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Date

**Associations Between Perinatal Exposures and Neonatal DNA Methylation**

By

James William Schroeder  
Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences  
Genetics and Molecular Biology

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## Abstract

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Several perinatal exposures have been linked with adverse health outcomes later in life. Fetal exposure to maternal depression, symptoms and treatment are associated with low birth weight, fetal cardiac defects, and impaired cognitive and emotional development. Likewise, gestational age, often in the form of preterm delivery, is associated with an increased rate of neonatal mortality and development of cerebral palsy, cognitive and behavioral issues. In this study, we examined whether there was an association between these perinatal exposures and differential methylation in >27,000 CpG loci in umbilical cord blood DNA of neonates born to mothers participating in a prospectively-characterized psychiatric cohort. Contrary to previous reports, we found no associations between neonatal DNA methylation and any psychiatric diagnosis or depressive symptom measures taken during pregnancy. However, maternal exposure to any antidepressant during pregnancy was associated with differential methylation at *TNFRSF21* and *CHRNA2*, though these findings were not associated with individual antidepressant classes. Gestational age was associated with differential methylation at 41 CpG loci in 39 genes, including genes previously implicated in labor and delivery (e.g., *AVP*, *CRHBP* and *ESR1*) or that may influence the risk for adverse health outcomes later in life (e.g. *DUOX2*, *TMEM176A* and *CASP8*). Twenty-six of these loci in 25 genes were then successfully replicated in an independent, non-psychiatric cohort. These combined findings will play an important role both in characterizing the epigenetic dynamics contributing to labor and delivery and in assessing potential impacts of these perinatal exposures on later-life health conditions.

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## **Chapter 1**

### **General Introduction**

## Epigenetics and DNA Methylation

Despite containing equivalent genomes, somatic cells differentiate to perform a variety of functions in response to stimuli by regulating the timing and magnitude of gene expression necessary for a multicellular organism to function. Epigenetic mechanisms are a broad category of molecular interactions that are potentially heritable (via mitosis, meiosis, or both) and contribute to the regulation of these dynamic phenomena by influencing gene expression without altering DNA sequence.

Genomic DNA interacts closely with a heterogeneous group of packaging proteins known as histones, which form an octamer wrapped by DNA creating the core particle of a nucleosome (Figure 1-1). Epigenetic mechanisms utilize this nucleosome structure by facilitating the covalent addition of signaling molecules such as acetyl, methyl, and phosphate groups to specific amino acids on the N-terminal tails of histone proteins. These epigenetic signals form a specified “histone code” that participates in gene regulation through a dynamic process of protein recruitment.<sup>1</sup> Histone modifications can also influence gene regulation by constricting or relaxing regional chromatin states, which alters the ability of regulatory proteins to bind corresponding DNA elements.<sup>2</sup>

DNA methylation is an epigenetic mechanism that covalently modifies DNA via addition or removal of a methyl group at the 5' position of the pyrimidine ring of cytosines within CpG dinucleotides (Figure 1-2). DNA methyltransferases (DNMTs), such as DNMT1, DNMT3a, and DNMT3b, catalyze these methylation reactions (Figure 1-1). CpG methylation marks both strands of DNA, with one methyl group added or removed from each cytosine of the complementary strands. This symmetrical marking

provides a signal for propagation of the methylation event to newly synthesized DNA strands by maintenance methyltransferases during the semi-conservative replication of DNA. Recent studies have also found that TET1, TET2 and TET3 convert 5-methylcytosine to 5-hydroxymethylcytosine (Figure 1-1).<sup>3,4</sup> This process has been shown to participate in the regulation of DNA methylation during embryonic development<sup>4-6</sup>, though the full role of this novel modification is still being characterized.

In more than half of all genes, CpG dinucleotides cluster into regions called CpG islands near the promoter region.<sup>7</sup> A CpG island is often defined as a region of DNA containing 200 or more base pairs where the ratio of observed to expected CpG dinucleotides is greater than 60%.<sup>8</sup> CpG islands generally associate with expression of the gene when hypomethylated and repression of the gene when hypermethylated.<sup>9-11</sup>

Methylation of cytosines regulates gene expression by influencing the recruitment and binding of regulatory proteins to DNA.<sup>12</sup> For example, methyl-CpG-binding protein 2 (MeCP2) binds specifically to methylated CpGs<sup>13</sup>, and methylation can directly block the binding of transcription factors.<sup>14,15</sup> DNA methylation also acts in concert with other epigenetic mechanisms such as histone modifications to regulate access of the transcriptional machinery to the DNA. Heterochromatin formation at *Oct3/4*, for example, is facilitated through a complex that contains the histone methyltransferase G9a, which recruits Dnmt3A and Dnmt3B increasing local DNA hypermethylation.<sup>16</sup> Histone modifying enzymes such as the histone deacetylases HDAC1 and HDAC2, as well as histone methyltransferase proteins such as SUV39, also interact with DNMT1.<sup>17-19</sup> Likewise, MeCP2 influences DNA methylation status with histone methylation via interactions with SUV39.<sup>20</sup>

## Epigenetic Mechanisms During Fetal Development

In mammals, DNA methylation plays essential roles during pre- and post-natal developmental processes. One of these processes is genomic imprinting in which the expression of certain alleles is affected by the parent that contributed those alleles through the alleles' methylation pattern.<sup>21</sup> In germ cells, these methylation patterns are removed and reprogrammed in accordance with the sex of the developing fetus.<sup>21</sup> DNA methylation also plays a major role in the establishment of gene dosage compensation by inducing a heterochromatic repression of gene expression on one of the X chromosomes in female cells.<sup>22</sup> Processes that affect genomic stability, such as repression of repetitive DNA elements<sup>23,24</sup> and maintenance of centromeric sequences and chromosomal condensation, are also influenced by DNA methylation.<sup>25,26</sup> As these processes demonstrate, early prenatal development is a crucial window for the establishment of long-term patterns of DNA methylation. Interruption of epigenetic programming during this critical period could have negative repercussions on post-natal outcomes.

Numerous animal studies have demonstrated that exposure of the fetus to differing *in utero* conditions can alter DNA methylation, which in turn can mediate variation in adult phenotype. A well-known example of such an epigenetically-mediated alteration of an adult outcome by differences in prenatal exposures is the Agouti mouse. In this instance, mice bred from fathers heterozygous for the yellow coat Agouti pigment ( $A^{vy}/a$ ) and black-coated ( $a/a$ ) mothers display an increase in darker coat pigmentation upon supplementation of the maternal diet with methyl donors and cofactors such as zinc during pregnancy.<sup>27</sup> This phenomenon results from a methyl-donor facilitated increase in CpG methylation at a transposable element associated with the  $A^{vy}$  locus.<sup>28</sup> Enhanced

CpG methylation and heterochromatinization at that element attenuates expression of the  $A^y$  gene, leading to increasing expression of the dark pigment over that of the yellow pigment.

Several other examples of prenatal maternal nutritional differences leading to epigenetically-mediated alterations in postnatal offspring phenotypes have been described. For example, there are aberrant patterns of *Dnmt1* regulation as well as the methyl-sensitive DNA-binding protein MeCP2 in macaques born to mothers with a high-fat diet.<sup>29</sup> Similarly, a study of pregnant mice exposed to 48 hours of hypoxic conditions bore offspring that displayed altered expression of *Dnmt3b* and another methyl-sensitive DNA-binding protein Mbd1.<sup>30</sup> Low levels of the insecticide dichlorodiphenyltrichloroethane (DDT) in young rats reduced DNA methylation levels and expression of genes involved in stress responses and DNA methylation maintenance, leading to speculation that these effects are a general reaction to oxidative stress.<sup>31</sup> Such widespread alterations in genes involved in the genome-wide maintenance and reading of DNA methylation patterns could lead to broad patterns of postnatal epigenetic dysregulation affecting many genes.

Genome-wide differential methylation has also been associated with uteroplacental insufficiency<sup>32</sup>, malnutrition<sup>33</sup>, and environmental toxins<sup>34-36</sup>. One study also reported intergenerational phenotypic effects of maternal exposure to environmental toxins, associated with altered DNA methylation in offspring.<sup>37</sup> In this study, the authors describe the co-occurrence of male infertility and differential DNA methylation patterns in genes known to influence the phenotype, which persist across multiple generations of rats derived from a pregnant rat exposed to the endocrine disrupter vinclozolin. These

findings did not replicate in a subsequent study<sup>38</sup>, although authors of the original study argue that this is likely due to the use of a more inbred CD-Sprague Dawley (Charles River) rat line in the follow-up study, which is less responsive to the effect than the outbred Harlan Sprague Dawley strain used in the original study.<sup>39</sup> These reports, which seek to relate adverse postnatal outcomes with differential methylation of genes, exemplify studies that can elucidate epigenetic mechanisms capable of altering postnatal phenotypes in response to prenatal exposures.

Associations between perinatal exposures and differential histone modifications in offspring have also been reported. For example, aberrant histone modifications associate with malnutrition<sup>40,41</sup>, cloning and *in vitro* fertilization.<sup>42</sup> However, while some suggestive evidence exists<sup>42</sup>, an association between perinatal stressors and differential histone modifications in combination with differential DNA methylation has yet to be established, though it remains a potential mechanism for inducing long-term changes that requires thorough investigation.

### **Early-Life Impacts on Phenotypes in Later Life**

Based on the observation that low birth weight associates with a higher risk for chronic adult conditions such as stroke, type II diabetes, heart disease, and hypertension, David Barker proposed that prenatal malnutrition or other factors leading to low birth weight predispose the fetus to be at greater risk for these later-life conditions.<sup>43,44</sup> Barker's developmental origin of adult health and disease (DOHaD) hypothesis has motivated a plethora of studies that have identified prenatal factors that associate with

differences in post-natal health outcomes such as overfeeding<sup>45</sup>, preterm birth<sup>46,47</sup>, delayed fetal growth<sup>48,49</sup>, and *in vitro* fertilization.<sup>50</sup>

Among the more famous examinations of the DOHaD hypothesis is the ongoing study of offspring born to women who were pregnant during the “Dutch Hunger Winter” (reviewed in 51). These mothers were undernourished during a well-circumscribed period from 1944-45, when the Nazi occupiers of the Western Netherlands retracted food supplies to the entire population of the region. The Dutch Hunger Winter offspring, who are now well into their seventh decade of life, exhibit elevated rates of coronary heart disease, an accelerated rate of decline in cognitive functions over time, and impaired glucose tolerance.<sup>52-54</sup> The Dutch Hunger Winter data, together with other evidence, suggests that *in utero* malnutrition leads to elevated risk for chronic metabolic disorders that overfeeding during adulthood further exacerbates. These effects could be mediated, in part, by epigenetic mechanisms such as DNA methylation.

### **Exposure to Maternal Mental Illness and Neonatal Health**

The first potential predictor of methylation in umbilical cord blood DNA that we examined was maternal depressive diagnosis and symptoms. Maternal depressive symptoms can persist throughout pregnancy and pose a potential threat to the developing fetus with depressed women being more likely to deliver prematurely or to deliver growth-restricted neonates.<sup>55,56</sup> The children of mothers depressed during pregnancy score lower on the Neonatal Behavioral Assessment Scale (NBAS)<sup>57,58</sup> and show reduced mental, motor and emotional development as infants.<sup>59,60</sup> Over time, the children of

depressed mothers are at higher risk of developing depression<sup>61</sup>, violent behavior<sup>62</sup> and anxiety.<sup>63</sup>

It is interesting to note that levels of maternally-derived cortisol, a hormone correlated with depressive and other stress-related symptoms, associates with neonatal temperament.<sup>64</sup> Maternal cortisol crosses the placenta and enters the fetal bloodstream<sup>65</sup>, suggesting the hormone could mediate effects of maternal depressive symptoms on adverse neonatal outcomes, possibly by influencing DNA methylation events during fetal development. In this study we hypothesized that maternal depressive diagnosis and symptoms will influence neonatal DNA methylation, a potential mediator of these later effects, though we will not here be examining associations with neonatal or maternal cortisol.

### **Neonatal Risks of Antidepressant Exposure**

During pregnancy many women with psychiatric disorders take psychotropic medications, another potential predictor of neonatal DNA methylation patterns.<sup>66</sup> Depressed women that discontinue medication during pregnancy are at high risk for relapse of depressive symptoms<sup>67</sup>, so there is a substantial *a priori* motivation for women to maintain treatment with antidepressants throughout pregnancy. Given the difficult choice between continuing antidepressant treatment throughout pregnancy and discontinuing medication given a possible relapse of depressive symptoms, it is unclear which option poses the greater risk, if any, to the developing fetus.<sup>68</sup> Several antidepressants and their metabolites cross the placenta and can be detected in umbilical cord blood at birth.<sup>69</sup> Reports describing the effects of *in utero* antidepressant exposure

on the developing fetus have been mixed; some studies conclude that there is little to no significant risk linked to antidepressant exposure,<sup>70</sup> and others suggest antidepressant exposure increases risks of adverse outcomes such as fetal cardiac defects,<sup>71</sup> attention deficit/hyperactivity disorder (ADHD)<sup>72</sup> and impaired motor development.<sup>73,74</sup>

A major difficulty confounding interpretation of the results of *in utero* effects of antidepressants in humans is that women taking such medications do so to alleviate symptoms. Due to the absence (for obvious ethical reasons) of studies where pregnant women are randomly assigned to antidepressants or a placebo in a double-blind manner, it is very difficult to distinguish the effects of maternal illness (or symptom severity) on human offspring from those of medication exposure. While studies in animal models are an obvious alternative, conclusions from such studies can themselves be confounded by factors such as inter-species differences in nutritional needs, the lack of clearly defined animal models of maternal depression, as well as by the uniquely-human features of certain post-natal outcomes (e.g., language development). Thus, despite several important limitations, studies of the impact of prenatal medication exposures on post-natal outcomes remain important, and differential DNA methylation may be a mediator of these impacts.

