclusion of the BPA outlier (p=0.00000752). Alternatively, in the seven CpG sites of

ZSCAN12P1, higher maternal first-trimester urinary BPA exposure was associated with lower percent methylation.

Pathway Analysis

BPA exposure associated DNA methylation sites were enriched for 38 pathways significant at FDR <0.05. Higher BPA exposure was associated with increased methylation for all enriched pathways (Table 2-4). The pathway or concept with the greatest odds of enrichment for differential methylation was type I interferon receptor binding; pathways related to type I interferon activity appeared four additional times. Other highly enriched pathways included JAK/STAT signaling and response; G-protein coupled receptor signaling, and immune response (Table 4). In general, the enriched pathways were associated with the nervous system, immune response, and neuroinflammation.

Results from the BPF exposure were enriched for smaller p-values in one pathway: systemic lupus erythematosus (q=0.0295). Higher BPF exposure was associated with increased methylation in genes of this pathway (Table 2-4). BPS exposure associated DNA methylation sites were not enriched for pathways at FDR <0.05.

Comparison of Results with Previously Published BPA Studies

42 of the 45 probes reported in Miura et al. as significantly associated with BPA exposure at p<0.0001 were included in our dataset. Pearson correlation between effect estimates at these 42 sites revealed a slightly positive correlation (cor=0.106) that was not significant (p=0.503) (Supplemental Table 5). The direction of the effect of BPA exposure on DNA methylation in *MEST* and *RAB408* in our results did not correspond to that detected by Junge et al. However,

Junge et al. modeled BPA as high versus low BPA exposure. The direction of the effect of BPA exposure on DNA methylation in *GRIN2B* also did not correspond to that reported by Alavian-Ghavanini et al. Again, this group chose to model BPA as an ordered categorical variable and as 4th quartile versus 1st quartile and reported odds ratios (Appendix Table 5).

Discussion:

In this chapter we evaluated the association of maternal first-trimester bisphenol exposure with differential DNA methylation in the infant cord blood. Utilizing the Illumina Infinium MethylationEPIC BeadChip ('EPIC') array to quantify DNA methylation in infant cord blood leukocytes at over 800,000 CpG sites, this study identified that maternal prenatal BPA exposure was associated with DNA methylation at 38 CpG sites while BPF and BPS in this same subset were not associated with specific CpG sites at $q<0.05$.

The preconception period and early pregnancy is a sensitive developmental time period for both physiological development and epigenetic reprogramming. During embryonic development, primordial germ cells and preimplantation embryos undergo two waves of methylation reprogramming (Messerschmidt et al., 2014; Reik et al., 2001). During the first wave, the paternal genome is actively demethylated and the maternal genome is passively demethylated, followed by reprogramming and remethylation of somatic embryonic stem cells and primordial germ cells in accordance with infant sex (Stein & Lee Davis, 2012). The interface of this essential reprogramming event with potential environmental or maternal exposures leaves the fetal epigenome extremely vulnerable to insult or alteration (Marsit, 2015). It is therefore possible that exposures experienced during this time period may alter DNA methylation in

somatic embryonic stem cells and primordial germ cells; changes which could be propagated to subsequent cells and possibly influence development and disease later in life (Marsit, 2015).

Considering very early in development is the most susceptible and a critical period for epigenetic effects (McCabe et al., 2017), the focus of this investigation centered on maternal exposure to bisphenols during the first trimester. Our single-site analysis revealed 38 individual CpG sites in infant cord blood leukocytes that were differentially methylated in relation to early maternal BPA exposure (Table 2) and three differentially methylated regions in the genes *HOXA-AS3*, *PRSS22*, and *ZSCAN12P1* (Table 3). However, the association of prenatal BPA exposure to DNA methylation in most of these genes was diminished when sensitivity analyses that excluded one outlying subject were performed. Associations with BPA and DNA methylation at CpG sites in *SLC2A1-AS1* and *RAD52* and the DMR in *PRSS22* remained ($p<0.001$) after outlier exclusion emphasizing the need to study these further in other birth cohorts with phenol exposures.

Using raw p-values, differentially methylated genes associated with BPA were enriched in pathways related to the nervous system, immune response, and neuroinflammation. For instance, a highly enriched set of concepts, as identified by the LRpath analysis, were JAK/STAT signaling and response. Previous literature has demonstrated an association between prenatal BPA exposure and disruption to genes involved in JAK/STAT signaling (Anderson et al., 2016b; Weinhouse et al., 2016). G-protein coupled receptors (GPCRs), which play an important role in the nervous system, were also enriched; 7 of 38 single sites were in genes that have functions related to this pathway (e.g., *KIF21B, DIRC2, SNAP25, PDE8A, CAMKK2, ERC2,* and *DIP2C*). Increasing evidence has demonstrated a connection between BPA exposure and dysregulation of the nervous system (Arambula et al., 2016; Jadhav et al., 2017; Martínez et

al., 2018). Lastly, the Interferon (IFN) 1 receptor and immune function pathways were also highly enriched with 5 of 38 single sites in genes with functions related to these pathways (e.g., *MSC, RNF181, USP15, KIAA0922,* and *XKR6*). Of particular interest as related to interferon 1 receptor binding is its role in the severity and manifestation of systemic lupus erythematosus (SLE) (Crow, 2014). *USP15, KIAA0922,* and *XKR6* are specifically related to this pathway and with SLE. It has been demonstrated that BPA can stimulate estrogen-receptor alpha (ERalpha) and IFN signaling in myeloid cells and immune pathways resulting in activation of innate immune sensors (Panchanathan et al., 2015), and increasing evidence supports the B-cell receptor pathway and IFN signaling in SLE pathogenesis (Järvinen et al., 2012). *USP15* has been documented to play a role in regulating the type 1 interferon response, particularly as it relates to pathogenic neuroinflammation (Torre et al., 2017). *TLR2* has also been demonstrated to be associated with arterial thrombosis in patients with SLE (Kaiser et al., 2014). Lastly, *XKR6* is associated with susceptibility and childhood onset of SLE in a variety of cohorts (Bin Joo et al., 2018; Yesim Demirci et al., 2017; You et al., 2015). Despite not reaching significance in the single-site models, urinary BPF exposure was also associated with the SLE gene set during enrichment analysis.

Currently published literature of prenatal exposure to BPA and its epigenetic impact present similar findings of genes and pathways related to neurological function and inflammation. Junge et. al detected hypomethylation at two CpG sites in infant cord blood in response to maternal prenatal exposure to BPA: cg17580798 in the *MEST* promoter region and cg23117250 in an intronic region of *RAB408* (Junge et al., 2018). These sites were not significantly associated with prenatal BPA exposure in our study; however, *MEST* expression in mesenchymal tissue and mesenchymal stem cells (MSCs) and its functional significance to

adipogenesis, particularly in the context of BPA exposure, is relevant to the role of *HOXA-AS3*, one of the DMRs detected in this study. *HOXA-AS3* has a distinct role as an epigenetic switch in the lineage specification of mesenchymal stem cells as either promoting the adipogenic or osteogenic induction of MSCs (Zhu et al., 2016). Although we did not detect differential DNA methylation in the same genes, there is concordance between our results and those reported by Junge et. al. in the potential for prenatal BPA exposure to impact genes related to MSCs, adipogenesis, and perhaps long-term body weight. Montrose et. al investigated the impact of maternal first-trimester urinary BPA exposure on DNA methylation in candidate genes in the same MMIP cohort (Montrose et al., 2018). Urinary BPA exposure was associated with a decrease in DNA methylation in *IGF2* and *PPARA* in female infants; highlighting both the sexually dimorphic response of exposure to bisphenols and its association with disruption of genes related to growth and, adipogenesis, and metabolism. Alavian-Ghavanini, et. al, *a priori* selected *GRIN2B,* a gene involved in neural function, and assessed associations between prenatal BPA exposure and DNA methylation at this gene in buccal DNA of 7-year old children (Alavian-Ghavanini et al., 2018). In the present study, prenatal BPA exposure was not associated with differential DNA methylation at the *GRIN2B* gene in infant cord blood leukocytes. However, there is evidence that expression of *GRIN2B* and two genes associated with BPA in the present study, *SLC2A1* and *HIF1A,* are related to one another via overlapping pathways (Alavian-Ghavanini et al., 2018; Bild et al., 2006; G. Wu et al., 2010). Miura et. al, utilized a Japanese cohort for whom they measured BPA concentrations in cord blood and evaluated cross-sectional epigenome-wide associations with cord blood DNA methylation (Miura et al., 2019). A principle element of the study involved sex-stratified analyses, which detected significant differences in the response of male and female infants. They detected 28 differentially

methylated sites ($q<0.05$) in male infants and 16 differentially methylated sites in female infants (Miura et al., 2019). While the same genes were not significant in the present study, there was concordance between the genes Miura et al., detected and the results presented in this study as related to gene families. For example, *PRSS* is a gene family for which we detected a differentially methylated region (*PRSS22*), and CpG sites within *SLC* and *KIAA* were associated with BPA in the Japanese cohort.

The discovery that neither BPF nor BPS maternal exposures were significantly associated with differential DNA methylation in the infant cord blood in this study was not surprising given the small sample size of the study, the necessity to model these exposures as categorical, and given that roughly half of mothers had undetectable levels of these bisphenols in their urine. Despite this, it was and is important to include BPF and BPS in the investigation of maternal exposure to bisphenols. BPF and BPS were first included in NHANES in 2013-2014, and Lehmler et. al found that exposures to BPA, BPF, and BPS among adults and children could be considered near-ubiquitous (Lehmler et al., 2018). We recommend assessment of other bisphenols in epigenetic studies in the future, because as the use of BPA substitutes in consumer products and manufacturing increases, it is pertinent to not only evaluate population exposure, but also to determine the impact of that exposure. Furthermore, we consider it valuable to simultaneously assess multiple bisphenols in human exposure studies so as to classify and categorize the similarities and differences of these toxicants.

Limitations and Future Directions

The MMIP cohort used in this study is based out of the University of Michigan Hospital in Ann Arbor, Michigan, and the majority of participants enrolled into the study were non-

Hispanic White. This may limit the generalizability of the results. The final number of mother-infant pairs included in this study was determined by the availability of samples with data (e.g., maternal first-trimester urine with exposure assessment and infant umbilical cord blood). This limited our statistical power to detect differentially methylated sites by all bisphenols, and broader pathways in association with maternal bisphenol exposure. However, in line with our recommendations for the inclusion of these bisphenols in exposure studies, the non-significant results that we detected for BPF and BPS still allow us to observe trends of exposure over time and a baseline to which we can compare future studies. We also acknowledge that the small sample size may lead to spurious effects from statistical outliers. Thus, we report results with and without one BPA outlier. Since we cannot determine in this study whether individuals with higher exposure levels would display similar associations with BPA, we recommend future studies of prenatal BPA exposure and the offspring epigenome be performed in populations with a wide range of exposure to better understand how families with increased toxicant burdens may be impacted.

We also acknowledge the potential limitations of using infant cord blood as a surrogate tissue for evaluating the impact of prenatal exposures. While we control for cell-type heterogeneity with the use of a cord blood-specific cell type reference panel, cord blood is still principally made up of immune cells. This may explain, in part, why some of the single sites that we detected were associated with immune function. However, we consider it a distinct strength of this study that we chose first trimester maternal exposure assessment, particularly because we expect changes induced early in pregnancy to propagate across all germ layers and tissues of the developing fetus.

Conclusion

This study examined the association between maternal first-trimester urinary bisphenol exposure and DNA methylation in infant cord blood. Maternal BPA exposure was associated with differential methylation at 38 single-sites in genes related to pathways of neurological function, inflammation, and in particular SLE. With mounting evidence of the consequences associated with exposure to endocrine disrupting chemicals comes the sincere need to evaluate a variety of exposures across many populations. BPA and its replacement chemicals, BPF and BPS, remain heavily utilized in manufacturing, and exposure to these chemicals is considered ubiquitous. This study adds to the body of evidence about prenatal exposure to bisphenols and its association with differential DNA methylation in infants. Furthermore, these data begin to elucidate the correlation between these chemicals and ultimately provide additional tools that may be integrated in risk assessment and mitigation in individuals or populations with higher bisphenol exposure levels.

Table 2-1: Descriptive statistics [median (25th, 75th percentiles) or n (%) for N=69 mother-infant pairs in the MMIP cohort.

Maternal age (years)	32 (30, 34)
Number of days to delivery (days)	277 (273, 282)
Maternal Race/Ethnicity	
White	66 (95%)
African American	1 (1.45%)
Asian	1 (1.45%)
Other or mixed race	1 (1.45%)
B cell proportion	0.0890 (0.0625, 0.111)
Monocyte proportion	0.0916 (0.0767, 0.105)
nRBC proportion	0.0696 (0.0481, 0.113)
CD4+cell proportion	0.151 (0.114, 0.189)
CD8+ cell proportion	0.124 (0.100, 0.148)
Natural killer cell proportion	0.00563 (0.0, 0.0270)
Maternal urinary BPA (ng/mL)	0.898 (0.349, 1.91)
Maternal urinary BPF (ng/mL)	0.298 (0.177, 0.820)
Maternal urinary BPS (ng/mL)	0.226 (0.145, 0.365)
Infant Sex	
Female	37 (53.6%)
Male	32 (46.3%)
Infant birth weight (gms)	3500 (3270, 3820)

Limit of detection (LOD) <0.2 ng/mL. Urinary bisphenol measures adjusted for specific-gravity.

Table 2-2: Differentially methylated CpG sites associated with maternal first trimester urinary BPA exposure.

Locus	Gene Name	Relation to CpG Island	Effect Estimate using Beta-values	Effect Estimate using M-values	q-value FDR
chr1: 43437674	*SLC2A1-AS1*	Open Sea	-0.10	-0.86	**0.00069**
chr1: 14591868		Open Sea	-0.060	-0.69	**0.00154**
chr1: 200992656	*KIF21B*	Island	0.027	0.35	**0.00154**
chr19: 36661673		Open Sea	0.031	0.29	**0.00155**
chr13: 21049223	*CRYL1*	Open Sea	-0.063	-0.75	**0.00166**
chr18: 33160855		North Shore	-0.0071	-0.47	**0.00239**
chr3: 122512541	*HSPBAP1*	Island	0.028	0.37	**0.00239**
chr8: 10622805		Open Sea	-0.027	-0.27	**0.00290**
chr2: 216237359	*FN1*	Open Sea	-0.057	-0.68	**0.00290**
chr16: 51184562	*SALL1*	Island	-0.00096	-0.27	**0.00290**
chr20: 10199434	*SNAP25*	North Shore	-0.0011	-0.29	**0.00290**
chr15: 85660361	*PDE8A*	Open Sea	-0.060	-0.70	**0.00290**
chr8: 72756155	*MSC*	Island	-0.00077	-0.27	**0.00333**
chr12: 121698404	*CAMKK2*	Open Sea	-0.058	-0.50	**0.00333**
chr19: 3180815		South Shore	-0.00096	-0.41	**0.00333**
chr2: 85822726	*RNF181*	Island	-0.0038	-0.43	**0.00370**
chr2: 239039182	*ESPNL*	North Shore	-0.045	-0.33	**0.00402**
chr8: 33342681	*MAK16*	Island	-0.00086	-0.27	**0.00402**
chr7: 142536625		Open Sea	-0.049	-0.36	**0.00402**
chr11: 26595206	*MUC15*	Open Sea	-0.060	-0.71	**0.00487**
chr2: 71017846	*FIGLA*	Island	-0.017	-0.26	**0.00712**
chr7: 1068244	*C7orf50*	Island	0.013	0.21	**0.00712**
chr5: 106879524	*EFNA5*	Open Sea	-0.030	-0.26	**0.00753**
chr2: 172957268		North Shore	-0.015	-0.40	**0.00913**
chr12: 11324011	*SMIM10L1*	Island	-0.00060	-0.26	**0.00963**
chr3: 46752152	*TMIE*	Open Sea	-0.037	-0.24	**0.01499**
chr16: 8735575	*METTL22*	Open Sea	-0.028	-0.28	**0.01543**
chr12: 62653559	*USP15*	North Shore	-0.0021	-0.34	**0.01769**
chr3: 56502021	*ERC2*	Island	-0.012	-0.36	**0.01797**
chr1: 111098247		Island	-0.00062	-0.16	**0.01906**
chr12: 123380878	*VPS37B*	Island	-0.0083	-0.44	**0.02749**
chr19: 2462065		Island	-0.00094	-0.27	**0.02749**
chr10: 636076	*DIP2C*	Open Sea	-0.027	-0.26	**0.02775**
chr14: 62210927	*HIF1A*	Open Sea	-0.017	-0.19	**0.03846**
chr4: 154400013	*KIAA0922*	Open Sea	-0.031	-0.27	**0.03846**
chr4: 154349775		Open Sea	-0.015	-0.18	**0.03846**

chr12: 1058965	*RAD52*	Island	0.011	0.25	**0.03990**
chr8: 11059042	*XKR6*	Island	-0.00049	-0.17	**0.04156**

Note: Results shown are for CpG sites associated with maternal urinary first-trimester BPA exposure below false discovery rate (FDR) significance of q<0.05. Model was adjusted for infant sex, nRBCs, Bcells, and sample plate (batch). Effect estimate is the unit change with each 1 ng/mL increase in BPA from the model of M-values (logit-transformed beta values). Beta is the effect estimate when modeling the proportion of methylation (beta value) at the same CpG site instead and represents the increase in proportion methylated per each ng/mL increase in first trimester BPA. The beta estimate is included for interpretation purposes; significance values are generated from the M-value analysis.

Table 2-3: Differentially methylated regions in association with maternal first trimester urinary BPA exposure. Significance was considered at q<0.05.

Chromosome	Gene Name	Start (bp)	End (bp)	Number of CpG Sites	P-value(a)	Max Beta Change per ng/mL BPA increase(b)
7	***HOXA-AS3***	27183794	27184375	18	1.79E-14	0.0190
16	***PRSS22***	2907517	2908715	13	6.83E-18	0.0376
6	***ZSCAN12P1***	28058802	28059208	7	2.25E-13	-0.0388

(a) Minimum FDR p-value for the region

(b) For interpretability, changes across the DMR are reported as proportion methylated (beta), though models used logit-transformed beta values (M-values).

Table 2-4: Gene-sets enriched for differentially methylated genes in cord blood leukocytes by maternal first trimester urinary BPA exposures using LRPath.

Panel A. Pathways associated with maternal first-trimester urinary BPA exposure.

Pathway ID	Pathway Name	Database with Concept	No. of Genes in Concept	FDR	Direction
GO:0005132	type I interferon receptor binding	GOMF	13	7.82E-07	up
GO:0000786	nucleosome	GOCC	86	1.53E-04	up
GO:0044815	DNA packaging complex	GOCC	92	1.53E-04	up
GO:0005549	odorant binding	GOMF	81	3.07E-04	up
GO:0033139	regulation of peptidyl-serine phosphorylation of STAT protein	GOBP	18	0.00206	up
GO:0033141	positive regulation of peptidyl-serine phosphorylation of STAT protein	GOBP	17	0.00206	up
GO:0002323	natural killer cell activation involved in immune response	GOBP	25	0.00258	up
GO:0042501	serine phosphorylation of STAT protein	GOBP	22	0.00258	up
GO:1900424	regulation of defense response to bacterium	GOBP	11	0.00258	up
GO:0002922	positive regulation of humoral immune response	GOBP	15	0.00359	up
GO:0001055	RNA polymerase II activity	GOMF	10	0.00570	up
GO:0007259	JAK-STAT cascade	GOBP	155	0.00584	up
GO:0042100	B cell proliferation	GOBP	81	0.00584	up
GO:0043330	response to exogenous dsRNA	GOBP	40	0.00584	up
GO:0097696	STAT cascade	GOBP	155	0.00584	up
GO:0006959	humoral immune response	GOBP	157	0.00631	up
GO:0042742	defense response to bacterium	GOBP	205	0.00837	up
GO:0071880	adenylate cyclase-activating adrenergic receptor signaling pathway	GOBP	18	0.00917	up
hsa04623	Cytosolic DNA-sensing pathway	KEGG	51	0.00982	up
GO:0016290	palmitoyl-CoA hydrolase activity	GOMF	11	0.0105	up
GO:0007189	adenylate cyclase-activating G-protein coupled receptor signaling pathway	GOBP	82	0.0164	up
GO:0007192	adenylate cyclase-activating serotonin receptor signaling pathway	GOBP	11	0.0164	up
GO:0071875	adrenergic receptor signaling pathway	GOBP	25	0.0184	up
GO:0032993	protein-DNA complex	GOCC	154	0.0209	up
hsa04630	Jak-STAT signaling pathway	KEGG	145	0.0213	up
hsa04140	Regulation of autophagy	KEGG	30	0.0213	up
GO:0033617	mitochondrial respiratory chain complex IV assembly	GOBP	13	0.0337	up

GO:0034340	response to type I interferon	GOBP	79	0.0337	up
GO:0060337	type I interferon signaling pathway	GOBP	75	0.0337	up
GO:0071357	cellular response to type I interferon	GOBP	75	0.0337	up
GO:0097034	mitochondrial respiratory chain complex IV biogenesis	GOBP	13	0.0337	up
hsa05320	Autoimmune thyroid disease	KEGG	44	0.0341	up
GO:0050830	defense response to Gram-positive bacterium	GOBP	65	0.0381	up
hsa05322	Systemic lupus erythematosus	KEGG	118	0.0403	up
GO:0007187	G-protein coupled receptor signaling pathway, coupled to cyclic nucleotide second messenger	GOBP	183	0.0457	up
GO:0002286	T cell activation involved in immune response	GOBP	84	0.0460	up
GO:0019731	antibacterial humoral response	GOBP	35	0.0460	up
GO:0005665	DNA-directed RNA polymerase II, core complex	GOCC	17	0.0497	up

Panel B. Pathway associated with maternal first-trimester urinary BPF exposure.