### **Gestational Age and Health Outcomes**

Another potential predictor of differential neonatal DNA methylation patterns is gestational age. Several previous studies have shown an association between age and DNA methylation patterns, though no studies yet exist examining the relationship between DNA methylation in a genome wide set of CpGs and gestational age.<sup>75-78</sup>

Term deliveries are defined as occurring after 37 weeks gestational age (GA) from the mothers last menstrual period (LMP), and although the majority of deliveries in the United States are term (37-42 weeks), nearly one in eight are preterm. GA is also closely related to birth weight, although they are not collinear, and birth weight can reflect a variety of factors other than GA.<sup>79</sup> There is a consistent trend of association of preterm delivery with significantly higher rates of neonatal morbidity and mortality<sup>80</sup> with children born preterm showing increased risk for cerebral palsy<sup>81</sup>, behavioral, emotional and cognitive disorders<sup>82,83</sup>, and other neurodevelopmental issues<sup>84</sup>, though the risks are increased with extreme preterm births.<sup>85</sup> Likewise, neonates born postterm (>41 weeks) associate with an increased risk for cerebral palsy<sup>86</sup>, epilepsy<sup>87</sup>, and mortality<sup>88</sup> than term births. Even within the period defined as “normal” term (37-41 weeks), there is a similar trend of increased risk for adverse neonatal outcomes, particularly respiratory complications, associated with declining gestational age.<sup>89,90</sup> There is, for example, a higher rate of morbidity and mortality during the first year of life for neonates born at 37 or 38 weeks compared to those delivered at 40 weeks.<sup>91</sup> These GA-associated effects might be mediated, in part, by DNA methylation, though, as previously stated, the association between DNA methylation and GA has yet to be adequately studied. The following sections will review the extant literature on associations between differential DNA methylation and early life exposures.

### **Animal Studies**

Several studies have reported an association between prenatal stress exposures or early life stress (ELS) and changes in DNA methylation in specific candidate genes

(reviewed in 92). These reports, which include both animal and human studies, also support the importance of epigenetic mechanisms as mediators of long-term outcomes associated with perinatal stress and, in particular, emphasize regulated methylation of specific CpG loci as a vital molecular mediator of later-life effects.

The first postnatal week to 10 days of a newborn rat pup is in many ways developmentally equivalent to the third trimester of a human fetus.<sup>93</sup> Researchers using rat models to study the effects of perinatal stress on offspring have taken advantage of this connection by exposing newborn pups to controlled, stress-inducing environments in order to gain insights into the potential impact of prenatal stress on human fetuses. Much of the early work on the association between ELS and DNA methylation was performed in rats and published in a series of reports beginning with a study by Weaver and colleagues.<sup>94</sup> This study examined the effects of ELS on methylation by using mothers that exhibited a high level of licking and grooming (LG) and arched back nursing (ABN) behavior compared with those that did not. These behaviors are part of a normal maternal nurturing pattern in rodents for mothers nursing neonates. Those rats raised by either a natural or surrogate mother with “low” LG/ABN behaviors show stronger HPA axis responses to stress than those raised by “high” LG/ABN mothers.<sup>95,96</sup> The rats raised by a “low” LG/ABN mother subsequently revealed an increased methylation pattern of the *Nr3c1* promoter in hippocampal DNA that included CpGs within the binding site of the transcription factor NGFI-A.<sup>94</sup> This increased methylation pattern likewise associated with both a decreased acetylation pattern of lysine-9 (K9) of histone H3, which is indicative of lower transcriptional activity<sup>97</sup>, and decreased NGFI-A binding to the *Nr3c1* promoter. *Nr3c1* is a prime candidate for mediation of long-term health effects due to

stress as it encodes the glucocorticoid receptor (GR), a hormone-activated transcription factor that mediates many of the downstream effects of the stress hormone corticosterone (which corresponds in rodents to cortisol in humans).

A follow-up study demonstrated that NGFI-A binding of the *Nr3c1* promoter sequence is itself responsible for loss of local methylation, and possibly plays a role in the epigenetic programming of *Nr3c1*.<sup>15</sup> Another report studied the effects of ELS on methylation status of the *Nr3c1* promoter region in offspring hippocampal DNA by using maternal separation to induce ELS.<sup>98</sup> The resulting observations included altered hippocampal nerve growth factor and corticosterone levels between rats exposed to ELS and controls, but no significant change in the methylation patterns of *Nr3c1*. The offspring of rats given a protein-restricted diet also exhibit reduced methylation of the *Nr3c1* promoter and a corresponding increase in the expression of hepatic GR.<sup>99</sup> The expression of *Dnmt1* in these offspring is also lowered, suggesting a potential causal mechanism for the altered methylation levels of the *Nr3c1* promoter.

Differential methylation of *Avp*, another HPA axis gene, has also been studied by inducing ELS in rodents. ELS in mice results in both hypomethylation of *Avp* and subsequently lowered *Avp* expression in the hypothalamus.<sup>100</sup> The hypomethylated region includes binding sites for the methyl CpG-binding protein 2 (MeCP2). Mice exposed to ELS also display a consistent increase in corticosterone secretion.<sup>100</sup> These studies highlight the long-term health outcomes that can be influenced by differential methylation patterns induced during a developmental stage in rats equivalent to late pregnancy in human fetuses.

## Human Studies

Studies in humans have claimed associations between prenatal stress and differential methylation patterns of stress-related genes. McGowan *et al.* (2009) compared the degree of CpG methylation in the human *NR3C1* promoter in DNA extracted from the hippocampus of suicide victims with a history of child abuse, suicide victims without child abuse, and individuals who died in accidents.<sup>101</sup> They reported increased methylation of the *NR3C1* promoter of the ELS sample (15% mean methylation vs. 40% vs. 10%, in the suicide-no abuse, suicide-abuse, and accidental groups, respectively), although this study was not specifically examining perinatal stress. A subsequent experiment demonstrated decreased NGFI-A binding to a putative binding site within methylated *NR3C1* promoter constructs as compared to unmethylated constructs. It therefore appeared that methylation decreased NGFI-A-inducible transcription.

Oberlander *et al.* (2008) reported an increase in the proportion of CpGs methylated in three CpG loci in the *NR3C1* promoter in umbilical cord blood DNA from offspring of women who experienced a depressive or anxious episode during the second or third trimesters of pregnancy, though these methylation differences were much smaller than McGowan (2009) observed.<sup>102</sup> On the other hand, they found no association between neonatal *NR3C1* promoter methylation and prenatal exposure to selective serotonin reuptake inhibitor (SSRI) antidepressants. Increased methylation at the third CpG locus also showed an association with decreased neonatal salivary cortisol levels at three months of age. This CpG is located in the putative NGFI-A binding site in the *NR3C1* promoter and these findings resemble results reported by Weaver *et al.* (2004) in

a similar motif of the orthologous gene in DNA from the hippocampus of rats exposed to ELS.<sup>94</sup> However, unlike the Weaver study, which made use of DNA derived from hippocampus, Oberlander *et al.* used DNA extracted from leukocytes in umbilical cord blood. These two cell populations might differ in their DNA methylation characteristics across the genome, although both are stress responsive (see below). The study can also be criticized for the very small magnitude of differences in methylation observed. Insufficient data are available to assess the biological impact of very small differences in DNA methylation.

Another study examined the effects of maternal depression during pregnancy on DNA methylation in umbilical cord blood in neonates from mothers with psychiatric illnesses.<sup>103</sup> That group reported decreased methylation in *SLC6A4*, which codes for the serotonin transporter, in neonates of mothers experiencing a depressive episode during the second trimester of pregnancy. It is important to note that the foregoing studies examined one or a few candidate CpG sites. The studies therefore did not provide a genomic context in which to evaluate either the statistical significance or magnitude of group differences relative to other regions in the genome. Nevertheless, these studies give evidence of human stress-related “programming” events associated with DNA methylation and extend the range of these events beyond the *in utero* period to early life exposure postpartum.

### **Epigenetic Patterns Vary by Cell Type**

Epigenetic differences are often cell-type specific. Cellular heterogeneity therefore makes DNA methylation studies in whole organisms much more challenging

than studies of DNA-sequence differences because, in principle, the epigenetic state of each individual cell can differ from those of all other cells. However, some epigenetic differences may be robust enough (i.e., are shared by a sufficient proportion of cells) to be detected in heterogeneous cellular preparations. The above rat and human studies utilized brain tissues when available, as with the studies in rats and victims of suicide, but relied on peripheral blood in remaining instances.

Umbilical cord blood is easily accessible and the leukocytes that the bulk of cord blood DNA derives, while heterogeneous, also represents a stress-responsive “tissue” that can be easily accessed without ethical concerns. The DNA extracted from umbilical cord blood is derived predominantly from stress-responsive fetal immune cells, which also express many of the same receptors and corresponding signaling machinery as cells in the central nervous system.<sup>104</sup> A disadvantage of using peripheral blood is that any given cellular extraction will be a heterogeneous mixture of varying types of leukocytes in which methylation signals in smaller cell populations within this mixture could be overwhelmed by the majority cellular signal for a particular CpG site. Despite these limitations, umbilical cord blood represents the most practical source of material for comparative studies of DNA methylation in neonates.

### **Study Objective**

The main objective of this study was to evaluate the association of specific perinatal exposures with differential DNA methylation patterns at >27,000 CpG sites across the genome in neonates born to women in a prospectively-characterized psychiatric cohort. We hypothesized that maternal mental illness and treatment during

pregnancy would associate with differential DNA methylation patterns in neonates. To test this, we examined DNA methylation in the children of women who sought psychiatric care during pregnancy and who were followed prospectively throughout the remaining pregnancy. While random assignment of medication treatment is not ethically feasible, it is possible to compare the offspring of women who elected to continue (or start) antidepressant medication during pregnancy to those who did not take such medications. In addition, the cohort we studied provided prospective data on a large number of exposures and health indices, which stands in contrast to the retrospective information some prior studies of medication exposure during pregnancy relied upon. Finally, as already noted, prior studies of anti-depressant exposure during pregnancy often did not account for the illnesses prompting treatment. In contrast, our cohort underwent prospective structured evaluations of psychiatric diagnosis and of symptom severity during pregnancy. While it remains impossible to disentangle medication effects from those of the illnesses themselves, the data available in our cohort allowed us to at least account for differences in illness parameters among individuals in specific medication exposure groups. Likewise, we hypothesized that neonatal gestational age would associate with differential DNA methylation patterns in neonates.

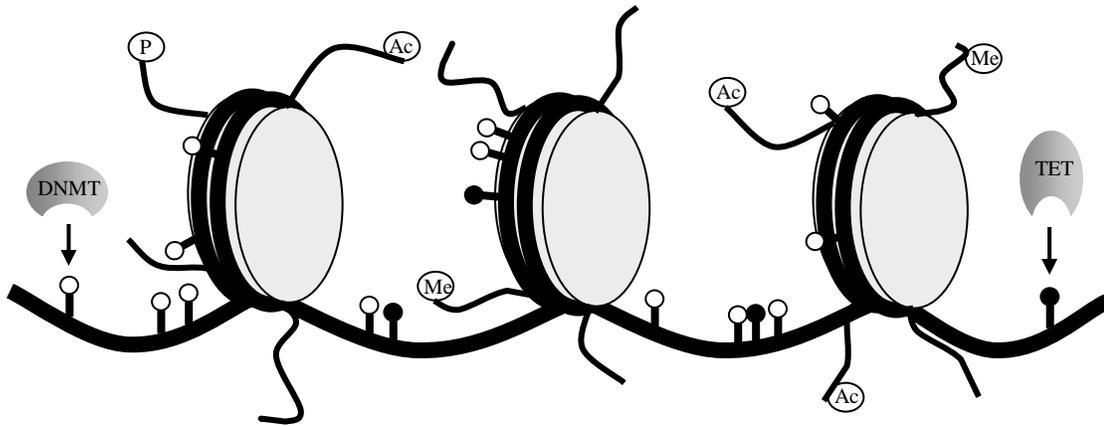
The discernment of whether there is an epigenetic impact on neonates due to gestational age at birth, or exposures to maternal psychiatric symptoms or treatment is a vital step in assessing treatment strategies for women with psychiatric symptoms during pregnancy. In addition, documenting molecular evidence of differences in neonatal DNA methylation associated with maternal illness, medication exposure or other prenatal factors could prompt future investigations into possible associations between such factors

and long-term post-natal health outcomes in the children. The results of this study could therefore be an important step in future assessments of whether there are epigenetic patterns in specific genes at birth, causal or otherwise, that associate with differences in post-natal outcomes. The remainder of this dissertation will overview the methodologies used in this dissertation as well as technical obstacles encountered therein (Chapter 2), examine the associations between both maternal psychiatric symptoms and treatment during pregnancy with differential DNA methylation (Chapter 3), and examine the association between neonatal DNA methylation and estimated gestational age (Chapter 4). The final chapter will outline summaries from these studies and give concluding remarks.

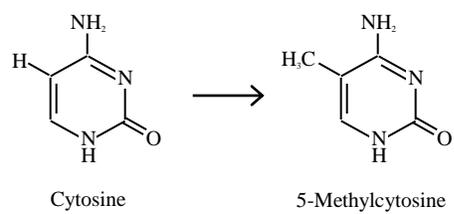
**Figure 1-1.** Representative model of epigenetic modifications of histone and DNA.

Histone modifications include acetyl (Ac), methyl (Me), and phosphate (P) groups.

Cytosine methylation at CpG dinucleotides is represented by a closed black circle, while open circles symbolize unmethylated cytosines at CpG dinucleotides. DNMT is shown targeting a CpG site for methylation, while TET is shown targeting a 5-methylcytosine for conversion to 5-hydroxymethylcytosine.



**Figure 1-2.** Conversion of cytosine to 5-methyl cytosine.



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## **Chapter 2**

### **Technical Methodologies**

## **Genome-wide Studies of Sequence Variation and DNA Methylation**

The candidate gene approach to genetic association studies is based on the rationale that genetic variation associated with a given phenotype can be identified through examining genes or regions supported by specific biological evidence. While candidate gene studies play an important role in genetic epidemiology, they are limited by the current understanding of the molecular underpinnings of the phenotype being studied. This limitation is amplified when examining complex traits that involve multiple genes that may interact with environmental factors to influence phenotypic expression. Successful candidate gene studies often yield variants that have modest effects on a given phenotype and are not readily replicated in subsequent investigations. Thus, the literature is replete with single-gene association studies that use lenient significance criteria that are not consistent with those used in genome-wide investigations. In fact, most genes identified in genome-wide studies are incongruous with prior biological evidence of the molecular underpinnings of those phenotypes.<sup>1</sup>

In contrast to candidate-gene studies, genome-wide association studies (GWAS) test loci across the entire genome, in an attempt to uncover genetic variants that are causal or are in linkage disequilibrium with potentially causal variants for a given phenotype. As such, these studies need not limit their range of genomic inquiry to candidate genes based on prior biological data of gene functions. GWAS of large numbers of individuals successfully identified numerous genes that were not previously suspected of being associated with complex diseases, including Crohns Disease<sup>2,3</sup>, Types 1 and 2 Diabetes,<sup>4-6</sup> and myocardial infarction<sup>7</sup>, as well as quantitative traits such as height<sup>8,9</sup> and skin pigmentation<sup>10,11</sup>. To date, GWAS have shown less success in

psychiatric disorders such as bipolar disorder and schizophrenia even though heritability estimates of the liability to those disorders range from 50-80%.<sup>12</sup> A practical challenge in genome-wide studies is that they require correction for conducting large numbers of statistical tests, and thus require larger sample sizes to maintain power due to stringent significance criteria. However, adherence to such stringent criteria has proven key to generating replicable results.

As reviewed in Chapter 1, methylation of DNA at CpG dinucleotides can associate with complex phenotypes, and several approaches exist for interrogating large numbers of CpG dinucleotides for methylation. Sampling DNA methylation on increasingly finer genomic scales has resulted in increased understanding of human development<sup>13,14</sup>, aging<sup>15,16</sup> and complex phenotypes.<sup>17,18</sup>

### **Laboratory Approaches to Genomic-scale Analysis of DNA Methylation**

Early genomic procedures used methylation-sensitive restriction enzyme digestion in combination with gel electrophoreses and Southern blotting to analyze differential DNA-digest patterns reflecting methylation patterns.<sup>19</sup> DNA can also be digested by methylation-sensitive enzymes and interrogated via 2-D gel electrophoresis and autoradiography in a process known as restriction landmark genome scanning (RLGS). This procedure has been used for larger-scale characterization of methylation patterns that are tissue-specific.<sup>20,21</sup> More recent protocols isolate and enrich methylated DNA via immunoprecipitation with antibodies specific for methylated cytosine.<sup>22</sup> Genomic areas enriched by such methylated-DNA immunoprecipitation (MeDIP) can be interrogated by downstream applications including real-time PCR, arrays specific for

particular loci (MeDIP-chip)<sup>22,23</sup> or next-generation sequencing technology (MeDIP-seq).<sup>24</sup>

Another technique for studying DNA methylation is through the use of bisulfite-converted DNA templates (described in Array Processing below). Platforms relying on bisulfite-treated DNA are capable of reliably querying high numbers of specific loci. Extensive bisulfite treatment required for full conversion of methylated cytosines can cause DNA degradation, though the quality of DNA required for techniques using bisulfite-converted DNA is not as high as it is for other applications. Indeed, bisulfite-based methodologies have a higher tolerance for sheared DNA fragments because preservation of flanking regions is usually not required. These techniques also require less overall DNA than affinity-enrichment methodologies and are less prone to contamination than are other methodologies because bisulfite-converted human DNA is no longer analogous to untreated human genomic DNA. However, bisulfite-based approaches are limited because they produce sometimes heterogeneous sets of DNA templates making it difficult to design reliable oligonucleotides targeting these templates for PCR and other applications.

This study used the HumanMethylation27 BeadChip array produced by Illumina, Inc. (San Diego, CA). This array utilizes BeadArray technology where probes with sequences complementary to specific to particular genomic loci are covalently bound to beads and assigned specific, known “addresses” on a BeadChip array.<sup>25</sup> The HumanMethylation27 BeadChip interrogates 27,578 CpG dinucleotides across 14,495 genes, or roughly 0.1% of the estimated 28 million CpG dinucleotides in the human genome. The array queries at least one CpG site in approximately 56% of annotated

human genes. In addition to the breadth of loci examined, the Infinium array also provides approximately a 30X redundancy for each locus investigated, which amounts to 60 probes of two varieties per locus. This array has performed reliably in previous studies, including those examining methylation in DNA extracted from blood.<sup>25-28</sup> Despite the large number of loci investigated, the HumanMethylation27 array only requires a small amount of input DNA for the procedure. The breadth of loci interrogated, depth of coverage, and limited input requirements make this array an appealing application for an unbiased study of methylation patterns in complex phenotypes.

### **Overview of the Infinium Protocol**

An initial genomic DNA sample is bisulfite converted, whole-genome amplified, sheared and hybridized to the BeadChip. The BeadChip utilizes one of two probe varieties specific for each particular CpG: a methylated and an unmethylated version (Figure 2-1). Both versions of the probe are complementary to the bisulfite-converted sequence flanking the CpG of interest with the exception of the 3' end. The final 3' end base of the methylated probe variety is complementary to a cytosine while this final base is complementary to a thymine in the unmethylated probe. Each bead is saturated by covalently-bound probes that are uniformly specific to one CpG site in only one of the two methylation-state varieties. These beads reside in well coordinates that are customized for each particular chip. The bisulfite-converted DNA fragments hybridize to their complementary probes and are extended by a single base downstream, which yields

a detectable signal after processing. These signals are then scanned and analyzed to yield a value analogous to the proportion of DNA methylated at a given locus.

### **Sample Preparation**

The Emory Women's Mental Health Program (WHMP) umbilical cord blood samples were collected at birth, stored on ice, and processed within 2 hours of delivery. Plasma was separated by centrifugation at 4<sup>0</sup>C, and the cellular fraction was frozen at -80<sup>0</sup>C until processing. DNA was extracted from the cellular fraction at the Emory Biomarker Service Center using a Qiagen Biorobot M48. The resulting DNA elutions were of varying concentrations and had to be normalized to a consistent concentration between them in order to ensure accuracy of pipetting during the bisulfite-conversion procedure. The initial quantification of the elutions were performed using Quant-iT PicoGreen (Life Technologies, Carlsbad, CA) that intercalates into double stranded DNA giving a detection signal that is proportional to the amount of DNA in the solution. The samples were then assayed the Synergy 2 Multimode Microplate Reader (BioTek, Winooski, VT). The concentrations of the DNA were verified alongside DNA standards of known quantity using agarose gels. Following completion of quantification, normalization and sample QC procedures, 500ng of each sample was used in the bisulfite conversion reaction.

### **Array Processing**

Prior to hybridization, each DNA sample was bisulfite-converted using the EZ DNA Methylation Kit (Zymo Research, Orange County, CA), whole-genome amplified

and sheared into fragments. Bisulfite treatment converts unmethylated cytosine to uracil by first sulfonating the 6 carbon of the pyrimidine ring of cytosine, which is then followed by a hydrolytic deamination of the 4 carbon and a 6 alkali-desulphonation producing uracil (Figure 2-2). Through subsequent amplifications, this uracil is replaced by thymine while methylated cytosines remain as cytosines after treatment. The bisulfite-converted DNA fragments were then hybridized onto more than two dozen 12-lane chips along with one technical control per chip, which was DNA from a single adult female.

A loading strategy was devised that sought to randomize the samples across chips according to a grouping of maternal drug exposures that is correlated with the diagnosis of the mothers as well as neonatal sex and race. The DNA fragments hybridize to the probes that each fragment is complementary to both in CpG locus and in DNA methylation state. These DNA fragments act as a template to extend the probes with a single dideoxynucleotide triphosphate, which cannot itself be extended because of the lack of a hydroxyl group on the 3' carbon of the deoxyribose sugar. These dideoxynucleotides were labeled with either biotin or 2,4-dinitrophenol (DNP) followed by fluorescent staining that were subsequently detected by the laser-based scanner BeadStation 500 (Illumina, Inc., San Diego, CA) after the chips were processed. The fluorescent hybridization signals representing relative levels of converted and unconverted CpGs were extracted from the Methylation Module of the BeadStudio software. In the graphic output, each well exhibits a signal of uniform fluorescent color, the magnitude of which reflects the percentage of either methylated or unmethylated template strands (depending on which variety of probe is bound to the bead in that well)

for that particular CpG locus. Signals collected from each probe variety for a particular CpG locus scattered across the chip were averaged together to create a mean methylated and mean unmethylated signal for that CpG locus. These values were used to create a  $\beta$  value that is computed as

$$\beta = \frac{\text{methylated signal}}{\text{methylated signal} + \text{unmethylated signal}} \quad (1)$$

yielding a result that is effectively the proportion of DNA methylated at a particular CpG locus. Three samples with probe detection call rates <90% or an average intensity value of either <50% of the experiment-wide sample mean or <2000 arbitrary units (AU) were then excluded from further analysis. Hierarchical clustering was also performed on the  $\beta$  values to identify samples that were extreme outliers based on their global methylation patterns; three samples were eliminated from further analysis (Figure 2-3B).

### **Chip and Experiment Effects**

Sizeable signaling discrepancies between two given samples can be observed if the two samples were run on two different chips. These “chip effects” can arise because manufacturing inconsistencies between any two chips at some time during their production were substantial enough to affect the final outcome of an experiment (Figure 2-3). Alternatively, chip effects can result from an inconsistency between the way two chips were processed during an experiment due to user technique, reagent usage, or other incidentals.

We applied a multi-pronged strategy for avoiding experimental error due to chip effects. As mentioned previously, samples were randomized by experimental group, and a technical control was included on all chips in this study. This control was utilized in a quality control assessment after the chips were run, which successfully identified two chips (Figure 2-3A) that were so divergent from the mean of all remaining technical controls that they were thrown out of the experiment and their samples rehybridized to new chips that yielded results comparable to the successful chips in quality (Figure 2-3B). Another strategy used to account for chip effects was to create a variable for chip numbers that could be used to control for chip effects in our statistical analyses. The resulting data set was adjusted for what Bolstad *et al.* describe as “obscuring variation” that interferes with an analysis of remaining, biologically-relevant variation arising from the data.<sup>29</sup> The combination of these precautions has increased the confidence in our results by reducing the likelihood of distortions due to chip effects.

Like chip effects, variation between experiments (sets of chips run at one time) could conceivably generate errors not due to biological variation between samples within each experiment. Experiment effects could arise for comparable reasons that chip effects would: differences in manufacturing consistency, or in the manner in which each experiment was performed. Although we initially statistically adjusted for both chip and experiment effects, we later realized that inconsistencies between experiments could be adequately accounted for by adjusting for chip effects alone due to the small number of experiments performed.

## HumanMethylation27 Probe Issues

The allelic composition of sequence variants can vary widely between diverse populations and ethnic groups.<sup>30,31</sup> Several technical complications may arise during the stages of hybridization and extension of the HumanMethylation27 probes if a SNP resides at or near the CpG of interest. If a SNP lies under the probe itself, the presence of an allele that does not match the probe will decrease the binding efficiency of the probe and likely altering the final signal strength that results from the single base extension (Figure 2-4). Likewise, the existence of a SNP at either the cytosine or guanine of a CpG dinucleotide may alleviate the CpG site entirely, highlighting the interesting complication that variation in the potential for methylation due to sequence differences can contribute to the overall differences in patterns of methylation among individuals. Finally, variation in the SNP used for the single base extension (SBE) reaction (one nucleotide downstream of the CpG site) may disrupt the expected fluorescent signals if the substitution of the SNP allele required extension by a nucleotide bound to the other fluorescent dye.