Pathway ID	**Pathway Name**	**Database with Concept**	**Number of Genes in Concept**	**FDR**	**Direction**
hsa05322	Systemic lupus erythematosus	KEGG	118	0.0296	up

Note: Significance was considered at FDR q<0.05.

Figure 2-1: Histograms of maternal first trimester urinary bisphenol measures (ng/mL) adjusted for specific gravity (SG).

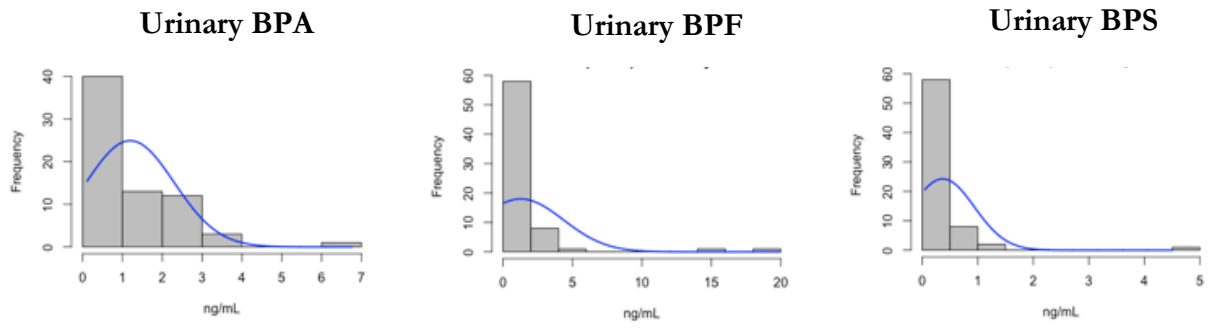
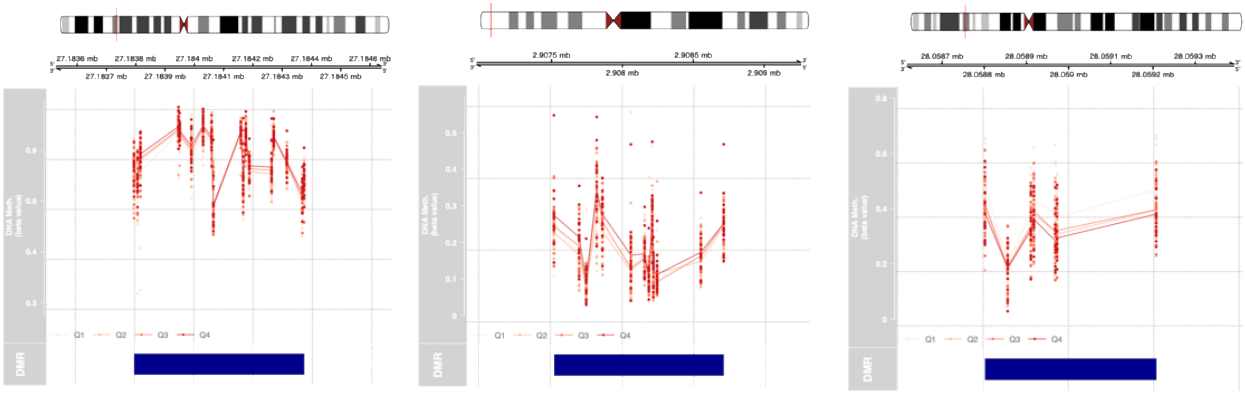


Figure 2-2: Differentially methylated regions associated with first trimester urinary BPA exposure.



Legend: Three DMRS in cord blood leukocytes in a) *HOXA-AS3*, b) *PRSS22,* and C) *ZSCAN12P1* were identified via DMRcate that were associated with first trimester BPA levels (modeled as a continuous variable and adjusted for infant sex, batch, and estimated nRBCs and B cells). Here, proportion of DNA methylation (beta values) at CpG sites within the DMR are displayed, averaged across quartiles of BPA. Quartile cut-offs are as follows: Q1 [<LOD, 0.348], Q2 [0.349, 0.897], Q3 [0.898,1.90], Q4 [1.91,6.76] in ng/mL BPA.

Chapter 3 Prenatal Timing of Exposure to One-Carbon Metabolites and the Epigenome-Wide Response in Infant Cord Blood Leukocytes

Introduction

Nutritional adequacy in women during pre-pregnancy, pregnancy, and post-partum is critical for both maternal health and the health of her child. The first 1000 days, or the time between conception and a child's second birthday (Schwarzenberg & Georgieff, 2018), is a time of such nutritional demand that rectifying preexisting nutritional deficiency is of immense importance. While consuming a well-rounded diet during pregnancy is foundational, there are many key nutrients that women are not sufficiently consuming and for which nutritional status is often not assessed. Furthermore, established requirements during pregnancy for many nutrients are either *insufficient* or are being reevaluated. The physiological changes that accompany pregnancy result in definitive changes in maternal nutritional needs.

In general, the literature acknowledges that requirements during pregnancy increase and that maternal nutrient deficits or excess can have measurable impacts on her growing offspring (Mousa et al., 2019). For example, excessive fat or protein intake during pregnancy is associated with metabolic perturbations of fetal and postnatal development (Mennitti et al., 2015; Symonds et al., 2009). This is of particular concern because nearly three-fourths of the US population consumes a diet low in vegetables, fruits, dairy, and oils; while also exceeding the recommendations for added sugars, saturated fat, and sodium (Mosher et al., 2016). Additionally, increasing evidence demonstrates that maternal diet is significantly associated with altered fetal epigenetic reprogramming (Hogg et al., 2012; Lau et al., 2011; Mathers, 2007; Saffery &

Novakovic, 2014; Szyf, 2009; Thornburg et al., 2010) and increased risk of later life disease (e.g. obesity, type 2 diabetes, and cardiovascular disease) (Ainge et al., 2011; Gluckman et al., 2007; McMillen & Robinson, 2005; Volpato et al., 2012). Nutrients like folate, choline, betaine, methionine, and their metabolic derivatives may play a critical role in the modification of the infant epigenome (Jiang et al., 2012; Kulkarni et al., 2011; Pauwels, Ghosh, Duca, Bekaert, Freson, Huybrechts, A. S. Langie, et al., 2017; West et al., 2013; Yan et al., 2013). This set of nutrients is highly correlated to and involved in the one-carbon metabolism pathway.

One-carbon metabolism (OCM) is principally mediated by folate, and it is a highly compartmentalized system of biosynthetic and catabolic reactions; the results of which produce and metabolize nucleotides and amino acids (Anderson, Sant, et al., 2012; Mahmoud & Ali, 2019). Purines, thymidylate, the regeneration of methionine from homocysteine, and transmethylation reactions are products and functions of the OCM network. The intersecting pathways of remethylating homocysteine to methionine and the formation of the universal one-carbon donor S-adenosylmethionine (SAM or AdoMet) are of particular focus in this chapter.

Methionine is a sulfur-containing amino acid and one of the nine amino acids essential to the diet (James D. Finkelstein, 1990). Within every cell, methionine use or function is divided into either protein synthesis or the formation of SAM (J. D. Finkelstein, 2000). Something to note here, and will be revisited shortly, is that amino acids, like methionine, and protein are very important for regulating energy metabolism in the body; protein is considered to be the second largest energy store in the body (George, 2006). The formation of SAM from methionine principally occurs in the liver; nearly 85% of the transmethylation reactions carried out by SAM and nearly 50% of methionine metabolism occurs in hepatic tissues (Lu & Mato, 2012). Methionine is converted to SAM with the addition of an adenosyl molecule by the enzyme

methionine adenosyltransferase (MAT) (J. D. Finkelstein, 2000). SAM is then poised to donate its one-carbon group (methyl group or -CH_3) to other molecules, including proteins, nucleic acids, carbohydrates, lipids, and small molecules in transmethylation reactions (e.g., the transfer of methyl-groups), which is catalyzed by a class of enzymes called methyltransferases (MTs) (Lu & Mato, 2012). MT enzymes include the DNA methyltransferase (DNMT) family, which uses SAM to transfer methyl groups on to C5 of the cytosine nucleotide ring. The establishment and maintenance of DNA methylation epigenetic marks is therefore highly dependent upon the availability of methionine and SAM in the cell, and by association, a myriad of additional cofactors and coenzymes in the OCM cycle. Coenzymes take the form of dietary micronutrients such as folate, vitamin B12, vitamin B6, betaine, choline, and methionine.

SAM-dependent reactions create S-adenosylhomocysteine (SAH) as a byproduct and competitive inhibitor of these reactions (J. D. Finkelstein, 2000). SAH is subsequently metabolized to homocysteine and adenosine by SAH hydrolase in a reversible reaction. SAH hydrolase favors the biosynthesis of SAH, so it is critical to OCM homeostasis for the homocysteine generated from hydrolysis of SAH be removed (Lu & Mato, 2012). Homocysteine can either enter the transulfuration pathway whereby it is converted to cysteine or it can be remethylated to regenerate methionine. The regeneration of methionine from homocysteine occurs through either methionine synthase (MS), which requires both folate and vitamin B12 as cofactors, or through betaine homocysteine methyltransferase (BHMT), which requires betaine—generated through the oxidation of choline (Kalhan, 2016). Micronutrient deficiencies and reduced protein intake have the potential to interfere with this pathway. So, baseline levels of given components of OCM are necessary, but because one-carbon metabolism is a *cycle*, a fundamental element of its integrity is negative feedback; levels and availability of micronutrient

cofactors influence the cycle's ability to generate, maintain, and replenish the components of the cycle, while also swiftly responding to the cell's needs.

Evidence suggests that disruptions of methionine metabolism and the related increased levels of plasma homocysteine are associated with metabolic abnormalities and health conditions in healthy adults and are of particular concern during pregnancy (Miller, 2012). Elevated homocysteine levels in adults is considered a risk factor for cardiovascular disease (Ganguly & Alam, 2015), neurodegeneration (A. D. Smith & Refsum, 2016), and other conditions (Fratoni & Brandi, 2015; Lai & Kan, 2015). In pregnancy, elevated levels of maternal plasma homocysteine are implicated in disorders such as preeclampsia (Gaiday et al., 2018; M. W. Kim et al., 2012), spontaneous abortion, and premature delivery (Bergen et al., 2012). Several studies exist that evaluate the normal fluctuation of OCM metabolites across human pregnancy (Cikot et al., 2001; Dasarathy et al., 2010; Gilley et al., 2020; Lindsay et al., 2015; Pinto et al., 2015), however, few studies have investigated a collective of OCM metabolites in association with epigenome-wide DNA methylation in the infant (Joubert et al., 2016; Knight et al., 2018). There is a genuine need to evaluate overall dietary quality and its impact on the fetal epigenome; such that improved insight into whole dietary patterns by newer dietary evaluation methods and the association with fetal epigenetic reprogramming will facilitate risk evaluation and development of better maternal nutrition counseling.

Protein is a highly studied macronutrient, and although most women in developed countries are consuming enough protein, cross-sectional data on the rates of sufficiency and/or insufficiency of protein intake are not plentiful. Furthermore, very little is known about amino acid requirements in humans. Studies indicate that during pregnancy, the requirements for some amino acids increase to a greater extent than others, such that it is likely inaccurate to assume

that amino acid requirements increase in proportion to protein requirements (Elango & Ball, 2016). Recent data derived through indicator amino acid oxidation, reveal that the current estimated average requirement (EAR) and recommended dietary allowance (RDA) are underestimates for many different groups, including pregnant women (Elango & Ball, 2016). Furthermore, studies in pigs show that requirements for threonine, lysine, isoleucine, and tryptophan increase during the later stages of pregnancy, but not at the same rate (Elango & Ball, 2016).

It is accepted that maternal diet during pregnancy is critical for normal fetal development. Evidence also supports the notion that maternal dietary requirements change for pregnancy to support mom and the various stages of fetal development. What requires elucidation is how nutrient requirements change at specific time points in pregnancy and the metabolic or physiologic fetal events that those changes are tied to and necessary for. OCM (proteins and the cofactor micronutrients) are such key compounds that understanding their concentrations from M1 to M3 opens the door to generating a map of 1) OCM dynamics during pregnancy, 2) linked developmental events relevant to those changes, and 3) the OCM with the greatest potential to alter the fetal epigenome or development. Interrogating these questions, with the endpoint of evaluating how OCM dynamics and concentration is associated with the availability of one-carbon groups for methylating an offspring's DNA, then allows for future investigations of whether current dietary recommendations are sufficient to support the metabolic changes at those timepoints. A study by Yan et al., eloquently demonstrates a portion of this process by investigating choline dynamics during late pregnancy with the use of stable isotope methodology and two choline intake groups. Yan et al., connected the concentration of a key OCM metabolite to metabolic processes and determined that current dietary recommendations for choline intake

during pregnancy were not sufficient to support the increased demand of those pathways. We considered it to be valuable and necessary to evaluate maternal levels of OCM at two time points and infant OCM levels at birth in association with fetal DNA methylation.

In this chapter we sought to identify genome-wide changes in infant cord blood DNA methylation associated with exposure to one-carbon metabolites. Untargeted metabolomic analysis via LC-MS/MS was performed on maternal first-trimester, third trimester, and umbilical cord plasma for N=89 maternal-infant pairs. From these data, we extracted one-carbon cycle metabolites betaine, choline, methionine, SAM, and SAH for further analysis. Multivariate regression models were used to investigate adjusted associations between these one-carbon metabolites and the cumulative distribution of infant cord blood DNA methylation across all CpG sites to evaluate the impact of maternal exposure on the infant epigenome and relative patterns of association between the timepoints. *We hypothesized that one-carbon metabolites would be associated with global shifts in the DNA methylation profile in infant cord blood.*

Methods

Study Population

The samples used in this study were derived from the Michigan Mother-Infant Pairs pregnancy cohort (MMIP), which initiated in 2011. Briefly, women providing informed, written consent were enrolled during their first prenatal visit to the University of Michigan Women's Hospital clinic. At this visit, maternal first trimester blood and urine were collected. Women also completed a questionnaire that gathered socio-demographic factors, health behaviors, food consumption and personal care product use, among other measures. Exclusion criteria included: age <18 years, prior infertility treatment, pregnancy with multiple fetuses, and pregnancy <8

weeks or >14 weeks gestation. Women were provided study materials between weeks 34-38 of gestation for blood and urine collection upon admission into labor. Maternal blood and urine were collected when admitted and umbilical cord blood samples were collected at delivery. At the time of writing, 331 mothers have enrolled in MMIP, and 200 have been followed-up through labor and delivery. For the analysis described here, a subset of MMIP families enrolled between 2011 and 2017 with DNA methylation analysis via the Infinium EPIC and with untargeted metabolomics at each time point (maternal first trimester N=93, maternal third trimester N=98, and cord blood N=96) were included. The University of Michigan Medical School Institutional Review Board approved all study procedures (HUM00017941).

Epigenome-Wide DNA Methylation Analysis of Infant Umbilical Cord Blood

Infant cord blood samples (N=111) were collected into PaxGene Blood DNA tubes (PreAnalytix) with the use of butterfly needles at the time of birth and stored at -80°C until processing. Total DNA was extracted with the PaxGene Blood DNA kit. DNA quality and concentration were assessed via Qubit at the University of Michigan Advanced Genomics Core. DNA was bisulfite converted using the EZ-96 DNA Methylation Kit (Zymo), wherein approximately 500ng of input DNA was used. The kit utilized sodium bisulfite to convert un-methylated cytosines to uracil and ultimately thymine, while methylated cytosines were protected. (Grunau et al., 2001) Following bisulfite treatment, DNA methylation at >850,000 CpG sites was evaluated using the Illumina Infinium MethylationEPIC BeadChip ('EPIC') at the University of Michigan Advanced Genomics Core according to standard protocols. Cord blood samples were run on three separate days, and these experimental batches are considered in statistical models.

Processing and Quality Control of Infinium MethylationEPIC Data

Arrays were assessed for quality of samples and probes using a standard pipeline. Briefly, the pipeline utilized the minfi package (Aryee et al., 2014) (R Project for Statistical Computing) to read in raw data image files. Quality control of samples was assessed by comparing estimated sex (from methylation values on the X and Y chromosomes) with known infant sex, detection p-values of probes, and intensity signals. Probes with poor detection (positions that failed detection in more than 10% of samples N=1475), cross-reactive probes, and probes that target polymorphic CpG sites in the Illumina HumanMethylation arrays were dropped. (McCartney et al., 2016) The Functional Normalization(Fortin et al., 2014) R package was used to correct for background and perform dye-bias normalization.

Using estimateCellCounts, the relative proportion of B-cells, CD4, CD8T, granulocytes, monocytes, neutrophils, and nucleated red blood cells (nRBCs) were estimated for each cord blood sample using an established algorithm based on DNA methylation profiles of sorted major cord blood cell types. (Bakulski et al., 2016)

These preprocessing steps resulted in 804,108 retained probes from N=111 cord blood samples that passed all quality control measures. Finally, M-values, defined as the log2 ratio of intensities of methylated probe versus unmethylated probes, were generated for each sample at these CpG sites and were used in downstream statistical analyses unless otherwise noted.

Metabolomics Profiling

Metabolites were extracted using an extraction solvent of MAA (Methanol : Acetonitrile : Acetone 1:1:1) with internal standards; for 200 samples 100 ml extraction solvent and 4 ml

internal standard mixture. Samples were reconstituted with the solvent: Methanol : H2O, 2 : 98. Untargeted shotgun metabolomics was performed on the reconstituted M1, M3, and CB samples. Samples in Ex00616 (N=56) were ionized in positive and negative ionization model using an Agilent Technologies 6530 Accurate-Mass Q-TOF with a dual ASJ ESI ion source. Samples from Ex00946 (N=) analysis was performed on an Agilent system consisting of an Infinity Lab II UPLC coupled with a 6545 QTof mass spectrometer (Agilent Technologies, Santa Clara, CA) using a JetStream ESI source in negative mode.

Raw data processing was done using Agilent software (Agilent MassHunter Qualitative Analysis). Metabolites participating in one carbon metabolism were classified using the National Institute of Standards and Technology (NIST) reference manual (Moorthy et al., 2017). Annotated metabolites were identified via comparing their MS/MS spectra to authentic standards, purchased internal or external standards ran on the same instrument.

Data normalization followed a recently described method (Fernández-Albert et al., 2014), using "pooled" reference samples that were analyzed repeatedly throughout each batch (M. Chen et al., 2014). Missing peak intensities were imputed by K-nearest neighbor-5 in features with at least 70% detection. R package "impute" was used for imputation. Features with less than 70% detection across samples were removed. Metabolites were log10 and z-score transformed. Z-score transformation was accomplished by using the scale function in R with centering. To center, the column mean of each metabolite is subtracted from its corresponding column. To scale, the centered columns of x are divided by their standard deviations. This was done for both first trimester and term maternal metabolites as well as for infant cord blood metabolites prior to using them in downstream analyses. Analyses performed on across timepoints that include M1,

M3, and CB include N=89 samples. For SS-association analysis and global, analyses were performed within-set numbers M1(N=93), M3 (N=98), CB (N=96).

Statistical Analysis

All statistical analyses were performed in R version 3.6.0 (Platform: x86_64-apple-darwin15.6.0 (64-bit) & Running under: macOS Mojave 10.14.6). We first performed univariate analyses on all metabolites and potential covariates of interest. We then assessed relationships between metabolites within and across timepoints using Pearson correlation. Equality of variances across time points were tested using Levene's Test (Fox et al., 2014). Following this, the distribution of metabolite concentration for each time point was visualized with boxplots. Subsequently, bivariate analyses were performed. Pearson correlation was used to test the correlation between metabolites, CB cell type proportions, and other covariates. T-test and ANOVA were performed to compare metabolite concentrations across categorical maternal and infant covariates and to compare average metabolite concentrations across timepoints.

Singular Value Decomposition (SVD) analysis was performed with the ChAMP package. (Tian et al., 2017) The correlation between principal components of the methylation data with biological and technical covariates was determined using linear regression (continuous variables) or Kruskal-Wallis (categorical variables). SVD analysis revealed that infant cord blood DNA methylation was significantly correlated with covariates of interest. Infant sex, B-cells, nRBCs, and sample-plate (batch) were selected as covariates to adjust for in final models at each time point due to their significant ($p<1x10^{-5}$) association with the DNA methylation data. Appendix 1.6 provides the SVD plots for each time point.

Cumulative Distribution

We performed unadjusted and adjusted logistic regression to assess the association between metabolite concentrations and global DNA methylation in infant cord blood. We calculated global methylation for each subject by first computing the mean methylation across all 804,108 CpG sites (or probes). We also calculated mean methylation for each subject across sites annotated to CpG islands, CpG shores, and CpG shelves. From the GAMP R package (Zhao et al., 2015), the TestCDF function was used to test the association between the DNA methylation distribution across all probes for each subject and exposure to one of the five metabolites at each of the three time points. The function uses a functional regression approach to approximate the cumulative distribution function (CDF) of the methylation values for each individual and a variance component test to assess significance.

Single-Site Association Analysis

Linear regression was used to identify differentially methylated CpG sites (using M-values) by each metabolite exposure, adjusting for covariates described above (infant sex, B-cells, nRBCs, sample plate). An empirical Bayes method in the limma (Smyth, 2005) R package was then used to shrink probe-wise variances towards a pooled estimate and calculate a moderated t-statistic. M-values were selected for statistical analysis given their advantages which include meeting the assumption of homoscedasticity and superior performance in Detection Rate (DR) and True Positive Rate (TPR), especially for highly methylated and unmethylated sites. (Du et al., 2010; Xie et al., 2019) P-value correction by the Benjamini-Hochberg false discovery rate (FDR) method was used, (Hochberg & Benjamini, 1990) and a 5% FDR (i.e. $q<0.05$) were considered significant.

Comparison to Previously Published Literature

Single-site results from Knight et al., 2018 (Knight et al., 2018) that derived from their analysis of the association of cord blood DNA methylation with one-carbon metabolites in infant cord blood were compared to our dataset. Specifically, we examined the association between one-carbon metabolites and DNA methylation in our data set using the m-values of five CpG sites, which Knight et al., identified as associated with metabolites. The sites tested were as follows: cg09238801, cg09501509, cg13753351, cg03527802, cg20694545, and $p<0.05$ was considered significant.

Differentially Methylated Regions (DMRs)

We utilized dmrcate (Peters et al., 2015) to test for differentially methylated regions by metabolite exposure. A differentially methylated region had to consist of at least 2 consecutive probes. Probes that were two nucleotides or closer to a single-nucleotide polymorphism (SNP) that had minor allele frequency greater than 0.05 were filtered out first. The model was adjusted for cell type (Bcell and nRBC), infant sex, and batch.

Pathway Analysis

LRpath (J. H. Kim et al., 2012) was utilized to perform gene-set enrichment across all probes annotated to genes (using Entrez Gene IDs) using concepts from KEGG and GO (Biological Process, Molecular Function, and Cellular Component). Raw P-values generated from the single-site linear model for the association between each metabolite and DNA methylation at sites within genes were used. Concepts from both Gene Ontology (GO) and

Kyoto Encyclopedia of Genes and Genomes (KEGG) databases were selected, and only gene-sets with a minimum of 10 and a maximum of 250 genes were used; a directional test was included. LRPath tests the odds that the genes in a concept have higher significance values (e.g., lower p-values from the differential methylation analysis) than expected at random.

Results

Table 3-1 contains the univariate descriptive statistics of the maternal-infant pairs included in this study. The mean maternal age was 32, and on average, the number of weeks to delivery was 39.7 weeks. The majority of participants in this subset identified as White (~94%). Mothers also identified themselves as African American (2%), Asian (2%), or Other (2%).

Average metabolite concentrations within timepoint were graphed to evaluate trends across time point. Trends between M1 and M3 differed across metabolites. For example, average concentrations of SAM and betaine decreased between M1 and M3, while average concentration of methionine, choline, and SAH increased (Figure 3-1). Mean metabolite concentration change across pregnancy (between M1 and M3) was significantly different for SAH, SAM, betaine, and choline at $p<0.001$. Alternatively, average concentration significantly increased between M3 and CB for each metabolite at $p<0.001$. Lastly, with the exception of betaine and SAM, metabolite concentrations also increased between M1 and CB (Figure 3-1). Next within-metabolite concentrations were graphed across time point (Figure 3-2). Levene's test revealed that the variance among timepoints for each metabolite was significantly different ($p<0.001$). CB metabolite concentrations had the greatest variance compared to M1 and M3.

Pearson correlations between metabolites and between metabolites and CB cell types were performed (Figure 3-3). M3 metabolites betaine, choline, and methionine were the only

metabolites to be significantly correlated with maternal/infant covariates of interest. M3 betaine was positively correlated with infant gestational age (r=0.235), M3 choline was positively associated with infant birth weight (r=0.290), and M3 methionine was negatively associated with maternal age (r=-0.222). Metabolites within- and across-timepoints displayed significant correlations with CB cell type covariates (Figure 3-3). Three M1 metabolites betaine, choline, and SAM had significant correlations with CB cell types: betaine:nRBC (r=0.211); choline:NK (r=0.284); SAM:CD8T (r=0.284), SAM:Gran (-0.289), SAM:NK (0.248). M3 SAH was significantly correlated with Mono (r=-0.253) and NK (-0.227), and CB SAH was significantly correlated with NK (r=-0.249) (Figure 3-3). Given the small sample size of this study, we chose to include those cell types with the strongest association with DNA methylation in our population.

Within-time point

Within M1, betaine and choline were positively correlated (r=0.50), betaine and SAM were positively correlated (r=0.22), and SAM and SAH were negatively correlated (r=-0.47) (Figure 3-3). Within M3, betaine and choline were positively correlated (r=0.47), betaine and methionine were positively correlated (r=0.33), choline and methionine were positively correlated (r=0.24) (Figure 3-3). Within CB, betaine was positively correlated with choline (r=0.65), methionine (r=0.91), and SAH (r=0.57). Choline was highly positively correlated with methionine (r=0.76), and methionine was positively correlated with SAH (r=0.47) (Figure 3-3).

Across time points

M1 to M3: M1 choline was correlated with M3 choline (r=0.289), and M1 methionine was correlated with M3 methionine (r=0.496). M1 SAH was correlated with M3 methionine (r=-0.257), M3 SAH (r=0.584), and M3 SAM (r=-0.267). M1 SAM was correlated with M3 choline

(r=0.235), M3 methionine (r=0.217), M3 SAH (r=-0.310), and M3 SAM (r=0.265). M1 SAH was significantly correlated with CB betaine (r=0.298) and CB SAH (r=0.756). M1 SAM was significantly correlated with CB SAH (r=-0.495). Three M3 metabolites were significantly correlated with CB SAH: M3 methionine (r=-0.253), M3 SAH (r=0.625), and M3 SAM (r=0.266).

Global Changes in DNA Methylation

M1 SAH metabolite concentration was significantly associated with the global distribution of DNAm in infant CB in both unadjusted (p=0.000139) and adjusted (p=0.0467) models (Table 3-2). This analysis indicates that the entire distribution of DNAm is shifted as metabolite concentrations increase. To infer the direction of this shift, we performed linear regression, adjusting for covariates, on mean DNA methylation levels across all CpG sites and within regulatory regions (e.g., CpG islands). While not statistically significant, the associations between DNA methylation and SAH were negative, indicating a shift towards less methylation. M1 SAM, methionine, choline, and betaine metabolite concentrations were not significantly associated with the global distribution of DNAm in infant CB. Similarly, M3 metabolites were not significantly associated with the global distribution of DNAm in infant CB.

CB SAH metabolite concentration was significantly associated with the global distribution of DNAm in infant CB in both unadjusted (p=0.00115) and adjusted (p=0.00110) models (Table 3-2). This analysis indicates that the entire distribution of DNAm is shifted as metabolite concentrations increase. Again, we performed linear regression to infer the direction of this shift, adjusting for covariates, on mean DNA methylation levels across all CpG sites and within regulatory regions (e.g., CpG islands). While not statistically significant, the associations

between DNA methylation and SAH were negative for three of four regions, indicating a shift towards less methylation. CB SAM, methionine, and choline metabolite concentrations were not significantly associated with the global distribution of DNAm in infant CB.

Single Site Association Analysis

M1 metabolites were not significantly associated with differential DNA methylation at specific loci in infant cord blood at $q<0.05$. M3 SAH was significantly associated with methylation at 272 sites in infant cord blood at $q<0.05$. The genomic inflation factor (lambda) for this analysis was 0.832, indicating a slightly underpowered model. Increasing maternal SAH concentrations were associated with higher DNA methylation at 74% of significant sites. M3 SAM, methionine, betaine, and choline were not significantly associated with differential DNA methylation in infant cord blood at $q<0.05$.

CB SAH, betaine, and methionine were significantly associated with differential DNA methylation in infant cord blood. CB SAH was significantly associated with 81 sites at $q<0.05$. The genomic inflation factor (lambda) for this analysis was 0.942. Increasing CB SAH concentrations were associated with higher DNA methylation at 93% of significant sites. Betaine was significantly associated with two sites at $q<0.05$ (lambda= l.28). Lastly, CB methionine was associated with one site at $q<0.05$ (lambda=1.01). A site located in the gene *FBXO16* was detected as the most differentially methylated site in both single site association analyses of CB betaine and of CB methionine. Increasing CB levels of betaine and methionine were associated with higher DNA methylation in this gene. CB SAM and CB choline were not significantly associated with differential DNA methylation in infant cord blood at $q<0.05$.

Comparison to Previously Published Literature

We found one published study that assessed the association between metabolites (in CB) and CB DNA methylation. We compared results from our models at 5 CpG sites from Knight et al., that were associated with metabolites. In our dataset, these 5 CpG sites were not significantly associated with any CB metabolite at $p<0.05$. We also tested the association of the five CpG sites with OCM metabolites in from M1 and M3 timepoints. Site cg13753351 (*PSMB7*) and cg09501509 (*unannotated*) were significantly associated with M1 SAM at $p<0.05$ and cg09501509 (*unannotated*) was close to significant in M1 choline. cg09238801 (*PNMA1*) was significantly associated with M3 choline.

Differentially Methylated Regions (DMRs)

Forty-four differentially methylated regions were detected in association with M3 SAH metabolite levels. Each region contained at least two sites, with a maximum of 12 (Table 3-3). The topmost differentially methylated region is within gene *ZNF559*, which also appears among the topmost differentially methylated sites. Thirty-one out of 40 regions displayed increasing DNA methylation with increasing M3 SAH. Eight differentially methylated regions were detected in association with CB SAH metabolite levels. Each region contained at least two sites, with a maximum of 12 (Table 3-4). Five out of eight regions displayed decreasing DNA methylation with increasing CB SAH. *ARGAP1* was also detected in the CB SAH single-site analysis. Four differentially methylated regions were detected in association with CB betaine metabolite levels. Each region contained at least two sites, with a maximum of 11 (Table 3-4). Three out of four regions displayed decreasing DNA methylation with increasing infant CB

betaine. One differentially methylated region was detected in association with CB methionine metabolite levels; however, it was not within an identified gene.

Pathway Analysis

M3 SAH associated DNA methylation sites were enriched for four gene-sets significant at FDR<0.05 (Table 3-5). For three of the four pathways, higher M3 SAH metabolite concentration was associated with decreased methylation in genes of the gene-set. CB SAH associated DNA sites were enriched for two gene-sets significant at FDR<0.05 and three gene-sets significant at q<0.10 (Table 3.6). Higher CB SAH levels were associated with decreased methylation. The gene-set or concept with the greatest odds for enrichment for differential methylation was linoleic acid metabolism (FDR=0.0438). Pathways related to fatty acid and lipid metabolism, amino acid metabolism, and carbohydrate metabolism were significantly enriched.

Discussion

This study examined the association of prenatal exposure to one-carbon metabolites at three time points with DNA methylation in the infant cord blood. By combining data from two high-dimensional platforms: LC-MS/MS untargeted metabolomics and the Illumina Infinium MethylationEPIC BeadChip ('EPIC') array, we identified (1) patterns of one-carbon metabolites within time points and across pregnancy, (2) one-carbon metabolites with the potential to influence global DNA methylation in cord blood leukocytes, and (3) maternal and infant one-carbon metabolites significantly associated with differential DNA methylation at specific genes in the infant cord blood.

Pregnancy is a time defined by significant physiological adaptations of the maternal system that are designed to ensure the adequate transfer of nutrients and oxygen to the growing fetus. Beginning around the time of implantation, the early embryo is fed by histiotrophic nutrition—the secretion products of the uterine gland, which are also known as 'uterine milk' (Moser et al., 2018). The formation of the definitive placenta and hemotrophic nutrition of the fetus begins around the start of the second trimester (*Pathology of the Human Placenta*). The second, and particularly the third trimester are periods of immense fetal growth, with increasing demands for carbohydrates, free fatty acids, and OCM (Lain & Catalano, 2007). The placenta is a critical mediator of nutrient transport between the maternal and fetal circulation; however, our knowledge is limited about OCM and how their levels in maternal circulation change across pregnancy trimesters, how they are transferred into fetal circulation, and how perturbations in plasma levels (both maternal and fetal) of those nutrients might impact the essential processes of DNA methylation and the establishment of the fetal epigenome. This study provides some key insight into these unknowns.

We detected that average maternal plasma concentrations of the OCM SAM and betaine decreased between M1 and M3, while methionine, choline, and SAH levels increased. This pattern of temporal change across pregnancy is similar to those documented in the literature. Gilley et al., performed a longitudinal evaluation of maternal OCM and amino acids from preconception, 12 weeks' gestation, and 32 weeks' gestation using dried blood spots and LC-MS/MS (Gilley et al., 2020). They determined that maternal methionine levels did not significantly change across pregnancy, while betaine significantly decreased, and choline significantly increased. These changes in betaine and choline were similarly detected by Yan et al., (Yan et al., 2012, 2013) and Visentin et al., (Visentin et al., 2015). In addition to evaluating

changes to maternal OCM concentrations across pregnancy, we were able to compare maternal OCM to CB OCM. We observed that infant CB levels of each metabolite were greater than maternal levels at M3. Reports by Visentin et al. and Molloy et al. found that cord plasma concentrations of free choline and betaine were 3 and 2 times maternal concentrations at birth (Molloy et al., 2005; Visentin et al., 2015). The study detailed in this chapter is unique in its measurement of SAM and SAH, and as it provides novel evidence of how these metabolites change across pregnancy as well as in comparison to CB concentrations.

Typical or uncomplicated pregnancies are associated with an increased rate of transsulfuration during early gestation (Dasarathy et al., 2010). Transsulfuration involves the transfer of the sulfur group of homocysteine to form cysteine; cysteine is a critical component in protein synthesis and in the production of the antioxidant glutathione (Sbodio et al., 2019). This pathway is not active in the fetal liver and therefore the fetus is entirely dependent upon maternal transsulfuration throughout gestation (Kalhan, 2016). SAH acts as a competitive inhibitor of the transmethylation of SAM and as an indicator of OCM, such that the metabolism of SAH into homocysteine and its subsequent use in either the transsulfuration pathway or transmethylation pathway is critical to ensure that the cycle is not interrupted (James D. Finkelstein, 1990). Accordingly, the detected association between increasing concentrations of M1 SAH and the decreased global methylation of the infant epigenome at birth potentially implies a disruption in the metabolism of methionine that resulted in decreased availability of SAM and consequently in DNA hypomethylation. The remethylation of homocysteine to methionine can be accomplished by two separate pathways—a folate-dependent pathway, which utilizes methionine synthase (MS) and requires vitamin B12 as a co-enzyme; and a choline-dependent pathway whereby betaine homocysteine methyltransferase (BHMT) requires betaine as a co-factor. Evidence

indicates that SAH is the critical determinant of cellular methylation capacity and global DNA methylation in lymphocytes (Yi et al., 2000); increases in homocysteine are reflected in concomitant increases in SAH concentration. Further, evidence in murine models demonstrates that deficiencies in both folate and choline are associated with increases in SAH and global hypomethylation (B. Li et al., 2019; Mehedint et al., 2010). Our analyses also revealed that OCM concentrations between M1 and M3 were highly correlated, that SAH concentrations were positively correlated across all three timepoints, and that M1 SAH and SAM were most strongly correlated to CB SAH and SAM concentrations.

In our analysis of the association between OCM and global changes in infant CB DNA methylation, M1 SAH and CB SAH were both significantly associated with the global distribution of DNA methylation in CB. From the linear regression analyses it was estimated that at both timepoints, increasing metabolite concentrations of SAH were associated with less methylation across the infant epigenome. The association between M1 SAH and a global shift in the infant epigenome is compelling, because not only is the first trimester an epigenetically vulnerable time (e.g., the critical period for fetal epigenetic reprogramming), but also because the fetus is directly dependent upon maternal dietary intake and nutrient stores for key metabolites like OCM to support DNA methylation reactions. That CB SAH was also significantly associated with a global shift in DNA methylation in the infant cord blood perhaps reveals the degree to which M1 OCM concentrations are predictive of CB concentrations and OCM potential (Molloy et al., 2005; Yan et al., 2012). It is of value to consider whether this association is reflective of conditions during the first trimester that influenced the fetal epigenetic microenvironment and perpetuated across pregnancy so as to be detectable at birth.

Alternatively, the association of M3 SAH and CB SAH with single-site DNA methylation in infant cord blood may be reflective of fine-tuning and changes in metabolism that are occurring as the fetus completes gestational growth. OCM concentrations are particularly important during this time period in relation to fetal tissue expansion, cellular growth, and the maintenance of DNA methylation marks (Yan et al., 2013). Analysis of the global distribution of DNA methylation provides some understanding of the metabolic environment during pregnancy overall. Single-site analysis of differential methylation at birth, on the other hand, aims to identify the most sensitive genes to change by one-carbon metabolite levels (Knight et al., 2018). We compared five CpG sites that were implicated as responsive to OCM by Knight et al., in their pilot study of OCM and infant CB DNA methylation, to M1, M3, and CB OCM and infant CB methylation in our cohort. Three of the five CpG sites were significantly associated with M1 and M3 metabolites in our cohort. There are few studies that evaluate the association between OCM concentration across pregnancy and DNA methylation such that, despite differences in study design, any replicability of detection of responsive genes across studies is promising and underscores the potential for OCM status to be a relevant exposure to fetal epigenetic reprogramming.

DMR analysis detected 44 DMRs in association with M3 SAH and 8 DMRs in association with CB SAH. These regions include and span genes involved in gene expression and regulation. Pathway analysis indicated a potential enrichment of gene-sets involved in immune response for M3 SAH, while CB SAH pathways implicated specific lipid metabolism pathways. These differential changes in DNA methylation that are occurring in infant cord blood in association with period-specific levels of SAH are likely connected to critical functional and expression changes needed during pregnancy.

The results of this chapter implicate modifications in methionine metabolism of the one-carbon pathway during pregnancy, which manifest as consistent associations between plasma SAH levels and both global and single-site DNA methylation in the infant cord blood. Methionine homeostasis is maintained not only through the cofactors B12, B6, and folate, but also through maternal dietary protein intake; the indirect and direct action of insulin and glucagon on methionine metabolism and whole-body protein turnover; and lastly through the redox state (Kalhan, 2009). Our global analyses and single-site analyses illuminate a portion of the relationship between OCM and gestational progress and the infant epigenome, while providing a foundation on which future investigations of OCM during pregnancy can build upon.

Limitations and Future Directions

While the use of the untargeted metabolomics platform is a powerful analytic tool, using a targeted approach would have allowed us to quantify metabolites accurately. In this analysis we were limited to data on relative abundance of the five metabolites of SAM, SAH, choline, betaine, and methionine. The addition of homocysteine, serine, and folate likely would have provided a more detailed picture of the OCM dynamics in this cohort of women and infants. For example, evaluating homocysteine and serine levels across pregnancy may have given an indication of methionine's utilization for transmethylation versus transulfuration. Furthermore, without dietary intake data for these mothers, our ability to detect associations between or make inferences about maternal sufficiency or insufficiency is finite. Our use of cord blood for epigenetics, as a surrogate tissue, limits our ability to draw conclusions about the association of OCM exposure on specific tissue development. Lastly, the sample size included in this study

limited our ability to detect all genes that were changing in response to the global shift in DNA methylation.

Future directions would include the addition of other metabolites in the OCM cycle, particularly cofactors and downstream metabolites of transulfuration. Evaluating maternal dietary intake of protein and foods high in methyl donors would allow for some understanding as to how intake correlates to plasma metabolite concentrations. Lastly, expanding epigenome-wide analyses to include the maternal timepoints of M1 and M3 in order to evaluate whether maternal epigenome or maternal OCM concentrations are more predictive of infant CB DNA methylation and OCM concentration.

Conclusion

Maternal dietary intake during pregnancy is a critical factor in the growth and health of her offspring in utero and their risk of developing disease later in life. Key maternal dietary components are foundational in the one-carbon metabolism pathway—one function of which is to support and maintain DNA methylation throughout the body. Therefore, disruptions to both maternal diet and subsequently DNA methylation during pregnancy have the potential to alter fetal epigenetic reprogramming. We sought to evaluate OCM concentrations at M1, M3, and in CB and to determine the association between concentrations at those timepoints with infant cord blood DNA methylation. We found that SAH at both M3 and in CB were most significantly correlated with both global and single-site measures of DNA methylation and associated with pathways related to metabolism and cell-signaling. These data provide insight into patterns of OCM across pregnancy in maternal and fetal tissues and add novel information about the

association between OCM concentrations during pregnancy and infant cord blood DNA methylation.

Table 3-1: Univariate descriptive statistics [median (25th, 75th percentiles) or n (%)] for N=89 mother-infant pairs in the MMIP cohort.

Maternal age (years)	32 (30, 34)
Number of days to delivery (days)	278.3 (274, 283)
Maternal Race/Ethnicity	
White	83 (93.725%)
African American	2 (2.24%)
Asian	2 (2.24%)
Other or mixed race	2 (2.24%)
Pre-pregnancy BMI	26.17 (22.04, 28.29)
Infant Sex	
Female	47(52.8%)
Male	42 (47.20%)
Infant birth weight (gms)	3533 (3270, 3820)
B cell proportion	0.0873 (0.0649, 0.104)
Monocyte proportion	0.0883 (0.0728, 0.105)
nRBC proportion	0.0823 (0.0462, 0.112)
CD4+ T cell proportion	0.150 (0.108, 0.188)
CD8+ T cell proportion	0.123 (0.0969, 0.144)
Natural killer cell proportion	0.0157 (0.0, 0.0230)

Table 3-2: Cumulative distribution (CDF) and linear regression analysis for one carbon metabolites across timepoints.