Due to the potential complications caused by SNP proximity to CpGs of interest, we sought to characterize the abundance of probes in the HumanMethylation27 array that fell into the aforementioned categories (Table 2-1). Because Illumina has reported that mismatches beyond 10 bases of the 3' end of a probe should not interfere with probe annealing, SNPs discovered within probe sequences were divided into those within 10 bases of the 3' end and those simply under a 50 base length probe. Of note is the preponderance of probes with SNPs under the CpG site of interest (273 probes with SNPs at the cytosine site and 273 at the guanine), with 206 (75.5%) C/T transitions at the cytosine site and 230 (84.2%) G/A transitions at the guanine site. These results reflect

the higher propensity of 5'-meth-C/T transitions observed at CpG dinucleotides, which are more likely to occur outside CpG islands. Using our methylation data from the HumanMethylation27 BeadChip, though not genotyping individual samples, we plotted the methylated vs. unmethylated signals in two probes (cg13284426 and cg22022041) that associated with race ( $p < 1.81 \times 10^{-6}$ ) and had a SNP located under the CpG site (rs56997225 and rs41289608, respectively; Figure 2-5). Both of these probes display a decreased or eliminated methylation signal in a portion of the samples. An examination of the racial constitution of these SNPs using the 1000 Genomes Project revealed that rs56997225 is polymorphic for C/T in African populations (minor allele frequency (MAF)=16%) and rs41289608 is polymorphic for G/A in both African and European populations, though at greatly different rates (MAF=34% vs. 1%, respectively). While the distorting influence that allelic variation in these probes warrant additional exploration, CpG sites associated with any outcome examined in this study do not appear to have SNPs that influence binding of the associated probes.

Similar technical issues could theoretically occur due to variation in the methylation status of CpG dinucleotides flanking the CpG of interest. While the design of the two varieties of probes (methylated and unmethylated) on the HumanMethylation27 chip does account for the methylation status of neighboring CpGs that may lay under the probe, this design assumes that the CpGs immediately flanking the CpG of interest will usually bear the same methylation status as the CpG of interest (Figure 2-4).<sup>32</sup> For example, if there are two CpG sites under a particular probe, the methylated variety of that probe will also contain bases that are complementary to the methylated (that is, cytosine-preserved) versions of those two flanking CpGs. If one or

both of these flanking CpGs are unmethylated, the methylated probe variety will be mismatched at one or both of those CpG sites in the same way it would be to a minor allele at a SNP. Although, as was stated previously, Illumina has reported that mismatches beyond a 10 base distance from the 3' end of the probe should not affect probe hybridization while those within 10 bases could possibly affect hybridization.

To assess the prevalence and influence of these flanking CpGs, we examined the full set of HumanMethylation27 probes to characterize the number of flanking CpG dinucleotides within 10 bases of the 3' end or under any probe bases. The results can be seen in Table 2-2. Of note is the finding that only 79% of the probes did not have a flanking CpG dinucleotide within 10 bases of the probe's 3' end. Likewise, only 26.3% of the probes had no flanking CpGs included in their sequence, which is reflective of the density of CpG dinucleotides in CpG islands.

Illumina has based their probe design on the fact that flanking CpGs that are within 50 bases of the CpG of interest (the length of a probe) will predominantly carry the same methylation status as the CpG of interest. Any stochastic fluctuation in the methylation status that does occur between two flanking CpGs suggests that the bias of both methylated and unmethylated probes will result in a reduction of signal strength instead of a systemic bias. This reduction in signal strength should not be problematic because our measure is not signal strength but  $\beta$ , which is calculated as a ratio of these two signals.

## Statistical Analysis

Associations between DNA methylation and each particular phenotype were analyzed by fitting separate linear mixed effects models for each probe using  $\log\left(\frac{\beta}{1-\beta}\right)$  as an outcome. Each analysis included covariates that have known effects on either DNA methylation or the phenotype of interest. Covariates that were included in all analyses were sex and race of the neonate, as well as a random effects term to account for chip effects. In addition to clear differences expected between the methylation patterns seen in each sex, sex also correlates with birth weight and gestational age.<sup>33,34</sup> Similarly, race is associated with both differential methylation and gestational age.<sup>33,35</sup> The false discovery rate for each analysis was controlled at .05 to account for 27,578 tests per analysis.<sup>36</sup>

The methods detailed in this chapter were applied to subsequent investigations of the association between DNA methylation of the HumanMethylation27 loci and maternal psychiatric illness and treatment (Chapter 3), and estimated gestational age (Chapter 4). Molecular and statistical methods specific to each study are detailed in the respective chapters.

**Table 2-1.** Prevalence of SNPs under the HumanMethylation27 probes or at the single base extension nucleotide.

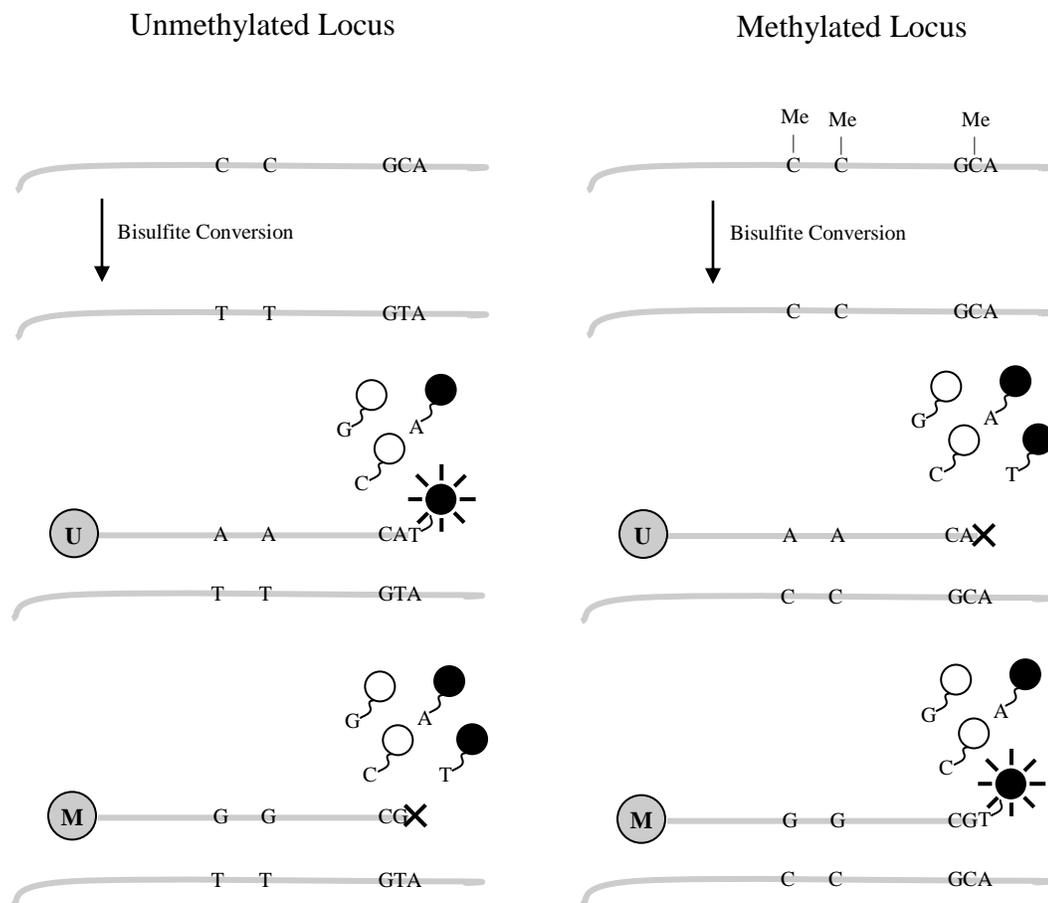
	<b>Number of Probes (%)</b>	<b>#Probes/base</b>
SNPs at SBE site	50 (.18)	50
SNPs at CpG site		
<i>SNPs at C</i>	273 (.98)	273
<i>SNPs at G</i>	273 (.98)	273
SNPs under bases 2-10 from 3' end	619 (2.2)	77.4
SNPs under bases 11-50 from 3' end	4741 (17.2)	118.5

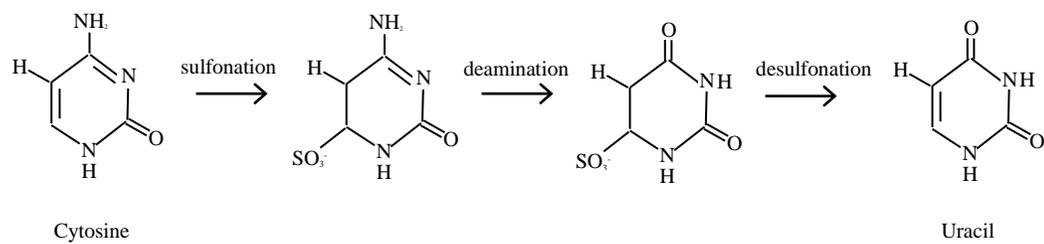
**Table 2-2.** Prevalence of flanking CpG dinucleotides under the HumanMethylation27 probes or at the single base extension nucleotide.

	<b>Number of Probes (%)</b>
<b># of flanking CpGs within 10 bases of 3' end:</b>	
<i>0 CpGs</i>	21800 (79)
<i>1 CpG</i>	5250 (19)
<i>2 CpGs</i>	502 (1.8)
<i>3 CpGs</i>	26 (.094)
<b># of flanking CpGs under any probe sequence:</b>	
<i>0 CpGs</i>	7253 (26.3)
<i>1 CpG</i>	8302 (30.1)
<i>2 CpGs</i>	5879 (21.3)
<i>3 CpGs</i>	3615 (13.1)
<i>4 CpGs</i>	1651 (6)
<i>5 CpGs</i>	628 (2.3)
<i>6 CpGs</i>	155 (.56)
<i>7 CpGs</i>	52 (.19)
<i>8 CpGs</i>	27 (.098)
<i>9 CpGs</i>	11 (.04)
<i>10 CpGs</i>	5 (.018)

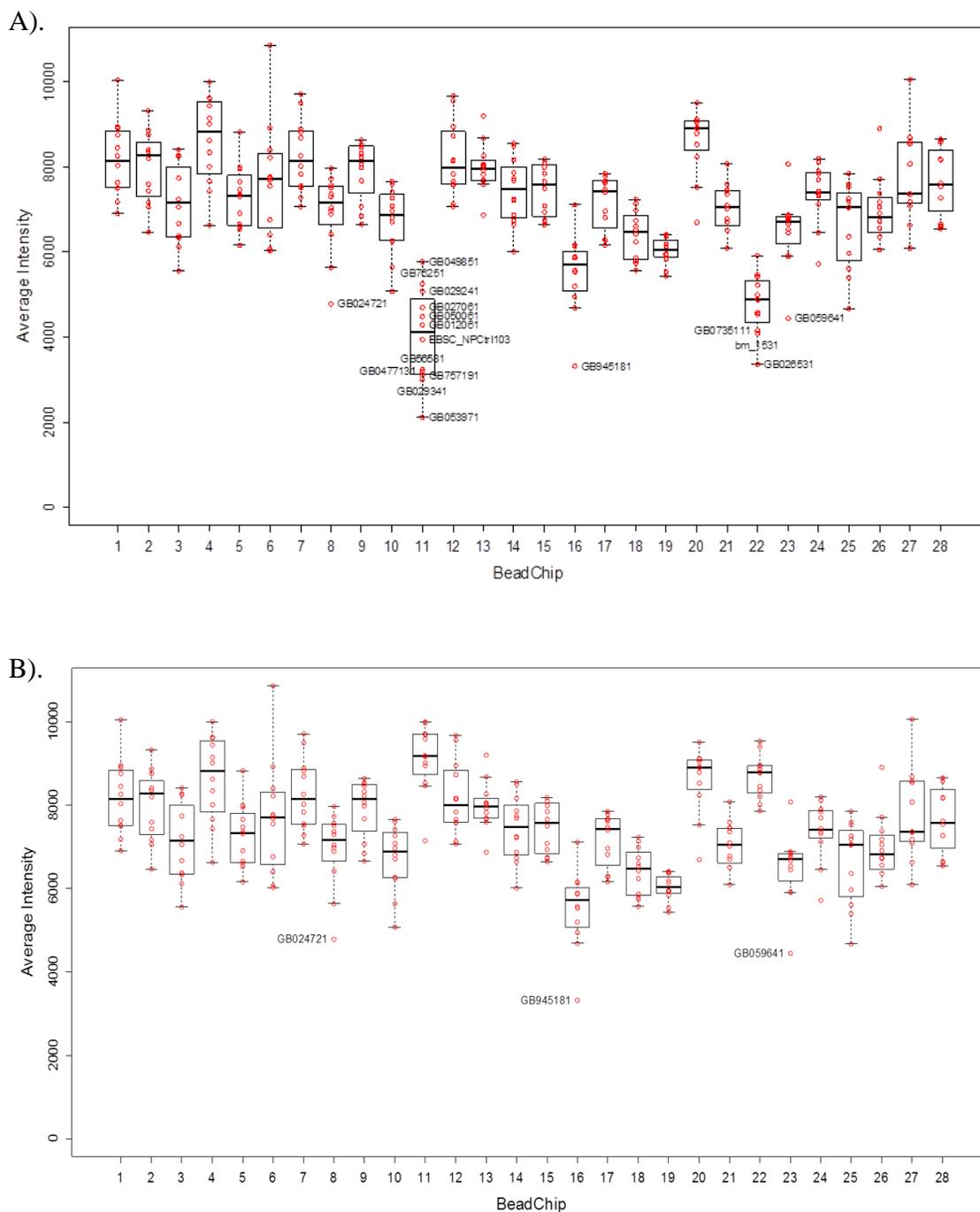
**Figure 2-1.** Model depicting the HumanMethylation27 BeadChip extension reaction.