Maternal First trimester		**Adjusted Model**	
Cumulative Distribution		**p-value**	
Methionine		0.446	
Betaine		0.551	
Choline		0.628	
SAH		**0.0467**	
SAM		0.200	
Linear Regression		**Estimate (SE)**	**p-value**
SAH	**All CpG sites**	-4.30	0.375
	Island	-2.69	0.880
	Shore	-3.69	0.530
	Shelf	-3.55	0.351
Maternal Third trimester			
Cumulative Distribution		**p-value**	
Methionine		0.261	
Betaine		0.546	
Choline		1.0	
SAH		0.116	
SAM		0.819	
Cord Blood			
Cumulative Distribution		**p-value**	
Methionine		0.437	
Betaine		0.110	
Choline		0.820	
SAH		**0.00110**	
SAM		0.498	
Linear Regression		**Estimate**	**p-value**
SAH	**All CpG sites**	-2.53	0.606
	Island	5.99	0.741
	Shore	-2.41	0.684
	Shelf	-2.51	0.515

Table 3-3: Differentially methylated regions associated with maternal third trimester SAH metabolite concentration.

Coordinates	Gene Name	Number of CpG Sites	P-value	Max Beta Change per 1ln-SD Increase in SAH Metabolite
chr19:9434270-9435397	*ZNF559*	12	3.01E-30	-0.0181775
chr6:19042722-19042842		3	2.70E-20	0.09493959
chr2:219697159-219697934		4	8.34E-17	0.08229797
chr16:68563546-68564532	*ZFP90*	8	1.77E-16	-0.0098396
chr10:134755601-134756707	*TTC40*	9	1.04E-15	-0.0373321
chr10:75504089-75504431	*SEC24C*	4	6.81E-14	0.0028381
chr1:32422024-32422214		2	1.20E-12	0.07419423
chr20:45530115-45530513	*EYA2*	5	5.09E-12	0.07743727
chr15:74466704-74467626		8	3.67E-11	0.07119991
chr13:53602856-53603286		3	4.79E-11	0.0459873
chr2:98612331-98612676		9	6.54E-11	0.00778542
chr12:1772230-1773108		4	8.91E-11	0.04393667
chr1:161697052-161697574		5	9.33E-11	-0.0016959
chr12:49318487-49319263		9	9.33E-11	0.00677816
chr15:90792223-90793056		10	9.40E-11	-0.0191133
chr16:71688526-71688761		2	1.01E-10	0.0756491
chr15:42213459-42213760		2	1.24E-10	0.04559198
chr10:112610085-112610100		2	2.38E-10	0.06934625
chr2:64978882-64979061		2	3.94E-10	0.00166852
chr5:44575014-44575156		2	1.19E-09	0.02669672
chr2:39493370-39493603		2	1.20E-09	0.06906493
chr16:57219487-57220272		11	1.28E-09	-0.0366368
chr9:140064540-140065057		8	2.85E-09	0.03047486
chr2:46843324-46843544		4	2.86E-09	0.00284786
chr13:43597263-43597736		6	7.52E-09	-0.0389196
chr8:102216949-102217569		6	1.49E-08	0.00280453
chr11:18433500-18434171		8	3.72E-08	0.03547202
chr7:960246-960771		9	5.21E-08	-0.0300732
chr19:55972646-55973338		11	6.50E-08	-0.022823
chr16:83986452-83986941		6	6.56E-08	0.01504186
chr12:76084308-76084805		4	9.07E-08	0.06835634
chr7:157129430-157130065		9	9.16E-08	0.00367949
chr12:124246917-124247223		7	2.92E-07	-0.0100377
chr19:46144962-46145190		2	3.33E-07	-0.0031374
chr6:20401608-20401796		3	3.83E-07	0.00171405
chr1:103572615-103572723		2	6.41E-07	-0.0081583
chr8:144653944-144653975		3	2.58E-06	0.00500794
chr2:145281519-145281622		3	2.65E-06	0.00224292
chr2:71162693-71162762		2	3.06E-06	0.03815554
chr15:59180671-59180732		2	3.32E-06	0.04298845
chr3:73046492-73046503		2	4.34E-06	0.00389077
chr17:80189962-80190154		8	4.34E-06	0.01386486
chr9:36136613-36136796		3	4.42E-06	-0.0017898
chr10:127512031-127512103		3	4.96E-06	0.00119984

Table 3-4: Differentially methylated regions associated with cord blood metabolite concentration.

	Chromosome	Gene Name	Start (bp)	End (bp)	Number of CpG Sites	P-value	Max Beta Change per 1 ln-SD Increase in SAH Metabolite
CB SAH	chr17	*ALOX12P2*	6797466	6797590	2	5.02E-07	0.0342
	chr22	*ANKRD54*	38244746	38244902	5	1.57E-10	0.0539
	chr20	*ARFGAP1*	61915590	61916279	6	2.78E-13	-0.0330
	chr16	*PDK1*	2652948	2653839	11	1.78E-15	-0.0533
	chr2:	*NRP2*	206628088	206629314	12	3.01E-15	-0.0632
	chr16	*CLDN9*	3062056	3062975	10	5.18E-10	-0.0464
	chr7	*VWDE*	12443529	12444115	10	7.78E-09	0.0581
	chr6	*CRISP2*	49681178	49681391	8	4.59E-07	-0.0389
CB Betaine	chr2	*CDKL4*	39471028	39471182	2	3.17E-10	-0.0686
	chr14	*LOC646548*	70690287	70690704	7	3.17E-10	0.111
	chr1	*S100A13*	153599671	153600156	11	3.17E-10	-0.0736
	chr6	*RNF39*	30039374	30039524	10	3.17E-10	-0.0544

Table 3-5: Gene-sets enriched for differentially methylated genes in cord blood leukocytes by maternal third trimester S-adenosylhomocysteine (SAH) levels using LRPath.

Pathway ID	Pathway Name	Database with Concept	Number of Genes in Concept	P-Value	FDR	Direction
hsa04650	Natural killer cell mediated cytotoxicity	KEGG	121	5.17E-05	0.0108	down
hsa04622	RIG-I-like receptor signaling pathway	KEGG	63	2.04E-04	0.0213	down
hsa05320	Autoimmune thyroid disease	KEGG	43	3.38E-04	0.0235	down
hsa00514	Other types of O-glycan biosynthesis	KEGG	41	5.35E-04	0.0279	up

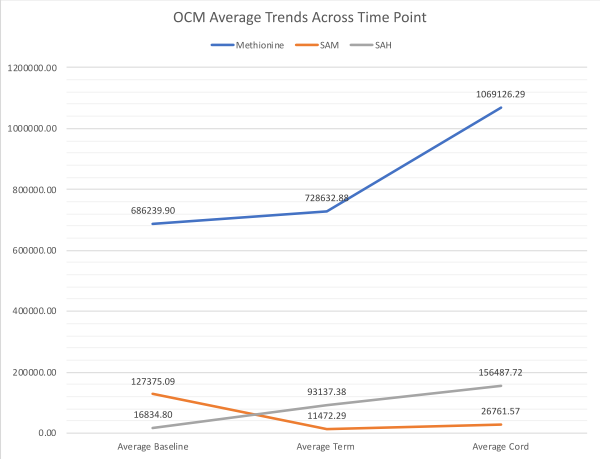
Note: Significance was considered at $q<0.05$.

Table 3-6: Gene-sets enriched for differentially methylated genes in cord blood leukocytes by cord blood S-adenosylhomocysteine (SAH) levels using LRPath.

Pathway ID	Pathway Name	Database with Concept	Number of Genes in Concept	P-Value	FDR	Direction
hsa05150	Staphylococcus aureus infection	KEGG	51	2.16E-05	0.00452	down
hsa00591	Linoleic acid metabolism	KEGG	30	4.20E-04	0.0438	down

Note: Significance was considered at q<0.05.

Figure 3-1: One-carbon metabolite trends across time points. Concentrations averaged within maternal baseline, maternal term, and infant cord blood.



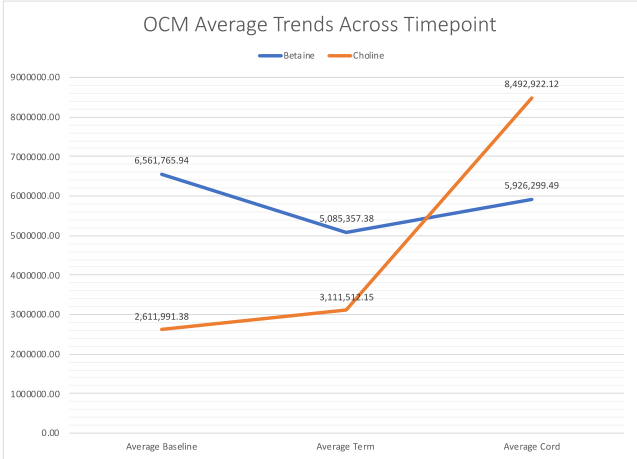
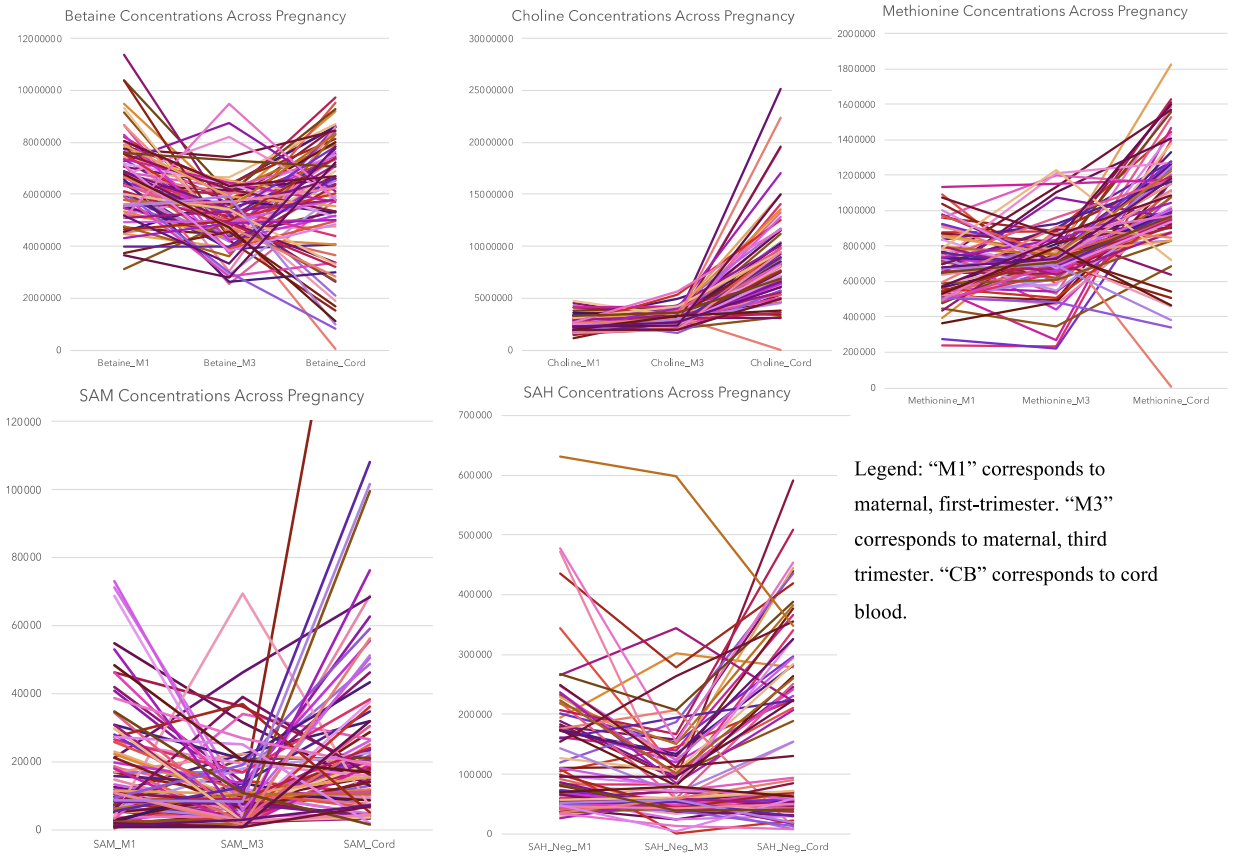
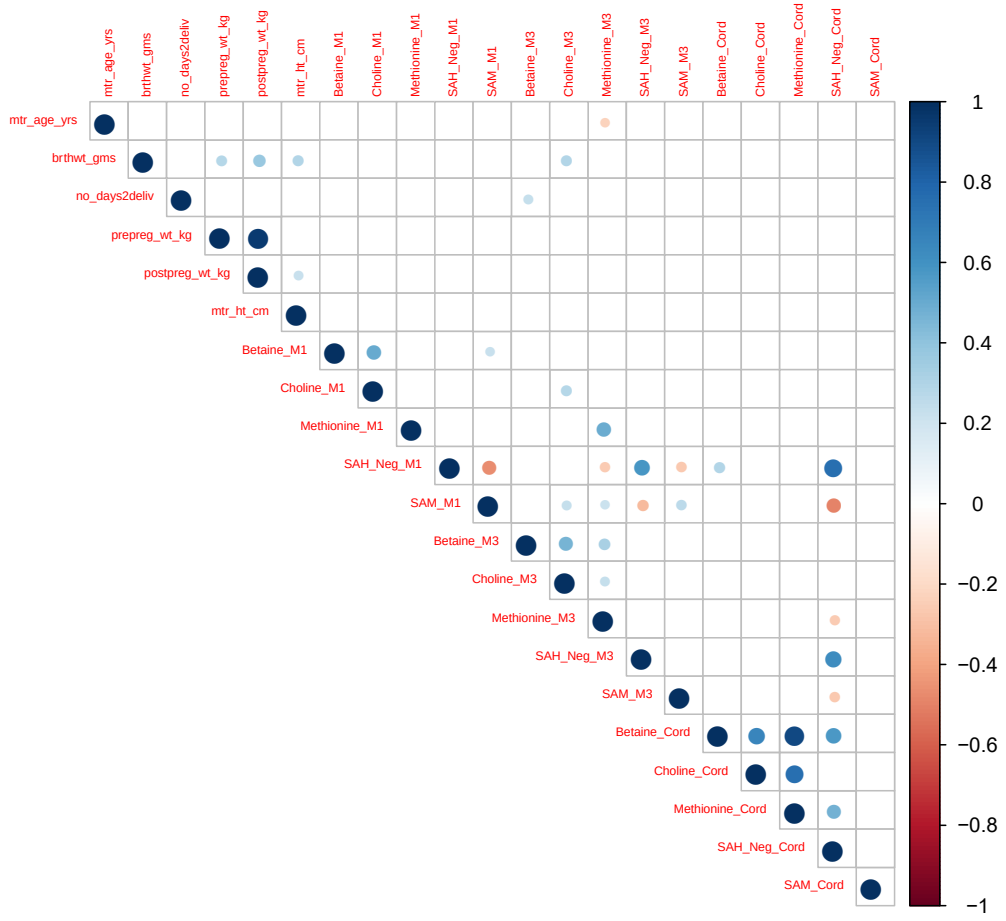


Figure 3-2: Intra-individual variability within metabolite concentrations across time points. Betaine, Choline, Methionine, SAM, SAH.



Legend: "M1" corresponds to maternal, first-trimester. "M3" corresponds to maternal, third trimester. "CB" corresponds to cord blood.

Figure 3-3: Matrix of Pearson correlations between metabolites, maternal and infant covariates, and cord blood cell types. Only statistically significant correlations ($p<0.05$) are colored in.



Chapter 4 DNA Methylation in Cord Blood, Cord Tissue, and Placenta: Comparison and Response to Prenatal Bisphenol Exposures

Introduction

The theory of DOHaD, as demonstrated thus far in and throughout this dissertation, is an important scaffold onto which investigations of early-life exposures can build. Chapters 2 and 3 aimed to expand upon the scientific understanding of the potential for maternal exposure to affect fetal epigenetic reprogramming, as one hallmark of DOHaD, by investigating changes in DNA methylation in cord blood leukocytes. Infant cord blood (CB), and whole blood in general, is commonly used to measure epigenome-wide DNA methylation in response to environmental exposure, because it is readily available, acceptable to collect (particularly in human pregnancy cohorts), and the DNA methylation profile in whole blood is associated with a variety of exposures and health conditions (Houseman et al., 2015). The epigenome differs in every tissue and even every cell type; a biological feature that assists in establishing tissue- or cell-specific gene expression and functions (Roadmap Epigenomics Consortium et al., 2015). Although advances have been made in elucidating how epigenomic signatures associated with cell types and tissues contribute to human disease (Campbell et al., 2020), additional studies are needed that address this concept. Epigenome-wide association studies (EWAS) can now integrate cell-type estimation algorithms that allow for the determination and control of cell-composition effects of whole blood—a method that, when combined with whole-blood analyses, offers the potential to assess many components of exposure response (Bakulski et al., 2016).

The use of CB is as a surrogate tissue; whereby changes in CB DNA methylation are evaluated as a proxy for target tissues like the brain or liver (Chadwick et al., 2015; Rakyan et al., 2011). Most epidemiological studies are limited to the collection of surrogate tissues, because of the clear, ethical restrictions of utilizing target tissues. As a result, pregnancy cohort studies most typically use CB, and some also include the use of placental tissue. This pipeline is standard for early-life exposure EWAS in epidemiology, however, there is a growing consensus within the field of environmental epigenetics for the quantitative and comprehensive interrogation of the association between early-life exposures and alterations in epigenome-wide DNA methylation in *multiple* neonatal or infant tissues; beyond and in addition to CB. It is compelling to use CB, umbilical cord tissue (UC), and placenta (PL) together within a single pregnancy cohort because of the potential to elucidate the way that these tissues interact and respond to prenatal exposures. Both UC and PL are tissues that are feasible to collect at the same time as CB, and due to their different embryonic origins, they offer the potential to gather additional information about the association of an exposure with differential DNA methylation in separate developmental lineages.

In a study by Lin et al., DNA methylation profiling of CB and UC samples from 295 neonates was performed with the IlluminaInfinium 450K array and compared between tissues (Lin et al., 2017). Hierarchical clustering analysis of infant UC and CB with 25 primary tissues and cells performed revealed that tissues and cells clustered in accordance with their germinal origin (Lin et al., 2017). Wherein CB clustered with hematopoietic stem cell (HSC)-derived mesodermic tissue (e.g. blood), while UC clustered with mesenchymal stem cell (MSC)-derived mesodermic tissue (e.g., muscle, heart, kidney) (Lin et al., 2017). Placenta, unlike CB and UC, is not composed solely of fetal cells, but rather it contains cells of both fetal and maternal origin.

These features of CB, UC, and PL, including that each tissue possesses its own epigenetic pattern of DNA methylation, establish a compelling incentive to not only add UC or PL to early-life EWAS, but also to compare their associations with exposures or outcomes of interest.

The PL is one of the most important tissues of pregnancy; engaging in multifaceted points of connection with the maternal and fetal system that require it to act as a master regulator to maintain the fetal environment. Proper formation and development of the PL is required for a successful pregnancy and is critical in fetal development. As such, research in fetal reprogramming and in human PL has turned its attention to the role of epigenetic regulation and modification in normal placental function and in the etiology of placental dysfunction (Maccani & Marsit, 2009). Recent evidence suggests that placentation, or the formation of the placenta, is regulated by epigenetic mechanisms and susceptible to dysregulation by environmental exposure (Strakovsky & Schantz, 2018). Not only is DNA methylation in the PL part of its normal physiology (Januar et al., 2015), but it also has varying characteristics of DNA methylation that are important for proper fetal development and progression through pregnancy. Currently, there is limited data detailing the effect of common human exposures like the bisphenols (BPA, BPF, and BPS) during pregnancy on placental or UC DNA methylation. However, birth cohort studies have reported associations between DNA methylation in PL and prenatal exposures including arsenic (Green et al., 2016), cadmium (Everson et al., 2018), copper (Kennedy et al., 2020), and maternal smoking (Morales et al., 2016). Both UC and PL are key tissues in fetal growth and development with evidence that UC and PL tissue-specific differentially methylated regions were enriched for functions in embryogenesis, vascular development, and the regulation of gene expression (Herzog et al., 2020). There is a critical gap in the literature of studies that compare CB, UC, and PL tissue DNA methylation both generally and in the context of prenatal exposure.

In this chapter, we compare DNA methylation across CB, UC, and PL at four genes. These genes were identified and selected from the study completed in Chapter 2—whereby prenatal exposure to BPA was associated with differential DNA methylation at 38 CpG sites and in three regions (DMRs) in CB. The candidate genes chosen include two genes from the single-site analysis and two DMRs - *FN1*, *SNAP25*, *HOXA-AS3*, and *PRSS22*. These genes were selected in part because of their functional relevance to biological processes.

FN1 (Fibronectin) is a high molecular weight glycoprotein and ubiquitous part of the extracellular matrix (ECM) (Dhanani et al., 2017). Fibronectin is involved in cell-adhesion and migration processes like embryogenesis and wound-healing; and expression levels of FN1 are correlated to pathologies like pre-eclampsia (PE) and intrauterine growth restriction (IUGR) (Wilson et al., 2015). *SNAP25* (synaptosomal-associated protein 25) is a key protein of the SNARE complex—a fundamental component and actor in neuronal signaling, in neurodevelopment, and neuroendocrine release (Kádková et al., 2019; Nazir et al., 2018). Disruption of the SNARE complex is linked to synaptic disfunction, neurodegeneration, and neurological disorders. SNAP25 has been distinguished as a potential neuropathological hallmark in identifying or treating disease (Karmakar et al., 2019).

HOXA-AS3 belongs to the Hox gene family, which are evolutionarily conserved and who's gene products are critical transcription factors (Quinonez & Innis, 2014). From developmental processes like anterior-posterior patterning to cell-lineage specification, Hox genes play a major role in development and oncogenesis. *HOXA-AS3* is a non-protein coding Hox member, and as a long non-coding RNA (lncRNA), it is also considered an antisense transcript (AST) (Zhang et al., 2018). ASTs or antisense lncRNAs have been demonstrated to control and play a role in each level of gene regulation (Villegas & Zaphiropoulos, 2015).