After the genomic DNA is bisulfite converted, the unmethylated probe (U) will only extend when bound to an unmethylated DNA strand while the methylated probe (M) will only extend when bound to a methylated DNA strand.



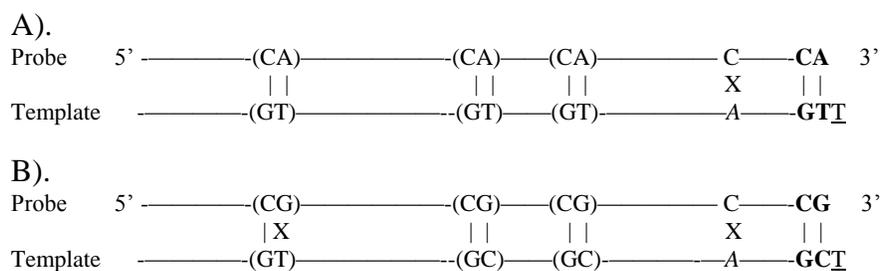
**Figure 2-2.** Bisulfite conversion of cytosine to uracil.

**Figure 2-3.** Chip effects shown by average sample intensity by BeadChip. A) the original scan of all 28 BeadChips with outlying chips 11 and 22, and B) the full set of 28 BeadChips with chips 11 and 22 re-hybridized.

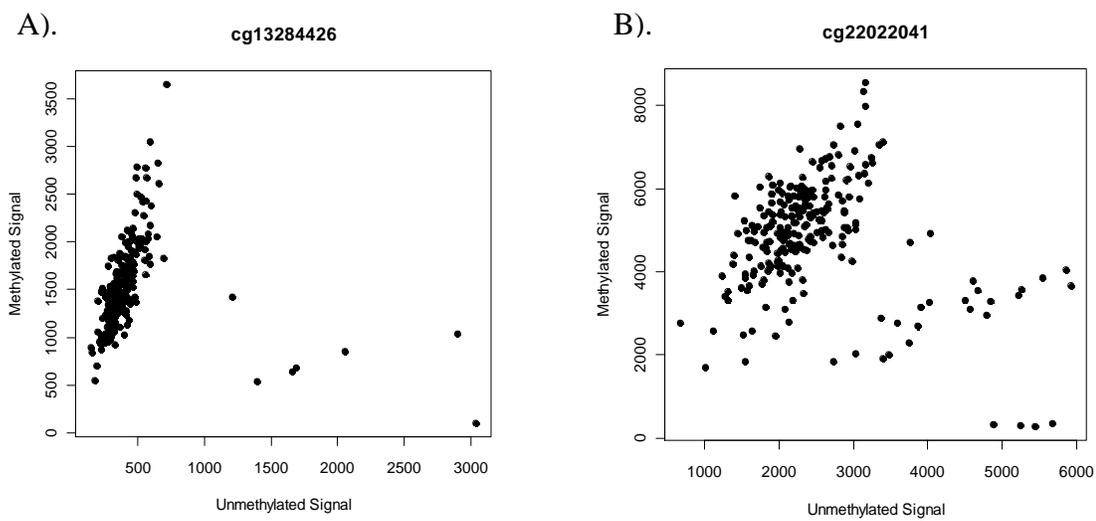


**Figure 2-4.** Representative HumanMethylation27 probe with mismatched nucleotides.

A) Unmethylated and B) methylated versions of a hypothetical probe and corresponding templates containing flanking CpGs (in parentheses), a SNP (in italics), and the single base extension nucleotide (underlined). Two mismatches are shown due to an unmethylated flanking CpG on the methylated strand template (B) and a SNP minor allele present in both strands.



**Figure 2-5.** Scatterplots of methylated vs. unmethylated signals. Probes A) cg13284426 and B) cg22022041 are shown containing SNPs rs56997225 and rs41289608, respectively.



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## Chapter 3

### DNA Methylation of Neonates Born to Women Receiving Psychiatric Care

Information presented in this chapter is currently under review as cited below:

JW Schroeder, AK Smith, PA Brennan, KN Conneely, V Kilaru, BT Knight, DJ Newport, JC Cubells, ZN Stowe. DNA Methylation of Neonates Born to Women Receiving Psychiatric Care. Submitted to Epigenetics.

## Introduction

Major depressive disorder is nearly twice as prevalent in women as it is in men and affects up to 12.8% of women during pregnancy.<sup>1</sup> Depressive symptoms do not abate during pregnancy, and children of depressed women are more likely to deliver prematurely and develop social and emotional problems as they develop.<sup>2-6</sup> Likewise, the children of women taking antidepressants during pregnancy have an increased risk of fetal cardiac defects and neurological development issues<sup>7-9</sup>, though some studies have suggested that these risks are not significant or are comparable to risks resulting from untreated depression.<sup>10,11</sup>

Prenatal exposure to psychotropic medications, psychiatric illness, or the combination of the two could influence offspring outcomes through changes in DNA methylation at CpG dinucleotides. Human and animal studies report links between prenatal or early life stress and differential DNA methylation of the *NR3C1* promoter.<sup>12</sup> In one such study, umbilical cord blood DNA methylation of *NR3C1* was associated with maternal mood during pregnancy, but not with SSRI exposure.<sup>13</sup> Similarly, another study by this group reported that neonates born to women depressed during the second trimester have significant methylation differences in the serotonin transporter (*SLC6A4*), though differential methylation of this gene did not associate with SSRI exposure or maternal depression in the third trimester.<sup>14</sup>

It is important to delineate the potential effects of psychiatric illness and symptoms from treatment on developing fetuses. Previous efforts in this regard have been limited to selected candidate genes. In this study, we examined the methylation patterns of >27,000 CpG sites across the genome in umbilical cord blood-derived DNA

from the offspring of women undergoing treatment for a mood disorder during the perinatal period.

## **Methods**

### *Subject Enrollment*

Subjects were recruited from the Specialized Center of Research for Sex and Gender Effects (SCOR) or the Translational Research Center for Behavioral Sciences (TRCBS) at Emory's Women's Mental Health Program (WMHP), a referral center for the treatment of perinatal psychiatric illness. Mothers were evaluated prospectively at 4-6 week intervals with serial measures of psychiatric symptoms and pharmacologic exposures throughout pregnancy.

The maternal inclusion criteria for this study included: 1) >17 years of age; 2) written and verbal fluency in English; 3) a live singleton delivery; 4) availability of DNA from umbilical cord blood collected at delivery; and 5) lifetime diagnosis of a mood disorder. Exclusion criteria included: 1) unstable non-psychiatric medical illnesses requiring pharmacological treatment during pregnancy (e.g. asthma, autoimmune disorders); 2) abnormal thyroid stimulating hormone (TSH); or 3) use of lithium, stimulants, antiepileptic, or migraine medications.

A total of 201 Caucasian and African-American neonates were included in this study (Table 3-1). Antidepressants were categorized by mechanism of action. Class 1 included selective serotonin re-uptake inhibitors (SSRIs), serotonin-norepinephrine reuptake inhibitors (SNRIs), and tricyclic antidepressants (TCAs), that act primarily through serotonergic pathways while Class 2 consisted of bupropion, which acts through

non-serotonergic pathways.<sup>15-17</sup> A Structured Clinical Interview for Diagnosis (SCID) was used to assess lifetime diagnosis according to DSM-IV criteria, and the SCID Mood Module was used to assess major depressive episodes at each visit during pregnancy.<sup>18</sup> No subject met criteria for a manic episode during pregnancy according to the SCID Mood Module. Depressive symptoms were assessed using the 17-item Hamilton Rating Scale for Depression (HRSD17)<sup>19</sup> and the Beck Depressive Inventory (BDI).<sup>20</sup>

All mothers provided written informed consent prior to study enrollment and the Institutional Review Board of Emory approved all procedures. This study was conducted in accordance with the Helsinki Declaration of 1975.

#### *Sample Collection and DNA Methylation Analysis*

Collection and processing of umbilical cord blood samples has been described previously in detail in Chapter 2. Briefly, umbilical cord blood was collected at birth, stored on ice, and processed within 2 hours of delivery. DNA was extracted from the cellular fraction at the Emory Biomarker Service Center. One microgram of genomic DNA was bisulfite-converted using a Zymo EZ DNA Methylation-Gold kit (Zymo Research, Orange, CA) and interrogated using the HumanMethylation27 BeadChip (Illumina, San Diego, CA). A single female genomic DNA sample was run on each BeadChip as a technical control. Three samples with probe detection call rates <90% or with an average intensity value of either <50% of the experiment-wide sample mean or <2000 arbitrary units (AU) were excluded from the analysis. We normalized the signal data to adjust for technical variability between samples by utilizing the information from sixteen negative control probes that are included on the Illumina BeadChip and are

designed to detect a true methylated and unmethylated signal at zero. The signals from methylated ( $M$ ) and unmethylated ( $U$ ) bead types were then used to determine a  $\beta$  value, calculated as  $\beta = M/(U+M)$ , or the proportion of DNA methylated at a particular CpG site.

### *Statistical Analysis*

All analyses used a logit-transformed  $\beta$  value equal to  $\log\left(\frac{\beta}{1-\beta}\right)$ . The associations between this function of  $\beta$ , the proportion of DNA methylated, and each maternal diagnosis, symptom, or exposure were evaluated by fitting a separate linear mixed effects model for each CpG site.  $\log\left(\frac{\beta}{1-\beta}\right)$  for each CpG site was then modeled as a function of the measures of maternal treatment exposure, psychiatric diagnosis, or symptoms.

Dichotomous variables examined included: prenatal exposure to any antidepressant or to specific antidepressant categories (Class 1 or Class 2); exposure to atypical antipsychotics, benzodiazepines, antiemetics, and hypnotics (each analyzed independently); lifetime and current principal diagnosis of major depressive disorder or bipolar disorder. Continuous variables examined included: the number of weeks of exposure to these antidepressant variables - both across the entire pregnancy and separately by trimester, and measures of depressive symptoms (HRSD17 and BDI). Additionally, depressive symptoms were dichotomized based on the maximum maternal score at any point pregnancy such that clinically-significant symptoms were present if a subject scored  $>15$  on the HRSD17 or  $>10$  on the BDI.

For each analysis, neonatal sex, race, and gestational age were included as covariates because they have been shown to exert independent effects on methylation patterns in this and other studies.<sup>21-24</sup> Random effects for chips were included in the model to allow for chip-to-chip differences in measurement of the proportion of DNA methylated. To account for the 27,578 tests performed for each diagnosis or exposure, the false discovery rate (FDR) was controlled using the method of Storey et al. at .05.<sup>25</sup> For CpG sites in two genes previously reported to be differentially methylated with maternal mood, we also considered nominal p-values ( $\alpha=.05$ ).

### *Power Calculations*

Power was calculated using Quanto (<http://hydra.usc.edu/gxe/>) to assess whether a lack of significant differences is likely due to insufficient sample size vs. an absence of differential methylation. For analyses of 201 individuals, there was >80% power to detect between-group methylation differences with an  $R^2 > .145$  after correction for 27,578 tests. For comparison with two genes previously reported to be differentially methylated with maternal mood, nominal p-values were also considered. For these analyses there was >80% power to detect differences with  $R^2 > .038$ , >90% power to detect differences with  $R^2 > .051$ , and >99% power to detect differences with  $R^2 > .088$ .

## **Results**

### *Maternal Diagnosis & Symptoms*

To assess the potential contribution of maternal psychiatric illness, both current and lifetime maternal diagnosis of a depressive or bipolar disorder was evaluated as well

as the presence (N=86) or absence (N=115) of maternal major depressive episodes during pregnancy. No association with neonatal DNA methylation was observed at any of 27,578 CpG sites, based on a false discovery rate (FDR) cutoff of .05. Similarly, the severity of maternal depressive symptoms (HRSD17; mean=16.3; SD=5.9 and BDI; mean=17.3; SD=10.8) and the presence of clinically significant depressive symptoms (HRSD17>15 or BDI>10) at some point during pregnancy did not show an association with neonatal DNA methylation at any CpG site.

### *Medication Exposure*

Exposure to an antidepressant (N=151 vs. 50) was associated with differential methylation of two CpG sites (FDR<.05). Methylation of a CpG site, cg22464186, in tumor necrosis factor receptor subfamily 21 (*TNFRSF21*) was decreased in the umbilical cord blood DNA of neonates exposed to antidepressants (mean=7.9% (SE=0.14%) vs. 9.8% (0.42%);  $p=2.8 \times 10^{-6}$ ; Figure 3-1A) while methylation of a CpG site, cg02953306, in cholinergic receptor, nicotinic, alpha 2 (*CHRNA2*) was increased (32% (0.36%) vs. 29% (0.60%);  $p=3.1 \times 10^{-6}$ ; Figure 3-1B). These results did not appear to be specific to either Class 1 (i.e. SSRIs, SNRIs, and TCAs; N=132) or Class 2 (bupropion; N=40) antidepressants. They likewise did not appear to be influenced by the number of weeks of exposure (mean=24.1; SD=17.4) or the timing of exposure.

Exposure to neurotropic medications taken concurrently with antidepressants was independently evaluated, but there was no association between any CpG site and exposure or duration of exposure to hypnotics (N=31), antiemetics (N=41) or benzodiazepines (N=39). Methylation of a CpG site, cg23034818, in BTB (POZ) domain

containing 6 (*BTBD6*), was associated with exposure to an atypical antipsychotic (N=32), but this association did not remain upon removal of a single influential outlier.

## Discussion

Using a genome-wide approach, we observed no association between neonatal umbilical cord blood DNA methylation and maternal psychiatric diagnosis or clinically-significant depressive symptoms. This finding was somewhat unexpected since previous studies have reported an association between maternal prenatal mood and umbilical cord blood methylation of glucocorticoid receptor (*NR3C1*) and serotonin transporter (*SLC6A4*).<sup>13,14</sup> We observed no association between neonatal methylation of any CpG site and maternal diagnosis or depressive symptom scales after accounting for multiple comparisons, nor did we observe even a nominal association of a CpG site in *NR3C1* or *SLC6A4* with any outcome. This disparity may reflect the limitations of candidate gene studies, as the magnitude of the effect sizes in the initial studies would not meet the significance criteria of this study following adjustment for multiple testing. Negative results can occur due to insufficient power; however, our analysis had >80% power to detect group differences explaining >14.5% of variation in methylation at a single CpG site even after applying the conservative Bonferroni correction. Although it is possible that we did not detect subtler effects due to limited power, we were generally well-powered to detect larger effects. Moreover, when we considered nominal p-values we had >80% power to detect differences explaining >3.8% of variation, >90% power to detect differences explaining >5.1% of variation, and >99% power to detect differences explaining >8.8% of variation in methylation in a single CpG site. Thus, we would have

had 90-99% power to identify an association with *NR3C1* or *SLC64* as strong as those previously reported (semipartial  $R^2$  ranged from .05-.11).<sup>13,14</sup>

Our sample was also restricted to women with a lifetime history of a mood disorder diagnosis, which may have resulted in a more restricted range of depressive symptoms experienced during pregnancy. Nevertheless, this study failed to replicate the initial findings that clinically-significant maternal mood state in pregnancy is associated with DNA methylation changes in cord blood. This suggests that the previously observed association between maternal psychiatric illness and adverse offspring outcomes may be due to genetic variants that remain undiscovered or to environmental or behavioral factors that do not correspond with DNA methylation changes present at birth.

Prenatal exposure to an antidepressant was associated with differential methylation of CpG sites in *TNFRSF21* and *CHRNA2*. *TNFRSF21*, also known as death receptor 6 (DR6), is expressed in both developing neurons<sup>26,27</sup> and developing lymphocytes.<sup>28,29</sup> Because of its role in refinement of neuronal connections during development,<sup>26,27,30</sup> alterations in *TNFRSF21* expression impact learning and memory as well as emotional responses to stressful events.<sup>31</sup> *CHRNA2* is a broadly expressed subunit of nicotinic acetylcholine receptors.<sup>32</sup> In addition to its role in nicotine dependence and neurocognitive functioning,<sup>33,34</sup> *CHRNA2* is located in a region of chromosome 8p that is suggested to contribute to psychiatric and neurodegenerative disorders.<sup>35</sup> Though these results are statistically significant, given the small magnitude of methylation differences between those exposed to AD and those that were not in both of these CpG sites (Figure 3-1), they may not be biologically significant. It is questionable whether the magnitude of methylation change observed in both of these

analyses is sufficient to reflect a substantial change in the regulation of *TNFRSF21* and *CHRNA2* expression. In addition, it is possible that these results are false positives since these results satisfy the false discovery rate threshold for significance but not the more conservative criteria for Bonferroni significance ( $p \leq 1.81 \times 10^{-6}$ ). As such, these results should be interpreted with caution pending replication or focused studies in relevant animal models.

A potential limitation to this study is the fact that the DNA was extracted from whole umbilical cord blood, and may not reflect changes in other relevant tissues within the neonatal central nervous system. However, both stress-responsive factors such as cortisol and the medications examined in this study cross the placental barrier and are detectable in umbilical cord blood<sup>17,36,37</sup>. The study is strengthened by the rich, prospective characterization of the course of psychiatric illness and treatment as well as other environmental exposures throughout pregnancy.

Delineation of the influence of maternal psychiatric illness and treatment on developing fetuses is vital for informing clinical care decisions of pregnant women. The results of this study suggest that there are no large effects of maternal psychiatric illness or depressive symptoms on neonatal DNA methylation though we cannot rule out subtle effects. The potential role of antidepressant exposure on neonatal methylation of *TNFRSF21* and *CHRNA2*, as well as the role of these genes on long-term behavior and neurocognitive development, warrant further attention, though, as it was noted, these effects are quite subtle.

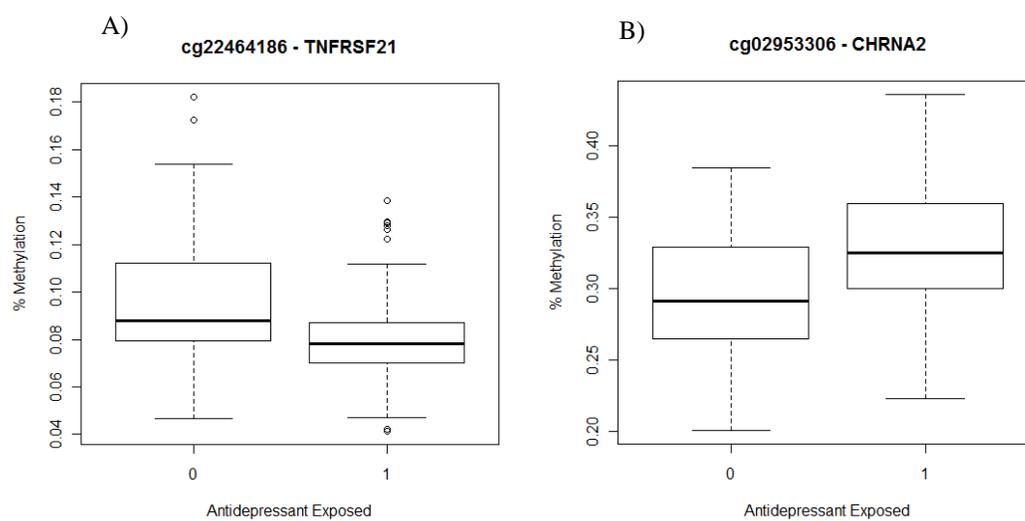
**Table 3-1.** Demographic and clinical characteristics of the WMHP subjects.

<b>Demographics</b>		<b>N (%)</b>
		<b>Total N=201</b>
<b>Sex</b>		
Male		100 (49.8)
Female		101 (50.2)
<b>Child Race</b>		
Caucasian		183 (91)
African-American		18 (9)
<b>Medications</b>		
No Antidepressants		50 (24.9)
All Antidepressants		151 (75.1)
Class 1 (SSRIs)		132 (65.7)
Class 2 (Bupropion)		40 (19.9)
Atypical Antipsychotics		32 (15.9)
Hypnotics		31 (15.4)
Benzodiazepines		39 (19.4)
Antiemetics		41 (20.4)
<b>Diagnosis and Symptoms</b>		
Lifetime MDD		146 (72.6)
Current MDD		118 (58.7)
Lifetime BPD		55 (27.4)
Current BPD		50 (24.9)
Other Current Diagnoses		33 (16.4)
Major Depressive Episode		86 (42.8)
Maximum Prenatal HRSD17		178
<15		69 (38.8)
≥15		109 (61.2)
Maximum Prenatal BDI		179
<10		54 (30.2)
≥10		125 (69.8)

\*Patient data was not available for all measures including 18 missing for Class 2 antidepressants and benzodiazepine, 20 for atypical antipsychotics and antiemetics, 16 for hypnotics, 23 for HRSD17, and 22 for BDI. The following abbreviations were used: major depressive disorder (MDD), all bipolar disorders (BPD), bipolar disorder 1 (BP1), bipolar disorder 2 (BP2) and bipolar – other (BPO). Other current diagnoses included schizophrenia (1), substance abuse (2), anxiety (29), and binge eating (1). Lifetime BPD

includes BP1 (78.2%), BP2 (12.7%) and BPO (9.1%), and current BPD has similar proportions.

**Figure 3-1.** Methylation of significant CpG sites. Methylation levels for probes: A) cg22464186 (*TNFRSF21*) and B) cg02953306 (*CHRNA2*) associate with exposure to any antidepressant.



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## Chapter 4

### Neonatal DNA Methylation Patterns Associate with Gestational Age

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## Introduction

The majority of deliveries in the United States occurs between 37 and 42 weeks gestational age (GA) and are classified as term deliveries. Whereas there is uniform agreement that preterm delivery (GA <37 weeks) associates with significantly higher rates of neonatal morbidity and mortality<sup>1</sup>, recent evidence also demonstrates an increased risk for adverse neonatal outcomes in term deliveries, particularly respiratory complications, with declining gestational age.<sup>2,3</sup> A higher rate of morbidity and mortality during the first year of life has been reported for neonates born at 37 or 38 weeks compared to those delivered at 40 weeks.<sup>4</sup> The importance of gestational age in determining neonatal outcome has been repeatedly emphasized and refined as reflected by the stringent American College of Obstetrics & Gynecology guidelines for elective induction.<sup>5</sup> Many reports suggest that a reduction in the rate of elective deliveries prior to 39 weeks reduces neonatal morbidity and mortality.<sup>6,7</sup>

While the mechanisms responsible for associations between GA and these adverse conditions remain unknown, an emerging body of evidence supports the role of epigenetic alterations, such as changes in DNA methylation, as molecular mediators of adverse postnatal phenotypes.<sup>8</sup> For example, evidence suggests that maternal nutrition can have dramatic effects on neonatal outcomes by altering offspring DNA methylation.<sup>9</sup>

To date most studies of neonatal DNA methylation have focused on premature or small for gestational age neonates.<sup>10-12</sup> Genome-wide DNA methylation in healthy, full-term neonates has not yet been examined. Therefore, despite reports of DNA methylation varying as adults age,<sup>13-16</sup> it remains unknown whether methylation varies as a function of GA. The current study addresses this critical gap in the literature,

examining genome-wide patterns of DNA methylation in umbilical cord blood from neonates of gestational ages ranging from 32–43 weeks, spanning the clinically significant periods of late preterm, term, and postdate pregnancies.

## **Methods**

### *Subjects and Sample Collection*

#### Discovery Cohort: Women’s Mental Health Program (WMHP):

Women with a history of neuropsychiatric illnesses who participated in prospective studies through Emory’s WMHP were screened for inclusion in the current study as stated previously. Mothers completed an intake questionnaire for demographic, socioeconomic, and medical and psychiatric history, were administered the Structured Clinical Interview for *DSM-IV* (SCID), and then evaluated prospectively at four to six week intervals to assess psychiatric symptoms and pharmacologic exposures throughout pregnancy.

We evaluated 259 DNA samples collected in the WMHP from neonates born to Caucasian and African-American women (Table 4-1). The inclusion criteria were: A) maternal age >17 years of age; B) maternal written and verbal fluency in English; C) a live singleton delivery; and D) availability of DNA from umbilical cord blood collected at delivery. The exclusion criteria used were described in Chapter 3.

#### Replication Cohort: Conditions Affecting Neurocognitive Development & Learning in Early Childhood (CANDLE):

Neonates were selected from CANDLE, a longitudinal cohort study of human development from pregnancy to age three being performed in Shelby County, Tennessee

(Table 4-1). Solicitation for inclusion occurs by advertising in local gynecological clinics, and 1303 women and their infants have been enrolled in the study. Maternal selection criteria for this analysis included: A) maternal age 18-40 years; B) singleton pregnancy; C) availability of birth weight and maternal pre-pregnancy weight; D) absence of pregnancy complications, specifically maternal sexually-transmitted disease, maternal diabetes mellitus, oligohydramnios, preeclampsia, placental abruption, and cervical cerclage; and E) availability of DNA from umbilical cord blood collected at delivery. Additionally, to make direct comparisons with the discovery cohort, we restricted analyses to Caucasian or African-American neonates. Based on the above criteria and availability of DNA methylation data, 194 neonates were included in this study. The parents of these 194 neonates were more likely to be married or cohabitating (66.0% vs. 55.5%;  $p < .001$ ), have received a high school diploma or beyond (65.9% vs. 39.8%;  $p < .001$ ), and have incomes  $>200\%$  of the poverty level (53.8% vs. 43.7%;  $p < .01$ ) when compared to the overall CANDLE cohort. The neonates were also less likely to be African American (56.7% vs. 67.7%;  $p < .01$ ) and had a higher gestational age on average (39.0 vs. 38.7 weeks,  $p = .03$ ).