PRSS22 (protease serine S member 22, brain-specific serine protease 4 (BSSP4), or tryptase epsilon) is a member of the human serine protease family (C. Y. Chen et al., 2014). It is an epithelial protein involved in ECM remodeling and in the urokinase plasminogen activator (uPA) system, which is implicated in a variety of cancers (Yasuda et al., 2005). Furthermore, *PRSS22* and the other members of the serine protease family, function to degrade proteins in pathways related to inflammation, immunity, and embryonic development (Wong et al., 2001).

The study presented in this chapter aimed to (1) compare average percent DNA methylation in candidate genes *FN1*, *SNAP25*, *HOXA-AS3*, and *PRSS22* across CB, UC, and PL; (2) evaluate the association between prenatal exposure to the bisphenols BPA, BPF, and BPS and DNA methylation by tissue type; and (3) identify additional genes that are associated with prenatal bisphenol exposure in placental DNA using an epigenome-wide approach (EWAS). *We hypothesized that DNA methylation at FN1, SNAP25, HOXA-AS3, and PRSS22 would be significantly different across tissue type, that prenatal bisphenol exposure would be significantly associated in a tissue-specific manner, and that placental EWAS would result in additional loci associated with bisphenol exposure.*

Methods

Study Population

The samples used in this study were derived from the Michigan Mother-Infant Pairs human birth cohort (MMIP), which initiated in 2011. Chapter 2 contains the details of participant recruitment, inclusion criteria, and study timeline. The study presented here utilized maternal urine collected at the first trimester visit and placenta, UC, and CB samples collected at the time of delivery from a subset of families. N=29 subjects had all three tissues (placenta, UC, and CB)

available—they are denoted as 'tissue triads.' N=23 maternal-infant pairs had maternal urinary bisphenol measures in addition to the tissue triads. The maternal and infant samples included in this study were collected between the years 2011-2017. Phenotypic data collected on and assessed in these samples included: maternal age, number of days to delivery, maternal height, maternal weight pre- and post-pregnancy, infant sex, and infant birth weight.

DNA Extraction and Quantification of Tissue Triads

DNA Extraction

Infant CB samples (N=29) were collected into PaxGene Blood DNA tubes (PreAnalytix) with the use of butterfly needles at the time of birth and stored at -80 until processing. Total DNA was extracted with the PaxGene Blood DNA kit. DNA quality and concentration were assessed via Qubit at the University of Michigan Advanced Genomics Core.

PL samples were collected shortly after birth; PL segments were dissected and stored at -80 until processing. Total DNA was extracted with the AllPrep DNA/RNA/miRNA Universal Kit (Qiagen). DNA and quality concentration were assessed via Qubit at the University of Michigan Advanced Genomics Core.

UC samples were collected by dissecting a portion of the umbilical cord that was closest to the infant side. Samples were stored at -80 until processing. Total DNA was extracted with the DNeasy Blood & Tissue Kit (Qiagen). DNA quality and concentration were assessed via Qubit.

Bisulfite Conversion

DNA for each tissue was bisulfite converted using the EZ-96 DNA Methylation Kit (Zymo), wherein approximately 500ng of input DNA was used. The kit utilized sodium bisulfite

to convert unmethylated cytosines to uracil and ultimately thymine, while methylated cytosines were protected (Grunau et al., 2001).

DNA methylation measurement

Pyrosequencing (Tost & Gut, 2007) was used to measure DNA methylation levels at CpG sites in regions of interest in the genes *FN1*, *SNAP25*, *HOXA-AS3*, and *PRSS22* in CB, UC, and placenta. PCR amplification was performed on the bisulfite converted DNA. Primers utilized for PCR amplification were designed using the PyroMark Assay Design Software 2.0 (Supplementary file 1). DNA methylation levels were then quantified using the PyroMark Q96 ID (Qiagen). Matched sample triads (e.g., CB, UC, and placenta) were run on the same plate for PCR amplification and pyrosequencing. All pyrosequencing plates included a 0% and 100% human bisulfite converted control, no-template controls, and one row of samples were run in duplicate.

Maternal Bisphenol Measurement

Chapter 2 provides the full procedure by which the bisphenols BPA, BPF, and BPS were measured in maternal first trimester urine samples. Briefly, samples were collected from mothers during their first trimester visit at the University of Michigan Hospital, which took place between weeks 8-14 of pregnancy. Quantification of bisphenol concentrations and specific gravity (SG) were completed by NSF International (Ann Arbor, MI) as previously described (Goodrich et al., 2019). Urinary bisphenol values below the limit of detection (LOD, 0.2ng/mL) were replaced with LOD/$\sqrt{2}$ (0.141ng/mL).

Statistical Analyses

All statistical analyses were performed in R version 3.6.0 (Platform: x86_64-apple-darwin15.6.0 (64-bit) & Running under: macOS Mojave 10.14.6).

Cross-tissue Comparison

We first evaluated cross-tissue differences in DNA methylation at each gene. We visualized DNA methylation region averages for each subject through a spaghetti plot. Spearman correlations were calculated between tissue pairs. Finally, paired t-tests were run between tissue pairs to determine whether mean percent methylation at a gene was different across CB, CT, and PL.

Bisphenol Exposure and Tissue Response

Additional univariate analyses were completed on those families for whom maternal bisphenol measures were available. Bivariate analyses were completed to classify the correlation between bisphenol exposures and candidate gene methylation, in addition to covariates of interest, utilizing Spearman correlation. Bisphenol measures were adjusted for specific gravity and natural log (ln) transformed. Mixed effects regression was utilized to assess associations between first trimester bisphenol exposures and DNA methylation in the tissue triads. BPA, BPF, or BPS were regressed on repeat measures of DNA methylation for each gene at the three tissues. Models included a random intercept representing each subject and also adjusted for sex and tissue type. An interaction term between tissue type and exposure was included to determine whether any tissue(s) relationship with the exposure differed from the rest. Given that this is a proof-of-concept study with a small sample-size, results with a p-value<0.20 were discussed.

Since several genes had evidence for differential response across tissues (interaction term with p<0.20) by at least one bisphenol, the mixed effect regression was followed by linear regression for each gene in each separate tissue type to estimate the tissue-specific association

between exposure and DNA methylation. These models adjusted for infant sex, and when available, estimated cell type proportions of nRBCs and Bcells for CB and two surrogate cell type variables for placenta. Using estimateCellCounts, the relative proportion of B-cells, CD4 T-cells, CD8 T-cells, granulocytes, monocytes, neutrophils, and nucleated red blood cells (nRBCs) were estimated for each CB sample using an established algorithm based on DNA methylation profiles of sorted major CB cell types (Bakulski et al., 2016). Using RefFreeEWAS, cell type proportions were estimated for placenta, which is explained in further detail below.

Epigenome-Wide DNA Methylation Analysis of Placenta

Following bisulfite treatment, PL DNA methylation at >850,000 CpG sites was evaluated using the Illumina Infinium MethylationEPIC BeadChip ('EPIC') at the University of Michigan Advanced Genomics Core according to standard protocols.

Processing and Quality Control of Infinium MethylationEPIC Data

Arrays were assessed for quality of samples and probes using a standard pipeline. Briefly, the pipeline utilized the minfi package (Aryee et al., 2014) (R Project for Statistical Computing) to read in raw data image files. Quality control of samples was assessed by comparing estimated sex (from methylation values on the X and Y chromosomes) with known infant sex, detection p-values of probes, and intensity signals. Probes with poor detection (positions that failed detection in more than 10% of samples N=1475), cross-reactive probes, and probes that target polymorphic CpG sites in the Illumina HumanMethylation arrays were dropped. (McCartney et al., 2016) The Functional Normalization(Fortin et al., 2014) R package was used to correct for background and perform dye-bias normalization.

Using RefFreeEWAS, the relative proportion of putative cell types were estimated for PL with a reference-free deconvolution algorithm. This method utilized the underlying PL methylome to estimate constituent cell types and the number of cell types (Houseman et al., 2016). After these steps, two 'cell types' were retained. These preprocessing steps resulted in 822,020 retained probes from N=28 PL samples that passed all quality control measures. Finally, M-values, defined as the log2 ratio of intensities of methylated probe versus unmethylated probes, were generated for each sample at these CpG sites and were used in downstream statistical analyses unless otherwise noted.

Single-Site Association Analysis

Linear regression was used to identify differentially methylated CpG sites (using M-values) by each bisphenol exposure, adjusting for covariates infant sex and two surrogate variables for placental cell type. An empirical Bayes method in the limma (Smyth, 2005) R package was then used to shrink probe-wise variances towards a pooled estimate and calculate a moderated t-statistic. M-values were selected for statistical analysis given their advantages, which include meeting the assumption of homoscedasticity and superior performance in Detection Rate (DR) and True Positive Rate (TPR), especially for highly methylated and unmethylated sites.(Du et al., 2010; Xie et al., 2019). Due to the small sample size and exploratory nature of this part of our study, we used the p-value cut-off of $p<0.0001$. Ln-adjusted bisphenol measures were used in statistical models.

Differentially Methylated Regions (DMRs)

We utilized dmrcate (Peters et al., 2015) to test for differentially methylated regions by bisphenol exposure. A differentially methylated region had to consist of at least 2 consecutive probes. Probes that were two nucleotides or closer to a single-nucleotide polymorphism (SNP)

that had minor allele frequency greater than 0.05 were filtered out first. The model was adjusted for placental cell type (here called X1 and X2) and infant sex. A significance cut-off of $p<0.0001$ was used.

Pathway Analysis

LRpath (J. H. Kim et al., 2012) was utilized to perform gene-set enrichment across all probes annotated to genes (using Entrez Gene IDs) using concepts from KEGG and GO (Biological Process, Molecular Function, and Cellular Component). Raw P-values generated from the single-site linear model for the association between each bisphenol and DNA methylation at sites within genes were used. Concepts from both Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases were selected, and only gene-sets with a minimum of 10 and a maximum of 250 genes were used; a directional test was included. LRPath tests the odds that the genes in a concept have higher significance values (e.g., lower p-values from the differential methylation analysis) than expected at random. Significance for gene-sets was considered at $q<0.05$.

Results

DNA Methylation Varies across Tissue Type

Initial trends in average percent DNA methylation in candidate genes across tissue, spaghetti plots were generated (Figure 4-1). Across candidate genes *FN1*, *SNAP25*, *HOXA-AS3*, and *PRSS22*, UC tended to have higher percent methylation compared to CB and patterns in methylation across tissue differed depending on whether a gene was highly or lowly methylated.

DNA methylation percent, averaged across the sites for a given gene in each tissue, were graphed in boxplots (Figure 4-2) and compared via t-tests. Correlations between tissues were

also assessed. Notably, *FN1* and *HOXA-AS3* displayed similar cross-tissue trends, as did *SNAP25* and *PRSS22*. UC and PL average percent methylation were negatively correlated ($p<0.05$) in *FN1* and *HOXA-AS3,* while CB and PL average percent methylation were positively correlated ($p<0.05$) in *SNAP25* and *PRSS22*. Furthermore, paired t-tests demonstrated that average percent methylation was significantly different between at least one tissue pair per candidate gene. For example, in both *FN1* and *HOXA-AS3,* UC had the highest average DNA methylation percent, while PL had the lowest (Figure 4-2). Lastly, *SNAP25* and *PRSS22* had two tissue pairs that were not significantly different upon comparison.

Environmental Exposure Response across Tissue Type

To further assess tissue-specific differences or similarities we sought to determine the degree to which tissue types responded to environmental exposure to bisphenols. Table 1 details the univariate statistics for maternal BPA, BPF, and BPS exposure for the subset of families for whom all three tissues and first-trimester exposure levels were available. 95% of samples (22/23) had BPA levels above the LOD and 83% of samples (19/23) had BPF and BPS levels above the LOD.

First, mixed effects regression was performed to evaluate whether tissue-specific DNA methylation was associated with BPA, BPF, or BPS exposure (Figure 4-3). An interaction term between tissue and exposure was also included. Significant interactions were detected between BPF and tissue type for both *FN1* ($p=0.0102$) and *HOXA-AS3* ($p=0.00882$). Mixed effects modeling was followed by linear regression of each separate tissue to estimate associations between exposure and DNA methylation.While none were significant at $p<0.05$, we discussed with $p<0.20$ to inform future research. BPA exposure was associated with PL DNA methylation in candidate genes *FN1*, *SNAP25*, and *PRSS22* (Figure 4-3). For example, for every ln-

transformed-unit increase in BPA exposure (i.e. 2.23ng/mL), average PL DNA methylation at *FN1* decreased by 5.45% (p=0.094) (Figure 4-3). BPA exposure was only associated with CB DNA methylation in *SNAP25* (p=0.169) and did not display an association with UC DNA methylation. BPF exposure was associated with PL DNA methylation in *FN1* (p=0.15) and *HOXA-AS3* (p=0.098). BPF exposure was also associated with CB DNA methylation in *HOXA-AS3* and UC DNA methylation in *PRSS22*. Lastly, BPS exposure was associated with UC DNA methylation in *FN1* (p=0.131) and CB DNA methylation in *PRSS22* (p=0.108). Although some candidate genes displayed similar effects regardless of tissue type (e.g., *SNAP25*), DNA methylation response to bisphenol exposure ultimately exhibited tissue-specific patterns.

Single-Site Epigenome-wide DNA Methylation in Placenta

We assessed DNA methylation at 822,020 CpG sites via EPIC and report associations in placenta with bisphenols at $p<0.0001$ in this exploratory analysis. Maternal first-trimester urinary BPA exposure was associated with 63 differentially methylated sites (DMS) at $p<0.0001$. The genomic inflation factor (lambda) for the analysis was 1.20. Increasing BPA concentrations were associated with increasing DNA methylation at 83% of these CpG sites (Appendix 1.6). BPF exposure was associated with 29 sites at $p<0.0001$ (lambda=1.03) and increasing BPF concentrations were associated with decreasing DNA methylation at 97% of these CpG sites (Appendix 1.7). Similarly, BPS exposure was associated with 32 sites at $p<0.0001$ (lambda=0.797). Increasing BPS concentrations were associated with decreasing DNA methylation at 90% of these CpG sites (Appendix 1.8).

Differentially Methylated Regions in Placenta

Six differentially methylated regions (DMRs) were detected in placenta in association with maternal first-trimester urinary BPA exposure at $p<0.0001$ (Table 4-2). Each region contained at least five sites, and four of the six regions displayed a decrease in DNA methylation with increasing BPA levels. Sixteen DMRs were detected in placenta in association with maternal first-trimester urinary BPF exposure at $p<0.0001$ (Table 4-2). Each region contained at least two sites, and 13/16 (81%) of sites displayed a decrease in DNA methylation with increasing BPF levels. Two genes, *RPS6KA2* and *CBFA2T3*, contained two separate DMRs in association with BPF exposure. Thirty-seven DMRs were detected in placenta in association with maternal first-trimester urinary BPS exposure at $p<0.0001$ (Table 4-2). Each region contained at least three sites, and 23/37 (62%) of sites displayed a decreased in DNA methylation with increasing BPS levels. *ADAMTS17* contained three DMRs while *LMF1* contained two DMRs.

Pathway Analysis of Placenta Single-Sites

BPA exposure-associated DNA methylation sites were enriched for three gene-sets significant at FDR <0.05. Higher BPA exposure was associated with decreased methylation for genes in two out of three enriched pathways (Table 4-3). The pathway or concept with the greatest odds of enrichment for differential methylation was RIG-I-like receptor signaling pathway. In general, the enriched pathways were associated with immune sensing and inflammation/inflammatory host defenses. BPF exposure-associated DNA methylation sites were enriched for 13 pathways significant at FDR <0.05. Higher BPA exposure was associated with decreased methylation for genes in 12/13 pathways (Table 4-3). The pathway or concept with the greatest odds of enrichment for differential methylation was ascorbate and aldarate metabolism.

In general, the enriched pathways were associated with metabolism and drug metabolism. No pathways were significant for BPS exposure at FDR <0.05.

Discussion

Increasing evidence demonstrates that prenatal bisphenol exposure is associated with the disruption of **many** biological systems (Kolatorova et al., 2017). Just as the organs and tissues of the body operate in separate but synchronous ways to respond to and manage 'typical' functions or insults, so too do these organs and tissues respond in a multi-dimensional way to environmental toxicant exposure (Shu et al., 2019). Therefore, organ- or tissue-specific biological effects after fetal exposure must be characterized to enhance understanding of the broader processes that are implicated in exposure studies. One fundamental feature of tissue-specific regulatory systems is the epigenome. The epigenome is known to differ across tissues (Zhou et al., 2017) and assist in establishing tissue- or cell-specific gene expression and functions (Roadmap Epigenomics Consortium et al., 2015). Although advances have been made in elucidating how epigenomic signatures associated with cell types and tissues contribute to human disease (Campbell et al., 2020), the extent to which tissues respond to environmental exposures in a similar or different manner is limited. This has only recently become an active area of research in the basic sciences (Wang et al., 2018) and epidemiology (Campbell et al., 2020). In this study, we found tissue-specific differences in DNA methylation across candidate genes and responses to prenatal bisphenol exposure. PL displayed the greatest number of associations with bisphenol exposure across candidate genes, and the epigenome-wide analysis of DNA methylation in the placenta further emphasized these modest associations.

The inclusion of matched CB, UC, and PL in this pilot study was an important first step in

deconstructing tissue-specific response to prenatal bisphenol exposure. Studies are needed that not only evaluate tissue-specific DNA methylation in multiple tissue types, but that also evaluate such differences in the context of response to exposure and offer this perspective in a paired fashion. The utility of UC and PL in early-life exposure studies is derived both from their separate embryonic origins from that of CB—specifically as it relates to assessing risk for target tissues for which CB, UC, or PL would serve as surrogates—and their differential physiological responses to environmental toxicants. For example, UC has an increased potential to accumulate lipophilic toxicants like BPA during gestation as compared to CB, and is suggested to be a better tissue in which to assess fetal toxicant exposure (Fukata et al., 2005). Despite this, currently no published studies have evaluated storage of bisphenols in UC. In a similar fashion, PL possesses mechanisms to protect the fetus from potentially toxic or harmful substances in maternal circulation (Gude et al., 2004) and likely increases its toxicant burden as a result. For example, Grandin et al. modeled perfused human PL exposure to BPA and BPS to determine the degree to which PL limits fetal exposure to these bisphenols (Grandin et al., 2019). They concluded that while the PL has the capacity to minimize transfer of both BPA and BPS into fetal circulation, it appears to be more efficient at limiting exposure to BPS—with higher clearance indexes and active efflux mechanisms (Grandin et al., 2019). These findings underscore the importance of evaluating multiple bisphenols in human cohorts and elucidating the tissue-specific response to prenatal exposure in these surrogate tissues.

Exposures were assessed in the first trimester, because this a highly relevant time in the context of epigenetic reprogramming. Because of the propagation of early gestational epigenetic changes across tissues in the growing fetus, peripheral tissues like CB, UC, or PL are of particular value. In particular, CB and UC originate from separate germ layers, and genome-wide

comparison of DNA methylation patterns reveal distinct epigenetic profiles in each tissue when collected at birth (Lin et al., 2018; Sakurai et al., 2019). Until recently UC has been overlooked as an epigenetically relevant and rich surrogate tissue in prenatal exposure studies. Even more so, the PL methylome and epigenetic response to bisphenols has limited documentation in these contexts.

Our initial assessment of DNA methylation at each candidate gene revealed baseline tissue-specific differences. Further inspection indicated that these patterns replicated across candidate gene sets. *FN1* and *HOXA-AS3* methylation for both UC and PL were inversely correlated, and DNA methylation for each tissue type was significantly different. In *SNAP25* and *PRSS22* CB and PL were positively correlated even though baseline (mean) methylation levels differed. However, the particular utility in identifying tissue pairs that are correlated in candidate gene DNA methylation is the potential to leverage this information in selecting appropriate surrogate tissues and tissue pairs for prenatal exposure studies. Knowing how tissues are similar or different in their epigenomic patterning, as a consequence of embryonic origin or physiological function underscores why investigations should include more than one fetal surrogate tissue in environmental exposure studies. CB cell types originate from the mesoderm and cell type-specific CpGs from CB tend to be enriched for immune pathways (Lin et al., 2018). Alternatively, UC cell types derive from the extraembryonic ectoderm as well as the mesoderm, and UC contains a rich population of mesenchymal stem cells (MSCs) within the Wharton's jelly substructure of the UC (Bharti et al., 2018). Including overlapping tissues in EWAS expands our ability to detect associations between exposure and biological pathways or tissues derived from those embryonic tissues—associations which can then be translated into detangling human disease.

Some work has been done to compare the DNA methylomes of UC and CB. Recently, Sakurai et al., reported that UC exhibits hypomethylation across the genome as compared to CB, with location-specific hypomethylation around transcription start sites (Sakurai et al., 2019). They also noted that the UC methylome possesses enrichment of DMRs associated with HOX gene clusters and genes related to developmental body patterning and lineage-specific cellular differentiation (Sakurai et al., 2019). The addition of UC to prenatal studies has revealed additional and novel epigenetic associations and biological pathways with prenatal exposures and disease. Wu et al., in their study of preterm birth, illustrated the complementary benefit of including UC methylome in their analyses; of the 994 CpGs associated with preterm birth, only 10 were detected in CB (Y. Wu et al., 2019). Moreover, Herzog et al. detected tissue-specific differentially methylated regions (tDMRs) associated with human umbilical vein endothelial cells and with PL (Herzog et al., 2020). The detectable differences in DNA methylation between CB and UC and PL are meaningful because of the measurable consequences to outcomes and these tissues should all be examined with respect to outcomes and exposures.