All mothers provided written informed consent prior to study enrollment following procedures approved by the Institutional Review Boards of Emory University (WMHP) or University of Tennessee Health Science Center (CANDLE). This study was conducted in accordance with the Helsinki Declaration of 1975.

### *Gestational Age and Birth Weight*

In the discovery cohort GA was estimated by each mother's obstetrician for all but three subjects (1.2%). For these, GA was determined by the time in weeks between the mother's last menstrual period (LMP) and delivery. Cook's distance was calculated to confirm that these 3 subjects did not influence the association between CpG sites and GA (Figure 4-3). In the replication cohort, a combination of obstetrician report (60%) or LMP (40%) was used to estimate GA. Birth weight in kilograms was assessed at delivery and extracted from the medical records of both cohorts.

### *DNA Extraction and Methylation Analysis*

WHMP (discovery cohort) umbilical cord blood samples were collected and DNA extracted as stated above. For the CANDLE study (replication cohort), whole umbilical cord blood samples were stored at 4<sup>0</sup>C and processed within 24 hours of delivery. DNA was isolated using a Maxwell 16 (Promega Corp.) automated nucleic acids extractor. The resulting DNA was processed and run on the HumanMethylation27 BeadChips (Illumina) from which  $\beta$  values were obtained as discussed previously.

### *Statistical Analysis*

#### Discovery Cohort

As stated previously, all analyses were based on linear models with  $\log\left(\frac{\beta}{1-\beta}\right)$  as the outcome and GA as the independent variable. In addition to the covariates discussed in Chapter 2, the gestational age analyses were also adjusted for parity, percentile birth weight with respect to GA and sex, maternal hypertension (chronic, pregnancy-induced,

and/or preeclampsia), and experiment. In the discovery cohort, the correlation between percentile birth weight and GA is substantially less ( $r=.15$ ) than the correlation between birth weight and GA ( $r=.49$ ). We also evaluated the potential effects of maternal psychiatric diagnosis and symptoms, maternal age, preconception and delivery body mass index (BMI), and method of delivery (standard and assisted vaginal delivery and planned or emergency cesarean section) as well as the area under the curve for the number of weeks of exposure to caffeine, alcohol, or tobacco on DNA methylation. None of these analyses yielded significant results and therefore these factors were not included as covariates.

Gene Ontology analysis was conducted using GeneCodis 2.0<sup>37,38</sup> for the 39 genes that demonstrated evidence of differential methylation in the discovery cohort to evaluate if specific biological processes were enriched in the dataset. This web-based application matches gene lists with common biological feature annotations. Significance is determined by enrichment of the genes of interest in the context of the known annotations, and a hypergeometric p-value is obtained through an FDR correction.

### Replication Cohort

Only the CpG sites that met experiment-wide significance in the discovery analysis were evaluated in the replication cohort. For each CpG site, we again regressed  $\log\left(\frac{\beta}{1-\beta}\right)$  on GA adjusting for neonatal sex, race, parity, maternal hypertension, and percentile birth weight, as well as a random effects term to allow for potential variation between batches. Similar to the results from the discovery cohort, percentile birth weight was less correlated with GA than birth weight was with GA ( $r=.10$  vs.  $r=.51$ ). Because

this was a replication study, we performed a one-sided test ( $\alpha=.05$ ) for each CpG site to test for associations between GA and methylation in the same direction as observed in the discovery cohort.

## Results

The discovery and replication cohorts were similar in terms of the distributions of GA at delivery, birth weight percentile, and parity (Table 4-1). In contrast, the discovery cohort had a higher proportion of female neonates, Caucasian neonates, women with prenatal hypertension, and a higher mean maternal age at delivery.

Forty-one CpG sites in 39 genes met experiment-wide criteria for significant association between neonatal DNA methylation and GA in the discovery cohort ( $FDR<.05$ ; Table 4-2; Figures 4-1, 4-2). Of these, 29 sites (70.7%) showed a decrease in methylation levels with increasing GA, while the remaining 12 (29.3%) showed an increase.

To further examine the potential functional significance of these results, we used gene ontology classifications to explore known biological processes for the 39 genes associated with GA (Table 4-3). Of the six biological processes implicated, three specifically involved pregnancy and development-related genes, while three involved more general cellular processes including transcriptional regulation, cell adhesion, and signal transduction.

We sought to replicate our initial findings from the discovery cohort in an independent replication cohort. Of the 41 CpG sites examined in the replication cohort, 26 sites in 25 genes were also associated with GA ( $1.6 \times 10^{-6} \leq p < .05$ ; Table 4-2). Among

these, we observed differential methylation of *AVP*, *OXT*, *CRHBP*, *CASP8* and *ESR1*, which are enriched in signal transduction and response to estradiol stimulus (Table 4-3).

It is not clear to what extent the onset of labor may have influenced these results. Therefore, we compared those born by spontaneous vaginal deliveries to those born by cesarean sections or induced vaginal deliveries in both cohorts. After comparing spontaneous to induced delivery types in an interaction analysis (data not shown), there was no evidence that the association between GA and methylation differed by delivery type (FDR<.05) for the 41 CpG sites differentially methylated in the discovery cohort. Also, there were no nominally significant differences (p<.05) consistent between the discovery and replication cohorts.

## **Discussion**

Using a genome-wide approach with replication in an independent cohort, we provide evidence for an association between GA at delivery and the differential methylation of CpG sites in 25 genes. Interestingly, several genes exhibiting GA-associated differences in methylation encode proteins that play putative roles in the timing of delivery or as regulators of postnatal outcomes known to associate with differences in GA.

Notably, we observed decreased methylation of the paralogs arginine vasopressin (*AVP*) and oxytocin (*OXT*). Maternal AVP and OXT impact the timing of delivery through the regulation of uterine contractions.<sup>17</sup> Estrogen has a pivotal role in the regulation of both AVP and OXT by altering DNA methylation in the promoter regions of *AVP* and *OXT*.<sup>18, 19</sup> Peripherally, AVP regulates renal water excretion and hemostasis,

in part through regulating von Willebrand factor (vWF) activity, and<sup>20</sup> we observed differential methylation of CpG sites in glycoprotein IX (*GP9*), a component of the vWF receptor.<sup>21</sup>

We also observed a decrease in the methylation of corticotropin releasing hormone binding protein (*CRHBP*). Maternal corticotropin releasing hormone (*CRH*) increases consistently throughout pregnancy but is neutralized by the binding of *CRHBP*,<sup>22</sup> which is released from the placenta.<sup>23</sup> *CRHBP* decreases dramatically towards the end of the third trimester releasing *CRH* prior to the onset of parturition.<sup>24</sup>

We observed increased methylation of estrogen receptor 1 (*ESR1*), which is largely increases prior to labor,<sup>25, 26</sup> although *ESR1* decreases in the lower uterine segment as labor progresses.<sup>27</sup> Estrogen in umbilical cord blood positively correlates with birth weight,<sup>28</sup> and neonates in both the discovery and replication cohorts were, on average, below the 50<sup>th</sup> percentile for gestational age-corrected birth weight. *ESR1* is activated by solute carrier family 30, member 9 (*SLC30A9* a.k.a. *GAC63*),<sup>29</sup> which is also differentially methylated in this study.

These results also reveal differential methylation of genes that have not previously been implicated in timing of labor and delivery but could contribute to the immunoendocrine axis and the development of health problems later in life. We observed differential methylation of CpG sites in genes that play a role in thyroid hormone synthesis (dual oxidase 2; *DUOX2*),<sup>30</sup> immune cell maturation (transmembrane protein 176A; *TMEM176A*),<sup>31</sup> and apoptosis (caspase 8; *CASP8*).<sup>32</sup> We hypothesize that fetal or neonatal DNA methylation of these genes may contribute to long-term

developmental outcomes, but further study is needed to address the stability of DNA methylation changes during subsequent developmental periods and in adulthood.

Several of the CpG sites differentially methylated in the discovery cohort did not replicate. Potential explanations for this include: 1) the initial associations in the discovery cohort were unreliable; 2) methylation patterns of some genes may be unique to the offspring of cohorts with neuropsychiatric illnesses; 3) differences in the umbilical cord blood processing procedures between the cohorts (<2 hours vs. <24 hours) may lead to differences in DNA quality and 4) methods to calculate GA may vary. Because maternal stress or medication use may increase the risk of preterm delivery,<sup>33,34</sup> it is unclear to what degree the observed methylation differences between findings in the discovery and replication samples reflect differences in maternal neuropsychiatric status or treatment. While the potential effects of maternal stress and medication warrant investigation, the replication of these findings in a distinct replication cohort provide further support for their association with GA. In the discovery cohort GA was estimated largely using obstetrician reports, which are primarily supported by an ultrasound, where GA at delivery in the replication cohort was estimated predominately using LMP. While gestational age determined by ultrasound correlates well with LMP, it can vary by some maternal and infant characteristics such as maternal age or low birth weight.<sup>35</sup> In the discovery cohort, we evaluated the correlation between GA determined by obstetrician report and LMP ( $r=.68$ ) and found that the estimates differed by 3.6 days on average. These subtle variations in gestational age estimates may have influenced the number of replicated findings.

This study was also limited by reliance on DNA extracted from whole umbilical cord blood. Thus, our results cannot be attributed to differences in specific cell lineages. It is also not clear whether the methylation changes observed reflect a causal mechanism or are merely observational. We examined the GA-associated DNA methylation patterns for the 41 original differentially methylated CpG sites in neonates born following natural labor compared to those who did not reach term because of induction of labor or a planned cesarean section and observed no differences in the relationship between GA and methylation for these delivery groups after correcting for multiple comparisons. It is unclear whether elective inductions and/or cesarean sections occurred after onset of labor. As such, future studies will be required to delineate these effects. The results of the gene ontology analysis should be interpreted with caution as the independent assumptions of the model may result in inflated test statistics.<sup>36</sup> However, as an initial examination in combination with a replication in a distinct cohort, these novel data warrant additional attention. While replication is not yet standard in large-scale studies of DNA methylation, the design of this study underscores the potential generalizability of these results.

This study suggests that DNA methylation patterns in umbilical cord blood vary continuously across a range of gestational ages. The results have broad implications warranting further investigations in preterm deliveries and the role(s) of DNA methylation in the relationship between gestational age and detrimental health outcomes. Future DNA methylation studies should include a wider range of gestational ages and incorporate longitudinal assessments of the stability of DNA methylation in neonatal and postnatal human DNA.

**Table 4-1.** Demographic and clinical characteristics of the discovery and replication cohorts.

Phenotype	Discovery Cohort N=259		Replication Cohort N=194		p-value*
	N (%)	Mean+SD	N (%)	Mean+SD	
Maternal Age		33.2 (5.2)		26.7 (5.2)	<.0001
Parity		0.9 (1.0)		1.1 (1.2)	NS
Sex					
Male	128 (49.0)		101 (52.1)		.635
Female	131 (51.0)		93 (47.9)		
Child Race					
Caucasian	232 (89.6)		84 (43.3)		<.0001
African Am.	27 (10.4)		110 (56.7)		
GA, weeks*					
<37	18 (6.9)	38.8 (1.2)	12 (6.1)	39.0 (1.3)	NS
37-41	240 (92.7)		182 (93.8)		
>42	1 (0.4)		0 (0.0)		
Birth Weight Percentile		47.6 (27.2)		47.1 (26.6)	NS
Hypertension During Pregnancy	32 (12.4)		10 (5.2)		.0088

\* The rate of preterm delivery is higher in both the overall discovery and replication cohorts than is represented in this sample, which were selected to over-represent term deliveries.

+ NS = non-significant

**Table 4-2.** CpG sites reaching experiment-wide significance in the discovery cohort compared to the replication cohort results.