We performed mixed effects and linear regression modeling to assess the similarities or differences by tissue in associations between prenatal bisphenol exposure and DNA methylation. Most commonly implicated were the bisphenols BPA and BPF, and they were consistently associated with PL DNA methylation across candidate genes using a relaxed p-value cut-off of 0.20 (Figure 4-3). Although UC and CB also displayed some significant associations with bisphenol exposure, the association within tissues and across candidate genes did not achieve the same magnitude as PL. These results suggest tissue-specific responses to exposure that also differ by bisphenol. As of this writing, no published studies have evaluated prenatal bisphenol exposure and UC DNA methylation. Similarly, only one human epidemiological study is

currently published that assessed placental DNA methylation in association with bisphenol exposure. Nahar et al. demonstrated the tissue-specific effects of prenatal BPA exposure on DNA methylation and found that repetitive element (LINE-1) percent DNA methylation in PL but not in fetal liver or kidney was significantly positively associated with total and free BPA concentrations (Nahar et al., 2015). Our analysis of epigenome-wide placental DNA methylation using the Illumina Infinium EPIC array revealed modest associations with maternal first-trimester BPA, BPF, and BPS exposure. Given the small sample size, we reported associations at $p<0.0001$ for each bisphenol; model results revealed that each exposure was associated with about the same number of differentially methylated sites. The gene family *DNAH* (dynein axonemal heavy chain) appeared in association with both BPA and BPS exposure, while the gene family *TRP* (transient receptor potential cation channel) appeared in association with both BPA and BPF exposure. However, no CpG sites were statistically significant at $q<0.05$, a common cut-off for statistical significance traditionally used in EWAS is a P-value correction by the Benjamini-Hochberg false discovery rate (FDR) (Hochberg & Benjamini, 1990).

We detected DMRs and biological pathways significantly associated with differential methylation in the PL in response to maternal exposure to BPA, BPF, and BPS. BPA-linked genes *EPHB4* (X. Liu et al., 2016, 2017; Shi et al., 2019); BPF-linked genes *RPS6KA2* (Kimball et al., 2015) and *CBFA2T3* (Fujiwara et al., 2013)*;* and BPS-linked genes ADAMTS-family (Namli Kalem et al., 2017; Namlı Kalem et al., 2018) revealed a consistent pattern of association with key developmental processes, like vascular development of the PL and disorders like preeclampsia and intrauterine growth restriction (IUGR). Gene-set analysis further emphasizes these associations and possibly delineates the way in which these bisphenols may differ overall. The three significant gene-sets associated with BPA exposure are implicated in immune-sensing,

the microbiome and inflammation, and inflammatory host defenses (A H Bryant, 2017) (Antony, 2015). Further, they are linked to initiating parturition through chromatin remodeling (Wang, 2019), gestational diabetes (Zhao, 2011), and epigenetic dysregulation of placental gene expression in preeclampsia (Leavey, 2018). This correlates to evidence that BPA exposure is associated with dysregulated placentation and elicits 'preeclampsia-like' features in mice (Ye et al., 2019). BPF-associated gene-sets include steroid and drug metabolism, a feature that is commonly linked to BPA exposure with increasing evidence also implicating BPF (Yang et al., 2017). Taken together, the DMR and gene-set analyses suggest a connection between prenatal exposure to bisphenols and increased inflammation, impaired vascularization, and endocrine disruption in the placenta. Although these births were uncomplicated and no pathology of the PL was detected, it is relevant to consider whether an increased sample size and a broader range of BPA exposure might validate these associations and increase our ability to detect complications. While there are studies that have evaluated BPA exposure and its association with changes in epigenetic marks like DNA methylation (Nahar et al., 2014), histone modifications and chromatin remodeling (Senyildiz et al., 2017), the specific mechanism of how bisphenol exposure elicits those changes is unclear. These data as a whole point to the need for the inclusion of a variety of tissues in prenatal exposure studies with larger sample sizes; the similar, although at times divergent responses in CB and UC, and the unique PL epigenetic response, demonstrates their utility in combination—as a means to capture a fuller picture of the developmental effect of an exposure through epigenetic programming. Future work is needed to characterize whether DNA methylation in these surrogate tissues correlates with health outcomes and of which health outcomes UC and CB are the best predictors. Determining the utility of surrogate tissues in epigenomic analyses will enable more effective use of population-based

studies to make connections between exposure, epigenetic changes, and the development of disease.

Limitations

The small sample size across our analyses limits the reliability and generalizability of our results. Reported results should be considered trends to explore in future studies as we lacked statistical power to detect all true associations with typical significance cut-offs. Furthermore, bisphenols exhibit non-monotonic dose responses and evaluating families with exposure level extremes would add key information about the risks posed for that portion of the population. It is also a limitation that we only evaluated four candidate genes and regions. It is possible that the differences in DNA methylation that we detected may not be reflective of broader changes across tissues.

Strengths and Future Directions

Despite the limited availability of families with all three tissues, we consider it a notable strength of this study that we compared DNA methylation at four candidate genes in matched tissue samples of CB, UC, and PL. As of this writing, this is the first study to include paired CB, UC, and PL in DNA methylation analyses as well as to evaluate tissue-specific differences and prenatal bisphenol exposures. Furthermore, this is the first study to perform epigenome-wide analysis in PL evaluating its association with prenatal bisphenol exposures. Despite the small sample sizes presented in this study, our evaluation of three bisphenols provides technical foundation for the inclusion of BPA's replacement analogs and emphasizes the need to evaluate their association with DNA methylation in multiple tissue types.

Future studies are warranted to examine whether the tissue-specific differences and associations can be replicated in a cohort with an increased number of subjects. Given the ease of collection of UC future studies are also needed that analyze epigenome-wide DNA methylation in UC in response to prenatal bisphenol exposure. The cross-tissue correlations detected underscore the importance of comparing epigenome-wide data across UC, CB and PL. The information gained through this Aim must be combined in future work with additional studies that evaluate health outcomes in order to enhance our understanding of changes in DNA methylation in these tissues as being representative tissues for diseases of interest.

Conclusions

This study is a proof-of-concept demonstrating similarities and differences in baseline DNA methylation across CB, UC, and PL, at four genes and in their associations with prenatal bisphenol exposures. Our exploratory analysis of epigenome-wide DNA methylation in placental tissue revealed modest associations with prenatal bisphenol exposure. We recommend that future investigations consider the addition of UC and/or PL as surrogate tissues in conjunction with CB to broaden the ability to detect or decipher biological pathways associated with environmental exposure.

Table 4-1: Descriptive statistics [median (25th, 75th percentiles) or n (%)] for N=28 mother-placenta pairs in the MMIP cohort included in this study.

Maternal age (years)	33 (31, 35)
Number of days to delivery (days)	275 (274, 278)
Maternal Race/Ethnicity	
White	28 (100%)
Cell type variable 1	0.502 (0.193, 0.827)
Cell type variable 2	0.248 (0.0425, 0.385)
Maternal urinary BPA (ng/mL)	1.01 (0.424, 1.30)
Maternal urinary BPF (ng/mL)	1.87 (0.411, 2.05)
Maternal urinary BPS (ng/mL)	0.331 (0.156, 0.375)
Infant Sex	
Female	14 (50%)
Male	14 (50%)
Infant birth weight (gms)	3452 (3195, 3698)

Limit of detection (LOD) <0.2 ng/mL. Urinary bisphenol measures adjusted for specific gravity.

Table 4-2: Differentially Methylated Regions in Placenta in Association with Maternal First-trimester Urinary Bisphenol Exposure. Significance considered at p<0.0001

A.

Location	Gene Name	Number of CpG Sites	P-value(a)	Max Beta Change per ln-ng/mL BPA increase(b)
Chr7: 100424355- 100425827	*EPHB4*	12	1.24E-12	-0.0620
Chr1: 55246867-55247408	*TTC22*	5	1.14E-08	0.0779
Chr1: 234367145-234367586	*SLC35F3*	5	6.36E-08	0.0642
Chr11: 46367725-46368295	*DGKZ*	5	9.81E-08	-0.0517
Chr2: 202125088-202125310	*CASP8*	5	1.22E-07	-0.0506
Chr11: 67170528-67171585	*PPP1CA*	7	5.15E-10	-0.0335

B.

Location	Gene Name	Number of CpG Sites	P-value(a)	Max Beta Change per ln-ng/mL BPF increase(b)
chr6:167275395-167276650	*RPS6KA2*	16	1.87E-09	-0.0512389
chr17:79098772-79099882	*AATK*	9	2.09E-08	-0.0249671
chr16:88948617-88950197	*CBFA2T3*	9	7.01E-07	-0.0434763
chr6:167190034-167190226	*RPS6KA2*	3	9.82E-07	-0.0317611
chr2:219135802-219135936	*PNKD*	2	4.04E-06	-0.0040822
chr6:31829644-31829960	*NEU1*	9	7.48E-06	-0.0296765
chr8:91013575-91014327	*DECR1*	6	1.97E-05	0.03289722
chr5:150618948-150619039	*GM2A*	2	2.10E-05	0.10775812
chr1:76080294-76080727	*SLC44A5*	5	2.10E-05	0.05970793
chr13:114111864-114112218	*DCUN1D2*	6	2.36E-05	-0.0407328
chr4:124232-124622	*ZNF718*	6	2.98E-05	-0.0183294
chr9:116225793-116225992	*RGS3*	4	3.11E-05	-0.0478295
chr16:88942335-88942358	*CBFA2T3*	2	4.88E-05	-0.0315858
chr11:3187511-3187939	*OSBPL5*	15	5.25E-05	-0.0165179
chr6:31478822-31478830	*MICB*	2	5.34E-05	-0.0444635
chr11:16761290-16761533	*C11orf58*	4	6.91E-05	-0.0272765

C.

Location	Gene Name	Number of CpG Sites	P-value(a)	Max Beta Change per ln-ng/mL BPS increase(b)
chr15:92398726-92399195	*SLCO3A1*	2	2.34E-06	-0.0519
chr16:983381-983870	*LMF1*	2	5.78E-05	-0.0506
chr17:77245306-77245327	*RBFOX3*	2	2.34E-06	-0.0656
chr6:29690766-29692183	*HLA-F*	26	7.23E-16	-0.0967
chr6:28983835-28985069		23	3.63E-13	0.0637

chr6:29648161-29649084	*ZFP57*	22	2.87E-16	-0.1067
chr10:102278918-102280155	*SEC31B*	15	4.31E-11	0.0859
chr2:70994758-70995607	*ADD2*	15	6.45E-11	0.1172
chr12:96183791-96185064	*NTN4*	12	2.72E-13	0.0830
chr9:33473445-33474350	*NOL6*	12	4.27E-08	0.0331
chr12:81331012-81331863	*LIN7A*	11	4.25E-07	0.0797
chr5:170735973-170736572	*TLX3*	11	1.73E-05	0.0323
chr6:32116653-32116963	*PRRT1*	10	5.78E-05	0.0619
chr3:185000208-185001026	*MAP3K13*	9	2.35E-09	-0.0740
chr6:32022929-32023409	*TNXB*	9	1.10E-05	0.0364
chr2:177042493-177043501	*HOXD-AS1*	8	4.10E-11	-0.1317
chrX:154842296-154842856	*TMLHE*	8	3.43E-05	0.0738
chr1:68517177-68517462	*GNG12-AS1*	7	1.76E-06	0.0353
chr1:161228203-161228877	*PCP4L1*	6	5.06E-07	-0.0603
chr16:1251787-1252484	*CACNA1H*	6	6.77E-06	-0.0663
chr17:28088578-28088749	*SSH2*	6	6.58E-05	0.0897
chr2:131792521-131793064	*ARHGEF4*	6	1.38E-05	-0.0862
chr15:100532781-100533336	*ADAMTS17*	5	1.56E-05	-0.0693
chr8:1814096-1814957	*ARHGEF10*	5	5.30E-06	-0.0357
chr8:2003810-2004488	*MYOM2*	5	1.96E-06	-0.0814
chr8:20831094-20831500		5	5.94E-07	-0.0427
chr15:100537304-100537761	*ADAMTS17*	4	4.11E-05	-0.0659
chr4:19756214-19756485	*RP11-608O21.1*	4	5.32E-06	-0.0798
chr6:13873924-13874251		4	5.73E-06	0.0500
chr1:158900384-158901032	*PYHIN1*	3	3.46E-07	-0.0915
chr10:131686425-131686574	*EBF3*	3	4.95E-05	-0.0537
chr15:72565016-72565039	*CELF6*	3	6.63E-05	-0.0170
chr15:99408804-99409194	*IGF1R*	3	3.55E-05	0.0507
chr15:100666162-100666305	*ADAMTS17*	3	3.27E-06	-0.0663
chr16:971556-971820	*LMF1*	3	1.28E-05	-0.0458
chr17:77657538-77657578		3	2.57E-07	-0.0535
chr8:13372453-13372491	*DLC1*	3	6.73E-05	-0.0672

A. BPA B. BPF C. BPS

(a) Minimum FDR p-value for the region

(b) For interpretability, changes across the DMR are reported as proportion methylated (beta), though models used logit-transformed beta values (M-values). Models adjusted for infant sex and two surrogate variable cell types.

Table 4-3: Gene-sets enriched for differentially methylated genes in placenta by maternal first-trimester urinary bisphenol exposures using LRPath.

A.

BPA

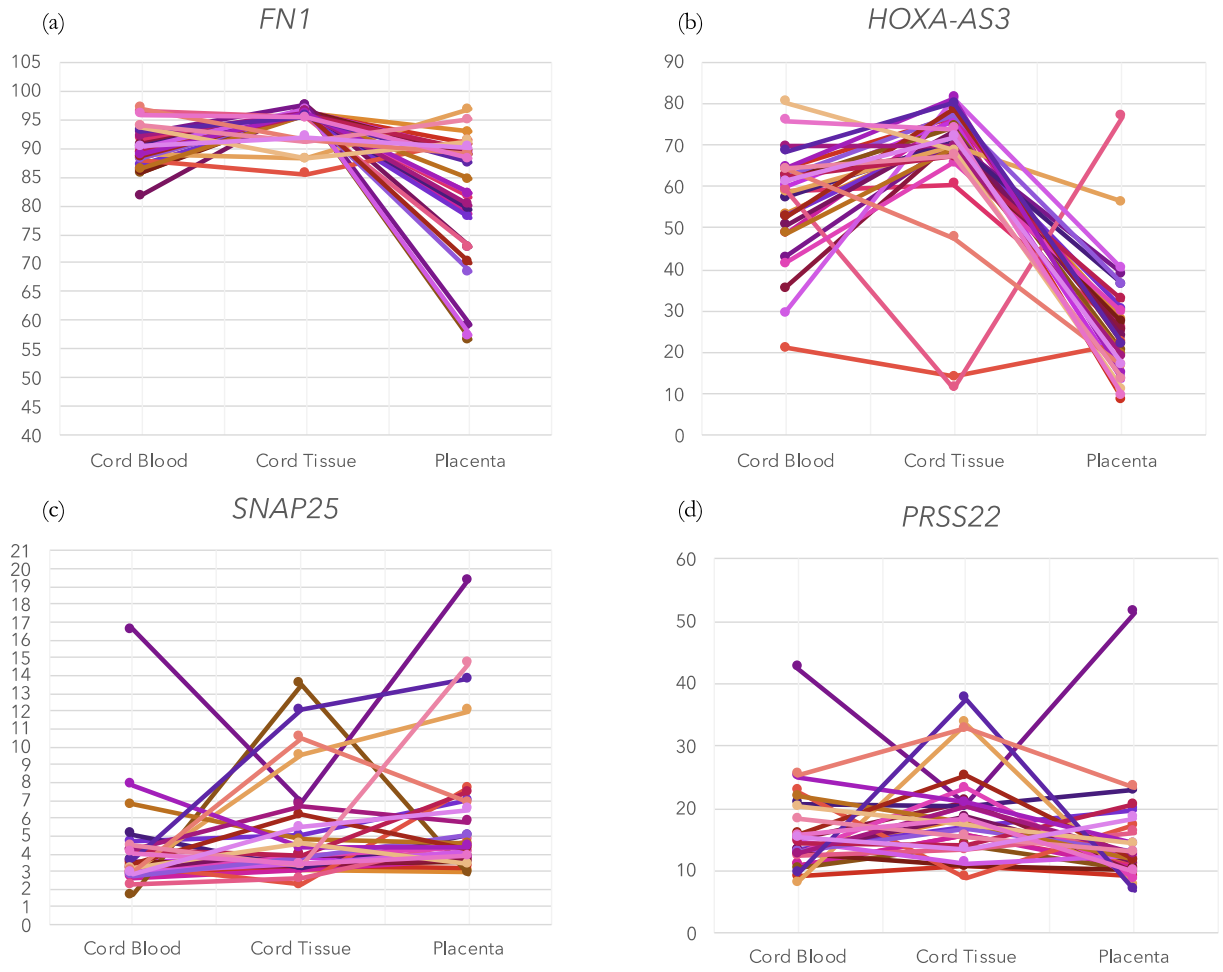
Pathway ID	Pathway Name	Database with Concept	No. of Genes in Concept	FDR	Direction
hsa04622	RIG-I-like receptor signaling pathway	KEGG	67	0.00576	down
hsa00650	Butanoate metabolism	KEGG	29	0.00576	up
hsa04060	Cytokine-cytokine receptor interaction	KEGG	249	0.0259	down

B.

BPF

Pathway ID	Pathway Name	Database with Concept	No. of Genes in Concept	FDR	Direction
hsa00053	Ascorbate and aldarate metabolism	KEGG	22	1.04E-10	down
hsa00040	Pentose and glucuronate interconversions	KEGG	27	2.83E-09	down
hsa00982	Drug metabolism - cytochrome P450	KEGG	67	2.50E-08	down
hsa00983	Drug metabolism - other enzymes	KEGG	46	4.81E-08	down
hsa00140	Steroid hormone biosynthesis	KEGG	52	4.81E-08	down
hsa00830	Retinol metabolism	KEGG	60	4.81E-08	down
hsa00860	Porphyrin and chlorophyll metabolism	KEGG	38	4.81E-08	down
hsa00980	Metabolism of xenobiotics by cytochrome P450	KEGG	66	4.81E-08	down
hsa00514	Other types of O-glycan biosynthesis	KEGG	42	1.37E-07	down
hsa00500	Starch and sucrose metabolism	KEGG	46	1.39E-07	down
hsa04742	Taste transduction	KEGG	50	0.0301	down
hsa04950	Maturity onset diabetes of the young	KEGG	24	0.0349	down
hsa04144	Endocytosis	KEGG	197	0.0491	up

Figure 4-1: Average percent methylation across tissue type for four genes in matched samples.

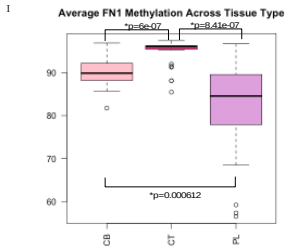


(a) *FN1* (b) *HOXA-AS3* (c) *SNAP-25* (d) *PRSS22*. Methylation was measured via pyrosequencing; methylation is the average of CpG sites for each gene and each line represents one individual. Y-axis shows percent methylation.

Legend: Spaghetti plots assist in identifying differences in DNA methylation as the graph is viewed (1) across tissue type; (2) within tissue type; and (3) within a single subject. Each graph represents one candidate gene, and average percent methylation in UC, CB, and PL was plotted for each subject (e.g., a subject is represented by a circle, and a subjects' measurements are connected with a line across tissues) in each gene.

Figure 4-2: DNA methylation comparisons across CB, UC, and PL.

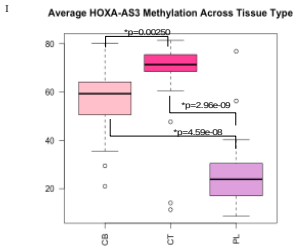
I



II

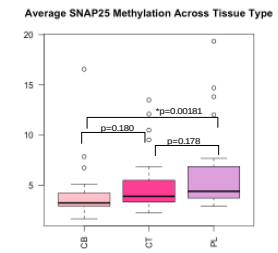
	Cord Tissue	Placenta
Cord Blood	-0.253	0.213
Cord Tissue		**-0.533**

I

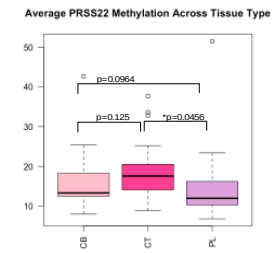


II

	Cord Tissue	Placenta
Cord Blood	0.261	-0.268
Cord Tissue		**-0.418**



	Cord Tissue	Placenta
Cord Blood	0.0263	**0.608**
Cord Tissue		0.344



	Cord Tissue	Placenta
Cord Blood	0.0348	**0.840**
Cord Tissue		-0.0315

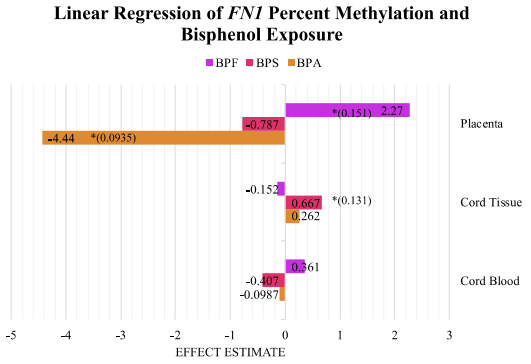
Panel I. DNA methylation percent averaged across the sites in a given gene for each tissue (N=29 subjects per tissue). Boxes represent the IQR with the line showing the median. Significance shown for paired t-test between each tissue and denoted with an asterisk (*) when $p<0.05$.

Panel II. Spearman correlation coefficients for DNA methylation at each gene are shown for matched tissues (N=29 subjects per tissue). Statistical significance denoted by correlation coefficients in **bold** ($p<0.05$).

Figure 4-3: Mixed-effects and linear regression analysis of DNA methylation and bisphenol exposure across CB, UC, and PL.

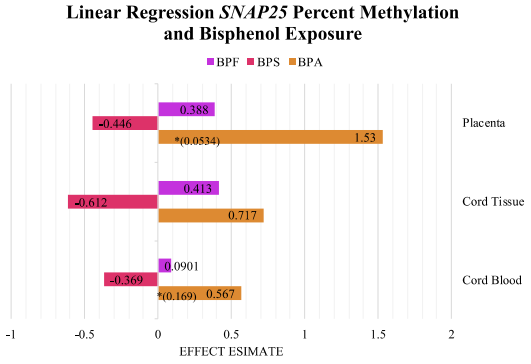
Mixed Effects FN1

	Estimate (se)	p-value
lnSGAdjBPA	-0.213 (1.33)	0.874
lnSGAdjBPA*Sample	-1.17 (1.09)	0.287
lnSGAdjBPF	-0.556 (0.786)	0.483
lnSGAdjBPF*Sample	**1.60 (0.601)**	**0.0102**
lnSGAdjBPS	-0.615 (1.54)	0.690
lnSGAdjBPS*Sample	0.781 (1.20)	0.518



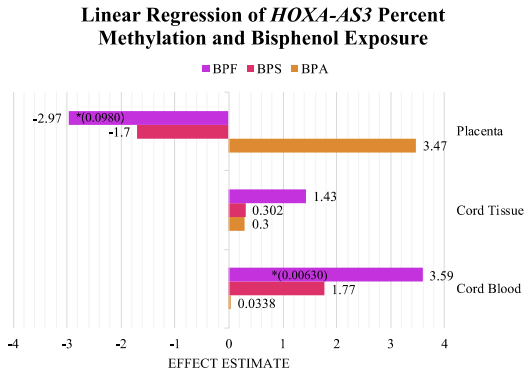
Mixed Effects SNAP25

	Estimate (se)	p-value
lnSGAdjBPA	-0.225 (0.465)	0.631
lnSGAdjBPA*Sample	0.408 (0.316)	0.205
lnSGAdjBPF	-0.0360 (0.280)	0.898
lnSGAdjBPF*Sample	0.0998 (0.193)	0.608
lnSGAdjBPS	-0.427 (0.510)	0.407
lnSGAdjBPS*Sample	0.109 (0.358)	0.763



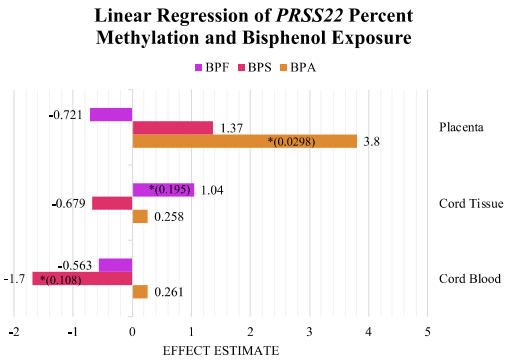
Mixed Effects HOXA-AS3

	Estimate (se)	p-value
lnSGAdjBPA	-0.376 (2.41)	0.877
lnSGAdjBPA*Sample	2.22 (1.95)	0.260
lnSGAdjBPF	**3.74 (1.41)**	**0.0102**
lnSGAdjBPF*Sample	**-2.96 (1.09)**	**0.00882**
lnSGAdjBPS	0.783 (2.71)	0.773
lnSGAdjBPS*Sample	-1.50 (2.15)	0.488



Mixed Effects PRSS22

	Estimate (se)	p-value
lnSGAdjBPA	-0.415 (1.05)	0.694
lnSGAdjBPA*Sample	0.341 (0.778)	0.663
lnSGAdjBPF	-0.171 (0.560)	0.761
lnSGAdjBPF*Sample	0.0518 (0.424)	0.903
lnSGAdjBPS	-0.722 (1.05)	0.492
lnSGAdjBPS*Sample	0.485 (0.798)	0.546



Chapter 5 Conclusions

Summary of Main Findings

The overall purpose of this dissertation was to examine DNA methylation in fetal tissues in response to maternal exposure to bisphenols and one-carbon metabolites. Specifically, we identified differential DNA methylation in infant cord blood leukocytes in association with prenatal exposure to BPA, BPF, and BPS. Additionally, we identified patterns of OCM across maternal and infant timepoints and OCM that were correlated with global shifts in the DNA methylation profile of infant cord blood. Lastly, utilizing matched CB, UC, and PL samples, we explored tissue-specific DNA methylation in candidate genes and examined both the differential tissue response across tissue triads and changes in epigenome-wide DNA methylation in placenta to bisphenol exposure.

Maternal Environmental Exposure to BPA is Associated with Changes in Epigenome-Wide DNA Methylation in Infant Cord Blood

In Chapter 2 we utilized the EPIC array to assess the association between maternal prenatal exposure to the bisphenols BPA, BPF, and BPS and epigenome-wide DNA methylation in infant cord blood. This study is one of the few human studies to leverage epigenome-wide DNA methylation technology to investigate early-life exposure to bisphenols. The focus of this investigation centered on maternal exposure to bisphenols during the first trimester, by virtue of early in development being the most susceptible and critical period for epigenetic effects (McCabe et al., 2017). The single-site analysis revealed 38 individual CpG sites in infant cord

blood leukocytes that were differentially methylated in relation to early maternal BPA exposure and three differentially methylated regions in the genes *HOXA-AS3, PRSS22*, and *ZSCAN12P1*. The association of prenatal BPA exposure to DNA methylation was diminished, however, when sensitivity analyses that excluded one outlying subject were performed. Associations with BPA and DNA methylation at CpG sites and the DMR in *PRSS22* remained ($p<0.001$) after outlier exclusion.

Gene-set analysis demonstrated the enrichment of gene-sets linked to the nervous system, immune response, and neuroinflammation. Prior research has established an association between prenatal BPA exposure and disruption to genes involved in these pathways (Anderson et al., 2016b; Arambula et al., 2016; Jadhav et al., 2017; Martínez et al., 2018; Weinhouse et al., 2016). Further, currently published literature of prenatal exposure to BPA and its epigenetic impact present similar findings of genes and pathways related to neurological function and inflammation (Alhomaidan et al., 2019; Kolatorova et al., 2017). Although we did not detect differential DNA methylation in the same genes, there is concordance between our results and those reported by Junge et al., Montrose et al., Alavian-Ghavanini et al., and Miura et al. in the potential for prenatal BPA exposure to impact genes related to MSCs, adipogenesis, and perhaps long-term body weight (Alavian-Ghavanini et al., 2018; Junge et al., 2018; Miura et al., 2019; Montrose et al., 2018). Neither BPF nor BPS maternal exposures were significantly associated with differential DNA methylation in the infant cord blood in this study, however ours is the first human study to evaluate the association of prenatal BPF or BPS exposure with infant DNA methylation. The results of this Aim suggest an association between prenatal bisphenol exposure and DNA methylation of key risk loci and biological pathways in infant cord blood.

Prenatal One-Carbon Metabolites are Highly Correlated across Pregnancy and Associated with Global Shifts in Infant Cord Blood DNA Methylation

Chapter 3 we examined the association of prenatal exposure to one-carbon metabolites at three time points with DNA methylation in the infant cord blood. We utilized both LC-MS/MS untargeted metabolomics and the EPIC array to generate a high-dimensional perspective of this correlation. We detected that average maternal plasma concentrations of the OCM SAM, SAH, and betaine decreased between M1 and M3, while methionine and choline levels increased across timepoints. Similarly detected changes are validated in the literature for choline, betaine, and methionine, but to our knowledge, this is the first report to detail the pattern of SAM and SAH between M1 and M3 of pregnancy in conjunction with epigenome-wide association analysis (Gilley et al., 2020; Visentin et al., 2015; Yan et al., 2012, 2013). We also compared metabolite concentrations at maternal timepoints to those in CB. We observed that average concentration of SAM, SAH, methionine, and choline increased between M1 and CB, whereas each OCM increased in concentration between M3 and CB. Our analyses also revealed that OCM concentrations between M1 and M3 were highly correlated, that SAH concentrations were positively correlated across all three timepoints, and that M1 SAH and SAM were most strongly correlated to CB SAH and SAM concentrations. These observations contribute to our understanding of OCM patterns across pregnancy and their role in establishing OCM concentrations in infant CB. M1 SAH and CB SAH were both significantly correlated with the global distribution of DNA methylation in CB whereby increasing metabolite concentrations of SAH were associated with less methylation across the infant epigenome. These associations reveal the degree to which M1 OCM concentrations may predict CB concentrations and OCM potential to support DNA methylation reactions in the growing infant (Molloy et al., 2005; Yan

et al., 2012). Single-site, DMR, and pathway analyses of OCM across pregnancy and differential methylation in the infant cord blood illuminated fine-tuning changes in OCM metabolism that may occurr as the fetus completes gestational growth. The enrichment of gene-sets and pathways associated with gene expression and regulation and the immune response underscore the importance of OCM across pregnancy and the potential for maternal OCM plasma concentration to shape infant growth and development.

DNA Methylation in Cord Blood, Cord Tissue, and Placenta: Comparison and Response to Prenatal Bisphenol Exposures

In Chapter 4 we performed pyrosequencing to quantify CpG-level DNA methylation in candidate genes *FN1*, *SNAP25*, *HOXA-AS3*, and *PRSS22* across three tissue types (CB, UC, and PL) from matched tissue samples. We utilized mixed effects and linear regression to determine the degree to which these tissues associated with prenatal bisphenol exposures in the same or different manner. Lastly, we performed an exploratory epigenome-wide analysis using the EPIC array to identify additional genes associated with bisphenol exposures in placenta. Our results indicated that DNA methylation at each candidate gene significantly differed at one or more tissue type. *FN1* and *HOXA-AS3*, and *SNAP25* and *PRSS22* displayed DNA methylation patterns across tissue pairs that were reflective of overlapping tissue-specific gene regulation. Mixed effects and linear regression models detected significant associations between BPA and BPF and CB and PL. PL displayed the greatest number of associations with bisphenol exposure across candidate genes. Epigenome-wide analysis of DNA methylation in the placenta revealed modest associations with prenatal bisphenol exposure. This Aim and the tissue-specific associations of DNA methylation in both cross-tissue and bisphenol exposure analyses spotlight the importance of expanding the number of surrogate tissues used in EWAS. Future studies are needed that

leverage tissue-specific DNA methylation and the embryonic lineage of surrogate tissues to identify whether these differences in DNA methylation across tissue types are correlated with health outcomes of human disease.

Comparison Across Studies

An integrated analysis of the studies presented in this dissertation revealed similar environmentally responsive pathways. For example, gene-sets related to the immune system and autoimmunity like "RIG-I-like receptor signaling pathway," "systemic lupus erythematous," and "autoimmune thyroid" were consistently enriched in CB (Aim 1 & 2) and PL (Aim 3) in association with prenatal exposure to BPA, BPF, BPS, and SAH. Notably, models of prenatal bisphenol exposure in both CB (Aim 1) and PL (Aim 3) also displayed enrichment for gene-sets related to amino acid metabolism. One-carbon metabolism is essential for both DNA methylation and immune function. In particular, methionine is a key nutritional factor for CD4+ T helper cell proliferation and function (Roy et al., 2020) and for the maintenance of histone methylation (Tang et al., 2020); SAM generation and availability is critical to inflammatory macrophage function (Yu et al., 2019); and lastly methionine is necessary to B cell differentiation and function (Lio & Huang, 2020). It is possible that this observed concordance of immune function and sensitivity between Aim 1, 2, and 3 provides mechanistic insight into prenatal bisphenol exposure and epigenetic reprogramming. We performed post-hoc analysis of model results across each aim to determine the correlation between BPA-DNA methylation associations and OCM-DNA methylation associations in our cohort. When we compared the full model results from prenatal bisphenol exposure and infant CB DNA methylation in Aim 1 with the model results from prenatal SAH exposure and infant CB DNA methylation in Aim 2, we detected significant ($p<2.2e^{-16}$) correlations between each bisphenol and CB SAH, M1 SAH, and M3

SAH (Table 5-1). Notably, the BPA:CB model was negatively correlated with each SAH model, while BPS:CB was positively correlated each SAH model. Furthermore, BPF:CB was positively correlated with SAH in M1, but negatively correlated with SAH in M3 and CB. These correlations highlight the differences between prenatal exposure to BPA, BPF, and BPS and in their association with OCM and DNA methylation. This result bears comparison with the foundational mouse study performed by Dolinoy et al., that utilized the Aguti A^{vy} mouse in a prenatal exposure model of BPA and BPA + a methyl donor supplement (Dolinoy et al., 2006; Dolinoy, Huang, et al., 2007). They discovered that upon maternal dietary supplementation with methyl donors or the phytoestrogen genistein, the BPA-induced hypomethylation observed at the A^{vy} IAP locus was counteracted (Dolinoy et al., 2006; Dolinoy, Huang, et al., 2007). The negative correlation between BPA and SAH indicate that these two exposures similarly have opposing effects on (a significantly sized portion of) the epigenome in this human cohort. In the context of the study by Dolinoy et al., the significance of maternal dietary supplementation negating the effect of prenatal BPA exposure on DNA methylation in the A^{vy} IAP locus is also in the correlating shift in the coat color distribution away from yellow. Yellow A^{vy} mice are the result of decreased methylation near the A^{vy} IAP locus, and they are prone to metabolic disorders and obesity as compared to their genetically identical, brown siblings (Waterland & Jirtle, 2003). In humans, differential DNA methylation in association with a prenatal exposure evinces pleiotropic effects on health and disease that we are only beginning to untangle. The corollary between our results and those of Dolinoy et al., and the patterns observed for BPF and BPS—that BPF and OCM are negatively correlated at the end of pregnancy but not at the beginning, while BPS displays the same direction of effect as SAH—are important to distinguish because of the

value in translating these findings across species in leveraging the diet to mitigate the effect of exposures to environmental toxicants.

We also compared model results from prenatal bisphenol exposure and infant CB DNA methylation in Aim 1 with the model results from prenatal bisphenol exposure and PL DNA methylation in Aim 2. We detected significant ($p<2.2e^{-16}$) correlations between the estimates for CB and PL BPA and BPF models (Table 5-1). These correlations provide some foundational information about how DNA methylation in these two tissues responds to bisphenol exposure and underscores the importance for future studies to examine bisphenol and epigenetic associations uncovered in this study in larger cohorts or meta-analyses.

Strengths and Limitations

Strengths of this dissertation are derived in part from the combination of two high dimensional datasets in our use of epigenome-wide DNA methylation from the EPIC array and untargeted metabolomics. The design of our human pregnancy cohort combines first-trimester exposure measures with DNA methylation in three fetal tissues at birth to closely capture and assess fetal epigenetic reprogramming. This dissertation was equally as innovative in our investigation of BPA along with its most common analogs BPF and BPS, providing pilot data of their association with DNA methylation in both CB and PL. In Chapter 3, the metabolomic evaluation of maternal plasma was an innovative way to reduce bias in the characterization of maternal diet & diet patterns. Furthermore, in Chapter 4, we compared DNA methylation at four candidate genes in matched tissue samples; this is the first study to not only include paired CB, UC, and PL in DNA methylation analyses, but also to evaluate the tissue-specific response to prenatal bisphenol exposures. Lastly, Chapter 4 is the first study to measure the association between prenatal bisphenol exposure and epigenome-wide DNA methylation in PL.

We equally acknowledge that this dissertation has limitations. The MMIP cohort used for each study is based out of the University of Michigan Hospital in Ann Arbor, Michigan, and the majority of the subjects eligible for inclusion in this dissertation were non-Hispanic White. This may limit the generalizability of the results. Further, the final number of mother-infant pairs included in this study was determined by the availability of samples with data. This limited our statistical power to detect differentially methylated sites by all bisphenols or OCM, and broader pathways in association with maternal exposure. Moreover, the small sample size in each of our studies may lead to spurious effects from statistical outliers.

Future Research

Future studies would consider the addition of subjects to bolster our findings, while also expanding the relative variation in maternal exposure levels. Such additions have the potential to improve our understanding of how families with more diverse prenatal environments may be impacted. For example, prioritizing the expansion of one carbon metabolites and methods to estimate maternal dietary intake. Future studies may also utilize transcription (RNAseq) analyses to determine the extent to which alterations in DNA methylation coordinate with alterations in the transcriptome, as well as identify additional candidate gene loci impacted by maternal exposure. This dissertation implicated a variety of biological pathways in association with maternal status. As such, it would be valuable to assess the extent to which such genes and pathways are associated with infant and childhood outcomes and the degree to which they mediate the relationship between exposure and outcomes.

Implications

The basic tenant of the DOHaD hypothesis on which this dissertation operates is the corollary between an adverse fetal environment and the increased risk of disease during

adulthood. The specific Aims address a small portion of this framework in evaluating two common maternal exposures. Figure 5-1 details this framework while also highlighting the particular contributions of the findings presented. Our cumulative investigation of maternal prenatal exposure to both bisphenols and OCM elucidated patterns of epigenome-wide DNA methylation and tissue-specific effects of exposure in a human pregnancy cohort. These studies impart initial data on which future studies can build to clarify the additional components connecting OCM and bisphenol exposures to both health and disease.

One-carbon metabolites are critical and fundamental dietary components contained within proteins, fruits, and vegetables. That diet has the potential to mitigate the effects of bisphenol exposure has been demonstrated by Dolinoy et al. Given the significant variety in dietary patterns displayed by human populations, the studies presented in this dissertation sought to evaluate the association between dietary pattern, BPA exposure and alterations in DNA methylation in a comprehensive and integrated approach. Ultimately, we believe that advances in epigenome-wide association studies, and the incorporation of specific suggestions made within this work, will ultimately enable researchers, clinicians, and policymakers to target risk factors (e.g. environmental and nutritional exposures that perturb the methylome and downstream birth outcomes) and to better understand a portion of the myriad of elements underlying the developmental origins of health and disease.

Table 5-1: Comparison of effect estimates from epigenome-wide association models across Aims.

(A) Bisphenol-DNA methylation associations (Aim 1) and SAH-DNA methylation associations (Aim 2)

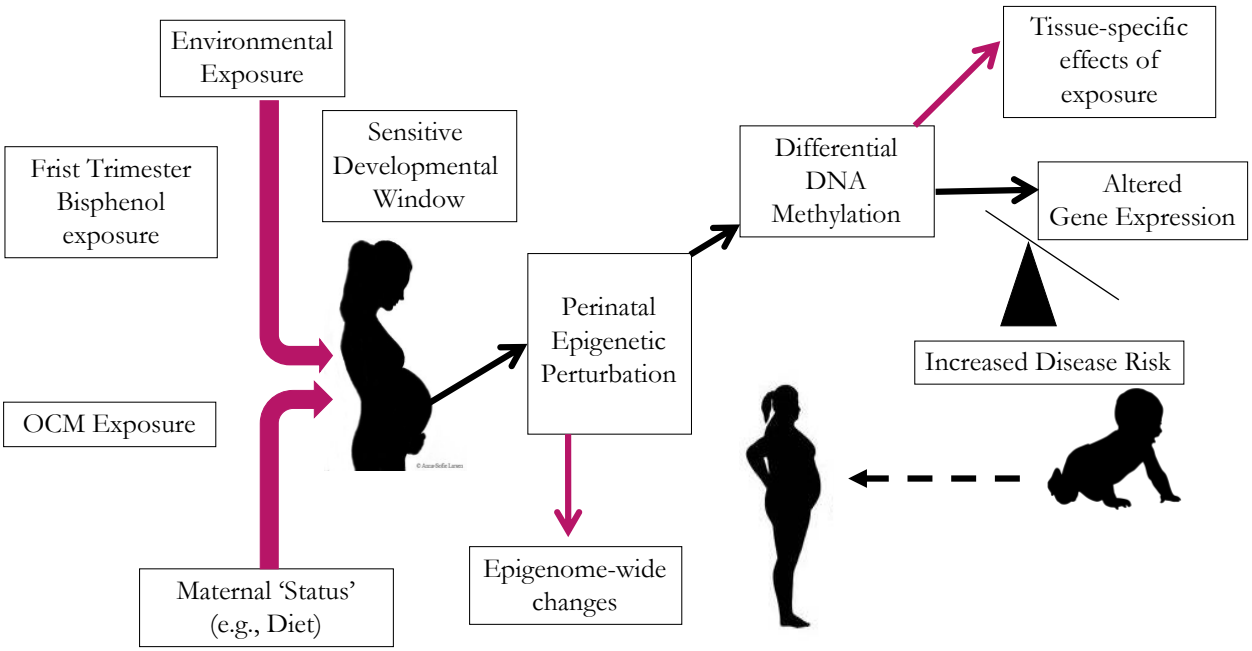
	CB SAH	M1 SAH	M3 SAH
BPA	-0.135	-0.088	-0.121
BPF	-0.161	0.180	-0.139
BPS	0.159	0.058	0.163

(B) Cord Blood Bisphenol-DNA methylation associations (Aim 1) and Placenta Bisphenol-DNA methylation associations (Aim 3)

BPA:BPA	BPF:BPF	BPS:BPS
0.0893	-0.0291	0.00205

Note: Represents Pearson correlation coefficients. Significant correlations are shaded, $p<2.2e-16$.

Figure 5-1: The theoretical framework of this dissertation highlighting those components to which it specifically contributes



Legend: The elements to which the studies presented in this dissertation addressed are highlighted with maroon arrows. Figure generated by Dr. Luke Montrose.

Appendix
Appendix Tables

Table A 1: Relationships between Bisphenol A and Covariates

A.

Mean (SD) in Female Group	Mean (SD) in Male Group	p-value
1.34 (1.21)	1.02 (0.96)	0.236

B.

Variable	p-value	Correlation Coefficient (rho)
Maternal Age	0.908	0.0141
Parity	0.489	-0.0845
Days to delivery	0.724	0.0435
Birthweight	0.912	-0.0135
nRBCs	0.14	0.179
Gran	0.488	0.0848
Mono	0.248	-0.14
Bcell	0.692	0.0484
NK	0.161	-0.17
CD4T	0.896	-0.0159
CD8T	0.949	0.0073
Pre-preg wt	0.323	0.12
Post-preg wt	0.26	0.137
Pre-preg BMI	0.269	0.134
Post-preg BMI	0.196	0.157
Preg wt gain	0.9221	0.0119

Panel A: T-test of maternal first-trimester BPA by infant sex, significance considered at $p<0.05$.
Panel B: Spearman correlations between covariates and BPA, significance considered at $p<0.05$.

Table A 2: Bivariate Analyses of Covariate vs Maternal First Trimester Urinary BPF

A.

	BPF <LOD	BPF >LOD	P-value from Chi-Squared test
Male	18 (26%)	14 (20.2%)	
Female	12 (17.3%)	25 (36.2%)	
Total	30 (43.4%)	39 (56.5%)	0.0806

B.

Variable	Mean (SD) in BPF <LOD Group	Mean (SD) in BPF >LOD Group	p-value from t-test
Maternal Age	31.93 (4.14)	31.78 (3.05)	0.887
Parity	1.5 (1.07)	1.08 (0.87)	0.165
Days to deliv.	277.13 (7.17)	279.82 (7.41)	0.182
Birthweight	3583.16 (393.3)	3557.82 (407.4)	0.818
nRBCs	0.0879 (0.04)	0.0944 (0.06)	0.669
Gran	0.454 (0.07)	0.481 (0.09)	0.274
Mono	0.0957 (0.02)	0.0901 (0.02)	0.338
Bcell	0.0954 (0.04)	0.083 (0.03)	0.217
NK	0.026 (0.05)	0.0216 (0.02)	0.688
CD4T	0.1526 (0.05)	0.1525 (0.05)	0.993
CD8T	0.132 (0.04)	0.115 (0.04)	0.114
Pre-preg wt	69.9 (14.81)	71.33 (19.7)	0.752
Post-preg wt	83.17 (13.4)	84.49 (17.9)	0.744
Pre-preg BMI	25.88 (6.05)	25.25 (6.75)	0.717
Post-preg BMI	30.76 (5.51)	31.01 (6.1)	0.577
Preg wt gain	13.27 (6.32)	12.71 (5.28)	0.948

Panel A: Chi-squared bivariate test of maternal first-trimester BPF and infant sex, significance considered at $p<0.05$.
Panel B: T-test comparing covariates of interest by maternal first-trimester BPF exposure category, significance considered at $p<0.05$.

Table A 3: Bivariate Analyses of Covariates vs Maternal First Trimester Urinary BPS

A.

	BPS <LOD	BPS >LOD	P-value from Chi-Squared test
Male	17 (24.6%)	15 (21.7%)	
Female	12 (17.3%)	25 (36.2%)	
Total	29 (42%)	40 (57.9%)	0.135

B.

Variable	Mean (SD) in BPS <LOD Group	Mean (SD) in BPS >LOD Group	p-value from t-test
Maternal Age	32.03 (4.17)	32.05 (3.05)	0.985
Parity	1.07 (0.96)	1.3 (1.02)	0.344
Days to deliv.	277.31 (8.16)	277.35 (6.63)	0.982
Birthweight	3613 (422.32)	3475 379.33)	0.169
nRBCs	0.0841 (0.05)	0.0861 (0.06)	0.872
Gran	0.487 (0.1)	0.462 (0.08)	0.223
Mono	0.0927 (0.02)	0.0869 (0.02)	0.57
Bcell	0.0879 (0.04)	0.0884 (0.03)	0.9522
NK	0.0163 (0.02)	0.0213 (0.04)	0.562
CD4T	0.143 (0.06)	0.159 (0.04)	0.215
CD8T	0.127 (0.04)	0.122 (0.04)	0.595
Pre-preg wt	67.11 (11.16)	75.04 (20.72)	0.0655
Post-preg wt	81.39 (10.61)	87.05 (18.82)	0.148
Pre-preg BMI	24.37 (4.16)	27.49 (7.42)	0.0452
Post-preg BMI	29.55 (3.94)	31.89 (6.74)	0.1
Preg wt gain	14.28 (5.85)	12.01 (5.51)	0.104

Panel A: Chi-squared bivariate test of maternal first-trimester BPS and infant sex, significance considered at $p<0.05$.
Panel B: T-test comparing cohort covariates of interest by maternal first-trimester BPS exposure category, significance considered at $p<0.05$.

Table A 4: Comparison of Effect Estimates for DNA Methylation of CpG Sites Associated with BPA with and without one BPA Outlier (latter in bold)

CpG Site Probe ID	Effect Size*	*p*-value	Chromosomal Location	Gene Name	Relation to CpG Island
cg14456173	-0.855248747	8.43E-10	chr1:43437674	*SLC2A1-AS1*	Open Sea
	-0.508035596	**0.00085458**			
cg11176519	-0.689096099	4.24E-09	chr1:14591868		Open Sea
	-0.013650245	**0.775885547**			
cg26228351	0.350294219	5.63E-09	chr1:200992656	*KIF21B*	Island
	0.003485497	**0.887710689**			
cg16660310	0.292487389	7.55E-09	chr19:36661673		Open Sea
	0.039065071	**0.262182476**			
cg05445263	-0.748809244	1.01E-08	chr13:21049223	*CRYL1*	Open Sea
	0.023916001	**0.600050046**			
cg21034201	-0.468096467	1.75E-08	chr18:33160855		North Shore
	0.004776233	**0.90339229**			
cg11735305	0.371378387	2.04E-08	chr3:122512541	*HSPBAP1*	Island
	0.020402941	**0.618477184**			
cg07935657	-0.268361981	3.04E-08	chr8:10622805		Open Sea
	-0.029054728	**0.408336608**			
cg16783576	-0.684849307	3.52E-08	chr2:216237359	*FN1*	Open Sea
	-0.038241384	**0.638840449**			
cg08526074	-0.270965932	3.58E-08	chr16:51184562	*SALL1*	Island
	0.006523332	**0.79499209**			
cg11687406	-0.294697987	4.02E-08	chr20:10199434	*SNAP25*	North Shore
	-0.068469805	**0.148438015**			
cg21589431	-0.698991603	4.24E-08	chr15:85660361	*PDE8A*	Open Sea
	0.031940099	**0.600410198**			
cg09734791	-0.270118659	5.42E-08	chr8:72756155	*MSC*	Island
	0.001470558	**0.959509732**			
cg03422016	-0.502120607	5.85E-08	chr12:121698404	*CAMKK2*	Open Sea
	0.028839504	**0.520154601**			
cg02997560	-0.410411086	6.07E-08	chr19:3180815		South Shore
	0.034457141	**0.283035883**			
cg25250853	-0.426976041	7.21E-08	chr2:85822726	*RNF181*	Island
	0.00093833	**0.984530915**			
cg04151762	-0.328374363	8.56E-08	chr2:239039182	*ESPNL*	North Shore
	-0.031163747	**0.504741557**			
cg11554525	-0.272608968	9.06E-08	chr8:33342681	*MAK16*	Island
	-0.002158948	**0.947584705**			
cg06983735	-0.35762919	9.28E-08	chr7:142536625		Open Sea
	-0.04395762	**0.411598405**			
cg22393694	-0.71070928	1.19E-07	chr11:26595206	*MUC15*	Open Sea
	0.085290582	**0.123760838**			
cg08834401	-0.264164034	1.86E-07	chr2:71017846	*FIGLA*	Island
	-0.024559793	**0.544016907**			
cg03870746	0.210032731	1.91E-07	chr7:1068244	*C7orf50*	Island
	0.047008132	**0.209474187**			
cg26966186	-0.259203052	2.11E-07	chr5:106879524	*EFNA5*	Open Sea

	-0.100581873	**0.055824125**			
cg08380477	-0.39742086	2.67E-07	chr2:172957268		North Shore
	-0.018438606	**0.754563608**			
cg11506835	-0.255103425	2.93E-07	chr12:11324011	*SMIM10L1*	Island
	0.024541218	**0.381556533**			
cg13011901	-0.235782236	4.74E-07	chr3:46752152	*TMIE*	Open Sea
	-0.018474281	**0.634141775**			
cg14384920	-0.281391368	5.07E-07	chr16:8735575	*METTL22*	Open Sea
	0.002287117	**0.955730675**			
cg15000998	-0.339853696	6.03E-07	chr12:62653559	*USP15*	North Shore
	-0.002538768	**0.961118696**			
cg02699336	-0.363145454	6.34E-07	chr3:56502021	*ERC2*	Island
	-0.030674738	**0.620292159**			
cg17725100	-0.163749266	6.95E-07	chr1:111098247		Island
	0.005055642	**0.833351931**			
cg10848724	-0.436669167	1.07E-06	chr12:123380878	*VPS37B*	Island
	0.024109212	**0.710797779**			
cg11879536	-0.270277725	1.07E-06	chr19:2462065		Island
	0.012939489	**0.750878207**			
cg11360973	-0.260694927	1.11E-06	chr10:636076	*DIP2C*	Open Sea
	-0.028694686	**0.550561143**			
cg01466219	-0.189413782	1.66E-06	chr14:62210927	*HIF1A*	Open Sea
	-0.03180952	**0.402173079**			
cg01490204	-0.271105221	1.68E-06	chr4:154400013	*KIAA0922*	Open Sea
	-0.080316045	**0.175214363**			
cg25720128	-0.176365055	1.68E-06	chr4:154349775		Open Sea
	0.004883887	**0.866295287**			
cg18715511	0.245313103	1.80E-06	chr12:1058965	*RAD52*	Island
	0.16115613	**0.009552043**			
cg02066409	-0.172426358	1.92E-06	chr8:11059042	*XKR6*	Island
	0.023933192	**0.319455394**			

Table A 5: Comparison of statistically significant CpG sites associated with BPA in published literature with results in our model

A.

Junge et al., 2019				
CpG	Chromosome	Gene	*p* value	Effect Estimate
cg17580798	7	*MEST*	1.35E-07	-1.80%
cg23117250	17	*RAB408*	1.55E-07	-2.00%
McCabe et al., current study				
cg17580798	7	*MEST*	0.231	0.06%
cg23117250	17	*RAB408*	0.523	0.03%

* Effect estimate is reported as deltaB, because they modeled high vs low BPA exposure. Our effect estimate represents the change in methylation (beta) per 1 ng/mL increase in BPA.

B.

Alavian-Ghavanini et al., 2019				
CpG	Chromosome	Gene	*p* value	Effect Estimate
cg10091102	12	*GRIN2B*	(0.91, 1.35)	OR 1.11
McCabe et al., current study				
cg10091102	12	*GRIN2B*	0.0509	-0.025%

*Reported BPA as 4th quartile vs. 1st quartile, and results reported as odds ratios. Results from our study represent the change in methylation per 1 ng/mL increase in BPA.

Table A 6: Differentially methylated CpG sites in Placenta Associated with Maternal First Trimester BPA Exposure

Locus	Gene Name	Relation to CpG Island	Effect Estimate using M-values	p-value
chr15: 27787181		S_Shore	0.498	6.28E-06
chr7: 95320969		OpenSea	-0.769	6.90E-06
chr18: 77171444	*NFATC1*	Island	0.638	9.17E-06
chr19: 49016629	*LMTK3*	N_Shore	0.405	1.39E-05
chr15: 24220187	*PWRN4*	OpenSea	-0.359	1.40E-05
chr4: 8689975		Island	0.486	1.42E-05
chr1: 227635558		OpenSea	0.781	1.91E-05
chr6: 29635692	*MOG*	OpenSea	0.311	1.97E-05
chr12: 132677645		S_Shelf	0.835	2.11E-05
chr8: 54507218		Island	0.335	2.26E-05
chr11: 1682006	*HCCA2*	OpenSea	0.610	2.48E-05
chr9: 140033911	*GRIN1*	Island	0.531	2.60E-05
chr1: 26198172	*PAQR7*	N_Shelf	0.432	2.69E-05
chr4: 114744568		OpenSea	-0.448	2.71E-05
chrX: 106984142	*TSC22D3*	OpenSea	0.336	2.74E-05
chr7: 67631771		OpenSea	0.545	2.79E-05
chr19: 53540845		Island	0.457	3.05E-05
chr1: 165853249	*UCK2*	OpenSea	-0.324	3.28E-05
chr1: 244143008		Island	0.184	3.58E-05
chr19: 51685227		Island	0.532	3.92E-05
chr18: 23805605	*TAF4B*	N_Shore	0.439	4.08E-05
chr19: 51141361	*SYT3*	N_Shore	0.607	4.08E-05
chr8: 96614915	*C8orf37-AS1*	OpenSea	-0.378	4.12E-05
chr5: 135688594	*TRPC7*	N_Shelf	0.512	4.18E-05
chr10: 133466918		OpenSea	0.586	4.18E-05
chr7: 98189557		OpenSea	0.569	4.23E-05
chr15: 94444099		OpenSea	0.281	4.42E-05
chr20: 52225459		OpenSea	0.549	4.45E-05
chr16: 56870473	*NUP93*	OpenSea	0.437	4.46E-05
chr16: 69457334	*CYB5B*	N_Shore	0.492	4.68E-05
chr6: 29425610	*OR2H1*	OpenSea	0.501	4.81E-05
chr2: 6616265		OpenSea	0.318	4.97E-05
chr20: 59594954		N_Shelf	0.290	5.42E-05
chr1: 181392832		OpenSea	0.425	5.49E-05
chr8: 689392		Island	0.517	5.69E-05
chr5: 50265443		Island	0.929	6.26E-05
chr3: 186819037		OpenSea	0.390	6.36E-05
chr19: 3286059	*BRUNOL5*	Island	0.542	6.91E-05
chr13: 113777045	*F10*	OpenSea	0.340	7.18E-05
chr1: 159175211	*DARC*	OpenSea	0.266	7.24E-05
chr17: 80836690	*TBCD*	OpenSea	-0.296	7.26E-05

chr1: 36565739	*COL8A2*	OpenSea	0.292	7.40E-05
chr17: 74100186	*EXOC7*	S_Shore	-0.424	7.42E-05
chr16: 87960072	*CA5A*	OpenSea	-0.432	7.56E-05
chr9: 124926741	*MORN5*	OpenSea	0.387	7.84E-05
chr12: 126969338		OpenSea	0.758	7.99E-05
chr22: 39369327	*APOBEC3A_B*	OpenSea	0.294	8.06E-05
chr11: 15057083		OpenSea	0.325	8.15E-05
chr6: 26240307	*HIST1H4F*	N_Shore	0.739	8.18E-05
chr2: 178938591	*PDE11A*	S_Shore	0.459	8.37E-05
chr12: 113443790	*OAS2*	OpenSea	0.437	8.39E-05
chr17: 74682676	*MXRA7*	OpenSea	0.403	8.47E-05
chr18: 58079137		OpenSea	0.498	8.53E-05
chr17: 4549127		OpenSea	-0.375	8.59E-05
chr3: 72027050		OpenSea	0.581	8.64E-05
chr18: 75774460		OpenSea	0.472	8.70E-05
chr13: 29166467		OpenSea	-0.280	8.93E-05
chr6: 38998315	*DNAH8*	OpenSea	-0.314	9.02E-05
chr13: 21872891		S_Shore	0.296	9.05E-05
chr1: 248100276	*OR2L13*	N_Shore	0.271	9.08E-05
chr10: 130122694		OpenSea	0.313	9.13E-05
chr4: 11369007	*MIR572*	N_Shore	0.357	9.29E-05
chr10: 28582533		OpenSea	0.532	9.79E-05

Table A 7: Differentially methylated CpG sites in Placenta Associated with Maternal First Trimester BPF Exposure

Locus	Probe	Gene Name	Relation to CpG Island	Effect Estimate using M-values	p-value
chr2:219135936	cg11036438	*AAMP*	S_Shore	-0.216	4.70E-06
chr8:139116005	cg07850450		OpenSea	-0.306	6.66E-06
chr2:241083975	cg00919014		OpenSea	-0.159	8.61E-06
chr6:167190034	cg01804233	*RPS6KA2*	OpenSea	-0.187	1.17E-05
chr10:72546264	cg23829193	*C10orf27*	OpenSea	-0.221	2.37E-05
chr1:114355693	cg00642679	*RSBN1*	S_Shore	-0.194	2.38E-05
chr7:150494998	cg07649114	*TMEM176B*	N_Shore	-0.317	2.48E-05
chr16:82191819	cg03804206	*MPHOSPH6*	OpenSea	-0.260	2.56E-05
chr17:189544	cg09652172	*RPH3AL*	OpenSea	-0.188	2.89E-05
chr6:19688777	cg08182409		N_Shelf	-0.238	3.33E-05
chr9:114602017	cg16066494		OpenSea	-0.141	4.70E-05
chr1: 85724328	cg09385306	*C1orf52*	N_Shore	-0.417	4.98E-05
chr3:158918327	cg02162900	*IQCJ*	OpenSea	-0.148	5.05E-05
chr1: 76174873	cg08541923		OpenSea	-0.254	5.14E-05
chr19:54415925	cg02726501	*CACNG7*	S_Shelf	-0.129	5.20E-05
chr7: 1819220	cg09911010		OpenSea	-0.371	5.35E-05
chr21:36448197	cg00367967		OpenSea	-0.310	6.21E-05
chr4:175133103	cg24018520		N_Shelf	0.262	6.30E-05
chr12:29762622	cg19240189	*TMTC1*	OpenSea	-0.262	7.02E-05
chr6:170398865	cg14468953		N_Shelf	-0.182	7.54E-05
chr8: 72941949	cg14667695	*TRPA1*	OpenSea	-0.224	7.55E-05
chr9:106005045	cg12307296	*LINC01492*	OpenSea	-0.330	7.80E-05
chr10:49916530	cg13125657	*WDFY4*	OpenSea	-0.258	8.11E-05
chr1:230118458	cg24605197		OpenSea	-0.231	8.49E-05
chr7: 48887421	cg22290893		N_Shore	-0.308	9.06E-05
chr11: 2444553	cg10955576	*TRPM5*	S_Shelf	-0.195	9.18E-05
chr3: 67345761	cg17478726		OpenSea	-0.190	9.26E-05
chr17:32427091	cg17995857	*ASIC2*	OpenSea	-0.313	9.28E-05
chr9 97491386	cg14163324	*C9orf3*	S_Shelf	-0.148	9.94E-05

Table A 8: Differentially methylated CpG sites in Placenta Associated with Maternal First Trimester BPS Exposure

Locus	Probe	Gene Name	Relation to CpG Island	Effect Estimate using M-values	p-value
chr19:57631384	cg05246057	*USP29*	S_Shore	-0.324	2.29E-06
chr18:75400720	cg02950608		N_Shore	-0.696	2.38E-06
chr3:116605431	cg18347332		OpenSea	-0.586	3.80E-06
chr7:156363743	cg23586788		OpenSea	-0.419	4.28E-06
chr9:127174352	cg07515961	*PSMB7*	N_Shelf	-1.427	8.88E-06
chr13:73112127	cg01086205		OpenSea	-0.432	1.32E-05
chr2: 52656979	cg15853771		OpenSea	-0.563	1.53E-05
chr8: 2197661	cg20299740		OpenSea	-0.468	2.01E-05
chr2:104668320	cg03407782		OpenSea	-0.442	2.58E-05
chr3: 77064282	cg27304415		OpenSea	-0.766	2.73E-05
chr8:106185570	cg14233855		OpenSea	-0.602	2.82E-05
chr17:77245306	cg24098990	*HRNBP3*	OpenSea	-0.602	3.66E-05
chr1:205557160	cg24435996	*MFSD4*	N_Shelf	-0.403	3.67E-05
chr1: 58126625	cg02058215	*DAB1*	OpenSea	-0.455	4.30E-05
chr2:168401402	cg22687547		OpenSea	-0.633	5.08E-05
chr15:92399195	cg12626076	*SLCO3A11*	S_Shore	-0.329	5.78E-05
chr6: 29648400	cg07134666		OpenSea	-0.794	6.06E-05
chr5: 13771787	cg24408918	*DNAH5*	OpenSea	-0.365	6.14E-05
chr5:155753616	cg03811411	*SGCD*	OpenSea	-0.452	6.37E-05
chr1: 7063174	cg15604777	*CAMTA1*	OpenSea	-0.551	6.38E-05
chr7: 26192199	cg07986525	*NFE2L3*	Island	0.208	7.09E-05
chr9: 17520350	cg01633078		OpenSea	-0.373	7.24E-05
chr10: 1338235	cg02319911	*ADARB2*	OpenSea	-0.461	7.54E-05
chr12:15038788	cg06601891	*MGP*	OpenSea	-0.711	8.21E-05
chr11: 2022324	cg16574793		S_Shelf	0.234	8.41E-05
chr8: 96619989	cg10712578		OpenSea	0.871	8.46E-05
chr13:66887072	cg12853300	*PCDH9*	OpenSea	-0.545	8.74E-05
chr11:133913868	cg17434901		OpenSea	-0.411	8.78E-05
chr5: 41059254	cg11718442	*MROH2B*	OpenSea	-0.441	9.16E-05
chr4: 155662375	cg27639408		N_Shore	-0.784	9.58E-05
chr15:100600822	cg19539605	*ADAMTS17*	OpenSea	-0.446	9.64E-05
chr5: 140561067	cg13394331	*PCDHB16*	S_Shore	-0.667	9.79E-05

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