Probe	Associated Gene	Discovery t-score	Discovery p-value	Replication t-score	Replication p-value
<b>Decreased Methylation in Both Cohorts</b>					
cg16536918	AVP	-5.39	$1.9 \times 10^{-7}$	-3.81	$9.4 \times 10^{-5}$
cg06051311	TRIM15	-5.08	$7.9 \times 10^{-7}$	-4.81	$1.6 \times 10^{-6}$
cg21842274	CRHBP	-4.95	$1.5 \times 10^{-6}$	-2.36	$9.7 \times 10^{-3}$
cg20994801	PIK3CD	-4.57	$8.3 \times 10^{-6}$	-1.80	.037
cg14409083	EMP1	-4.52	$1.0 \times 10^{-5}$	-2.36	$9.6 \times 10^{-3}$
cg25551168	AVP	-4.52	$1.0 \times 10^{-5}$	-4.22	$2.0 \times 10^{-5}$
cg13813391	CMTM2	-4.41	$1.6 \times 10^{-5}$	-2.22	.014
cg26267561	OXT	-4.35	$2.1 \times 10^{-5}$	-2.38	$9.1 \times 10^{-3}$
cg20291222	CAPS2	-4.33	$2.3 \times 10^{-5}$	-2.12	.018
cg14423778	MBNL1	-4.28	$2.9 \times 10^{-5}$	-1.81	.036
cg22417398	SCYL1	-4.26	$3.0 \times 10^{-5}$	-1.91	.029
cg01143454	C20orf141	-4.22	$3.6 \times 10^{-5}$	-4.31	$1.3 \times 10^{-5}$
cg09244244	KIAA0372	-4.21	$3.8 \times 10^{-5}$	-3.09	$1.2 \times 10^{-3}$
cg15561986	POMT2	-4.20	$4.0 \times 10^{-5}$	-2.15	.017
cg09523691	ATG12	-4.10	$5.7 \times 10^{-5}$	-1.89	0.03
cg10652277	SLC30A9	-4.03	$7.7 \times 10^{-5}$	-2.25	.013
cg26799474	CASP8	-4.01	$8.2 \times 10^{-5}$	-3.45	$3.46 \times 10^{-4}$
cg00411097	MGC9712	-4.01	$8.2 \times 10^{-5}$	-2.78	$3.1 \times 10^{-3}$
cg16301617	TMC6	-4.00	$8.6 \times 10^{-5}$	-2.16	.016
<b>Decreased Methylation in Discovery Cohort Only</b>					
cg20337106	C6orf139	-5.18	$4.9 \times 10^{-7}$	-1.4	.081
cg04536922	FAM13A1	-4.41	$1.6 \times 10^{-5}$	-1.07	.14
cg22854223	CD82	-4.42	$1.6 \times 10^{-5}$	-1.54	.063
cg16545105	CRHBP	-4.33	$2.2 \times 10^{-5}$	-0.66	.25
cg05664072	PER2	-4.33	$2.3 \times 10^{-5}$	-0.57	.29
cg17530977	GLI3	-4.30	$2.6 \times 10^{-5}$	0.88	.19
cg13393195	IQCD	-4.23	$3.5 \times 10^{-5}$	-0.089	.47
cg08996521	CISH	-4.12	$5.3 \times 10^{-5}$	-0.38	.35
cg03395898	TGFB3	-4.06	$6.8 \times 10^{-5}$	-1.27	.1
cg14011734	TATDN2	-3.98	$9.3 \times 10^{-5}$	-0.02	.49

Table 4-2. (cont).

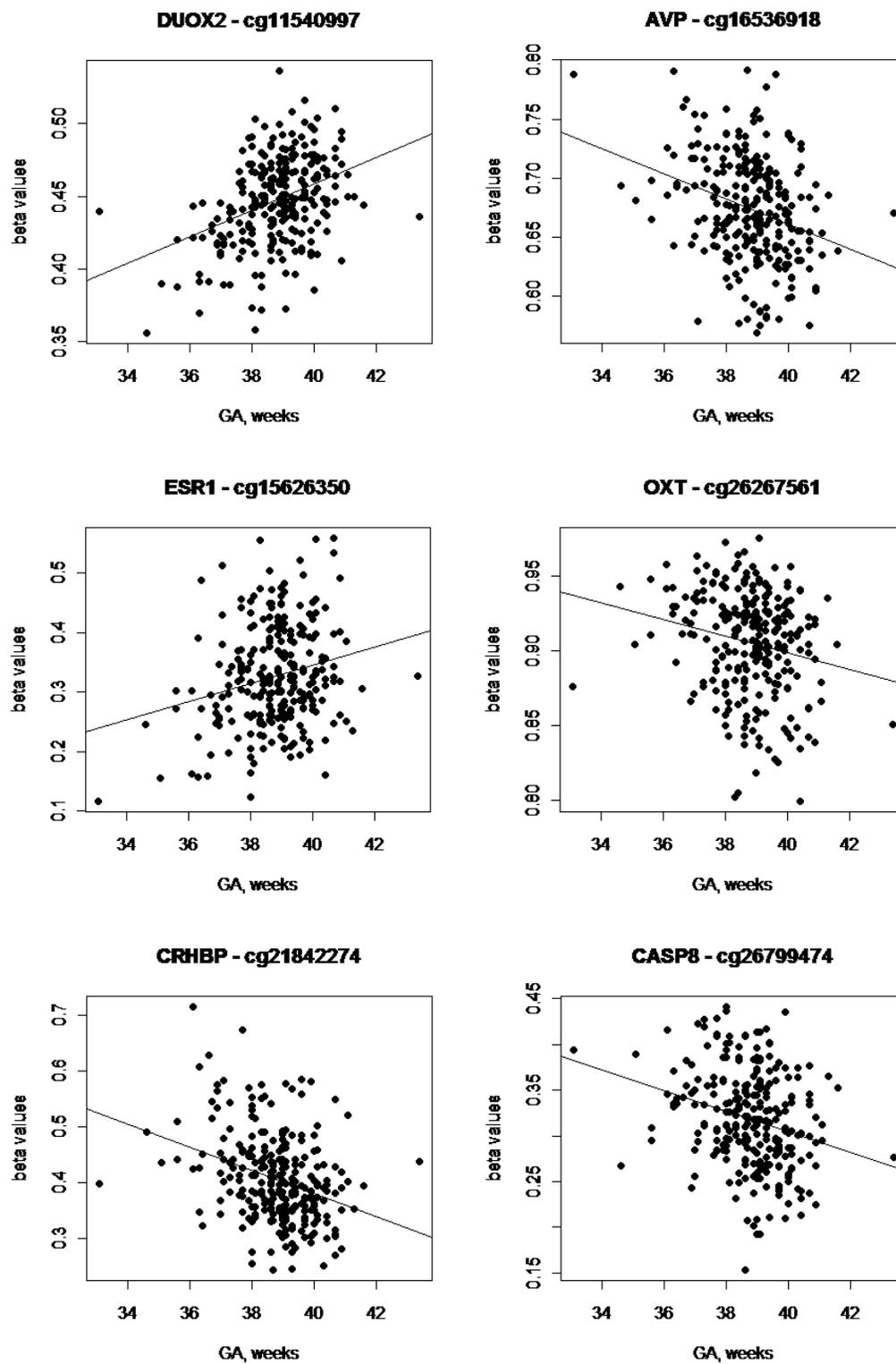
Probe	Associated Gene	Discovery t-score	Discovery p-value	Replication t-score	Replication p-value
<b>Increased Methylation in Both Cohorts</b>					
cg11540997	DUOX2	5.46	$1.3 \times 10^{-7}$	2.78	$3.0 \times 10^{-3}$
cg16098726	GP9	5.21	$4.5 \times 10^{-7}$	2.67	$4.2 \times 10^{-3}$
cg05294455	MYL4	5.16	$5.4 \times 10^{-7}$	4.03	$4.2 \times 10^{-5}$
cg27210390	TOM1L1	5.02	$1.1 \times 10^{-6}$	3.45	$3.5 \times 10^{-4}$
cg26385222	TMEM176A	4.86	$2.3 \times 10^{-6}$	3.29	$6.1 \times 10^{-4}$
cg15626350	ESR1	4.24	$3.2 \times 10^{-5}$	2.36	$9.8 \times 10^{-3}$
cg00594952	RIMS3	4.06	$6.8 \times 10^{-5}$	3.58	$2.2 \times 10^{-4}$
<b>Increased Methylation in Discovery Cohort Only</b>					
cg03098721	TLL7	5.26	$3.4 \times 10^{-7}$	1.06	.15
cg01919208	LAMB2	4.19	$4.1 \times 10^{-5}$	-0.25	.4
cg12188416	TP73L	4.13	$5.2 \times 10^{-5}$	0.64	.26
cg12564962	DSCR6	4.06	$7.0 \times 10^{-5}$	0.23	.41
cg05726109	GP1BB	4.02	$8.0 \times 10^{-5}$	1.21	.11

**Table 4-3.** Co-occurring gene ontology results for the 41 experiment-wide significant genes in the discovery cohort. Gene names in bold associated with GA in the replication cohort.

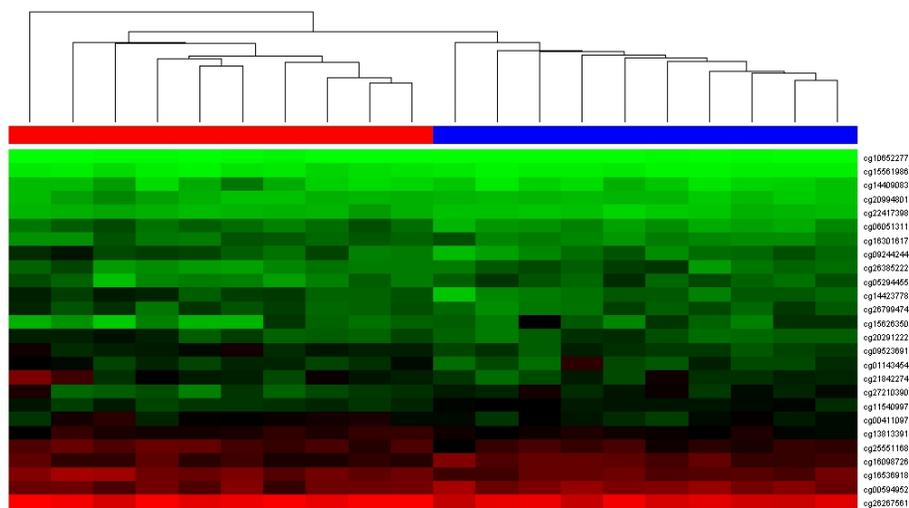
<b>Annotations</b>	<b>GO Term</b>	<b>Corrected p-value*</b>	<b>Genes</b>
Inner ear development	GO:0048839	$2.78 \times 10^{-5}$	<i>GLI3, TGFB3, DUOX2</i>
Female pregnancy	GO:0007565	$5.66 \times 10^{-4}$	<i>TGFB3, CRHBP, OXT</i>
Response to estradiol stimulus	GO:0032355	$5.86 \times 10^{-4}$	<i>ESR1, OXT, CASP8</i>
Heart development	GO:0007507	.0022	<i>CASP8, GLI3, OXT</i>
Blood coagulation & Platelet activation	GO:0007596 & GO:0030168	.0057	<i>TGFB3, GP1BB, GP9</i>
Signal transduction	GO:0007165	.0056	<i>ESR1, FAM13A1, CRHBP, AVP, PIK3CD, OXT</i>
DNA-dependent transcription	GO:0006351	.0076	<i>GLI3, TP73L, ESR1, SLC30A9</i>
Positive regulation of transcription	GO:0045944	.0107	<i>TGFB3, GLI3, TP73L</i>
<i>In utero</i> embryonic development	GO:0001701	.0027	<i>TGFB3, GLI3, MBNL1</i>
Negative regulation of transcription from RNA polymerase II promoter	GO:0000122	.013	<i>GLI3, TP73L, PER2</i>
Positive regulation of transcription from RNA polymerase II	GO:0045944	.022	<i>TGFB3, GLI3, TP73L</i>
Cell adhesion	GO:0007155	.033	<i>GP1BB, GP9, LAMB2</i>

\*p-values indicate the significance between the number of genes differentially methylated in this analysis and the total number of genes in each annotated biological process.

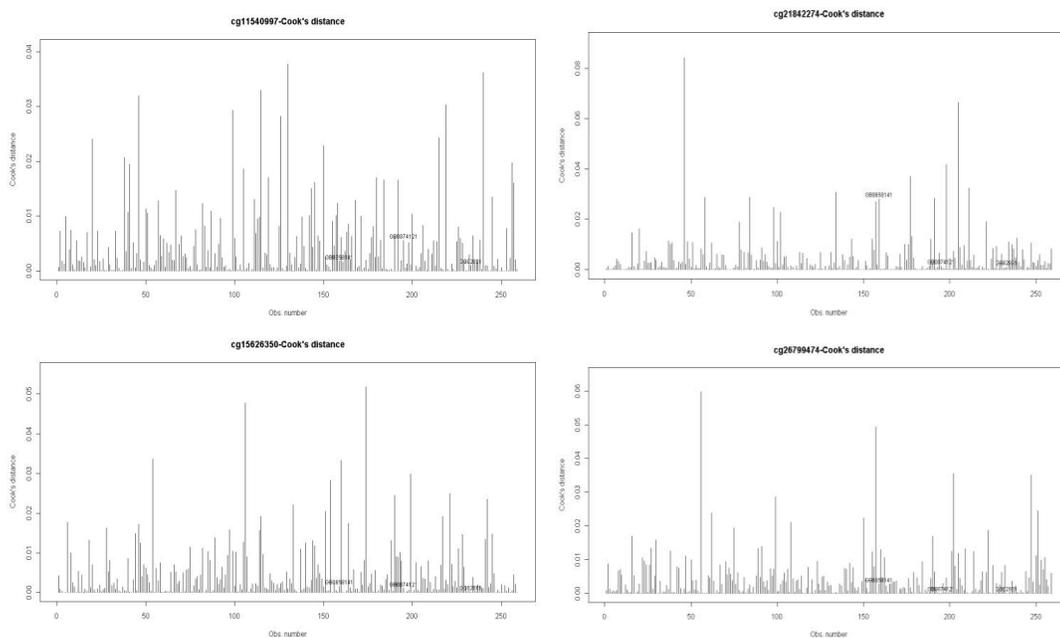
**Figure 4-1.** Scatterplots of representative CpG sites associated with GA with regression lines.



**Figure 4-2.** Heat map of beta values from subjects with the lowest (red; N=20) and highest (blue; N=20) gestational ages from the discovery cohort.



**Figure 4-3.** Samples in the discovery cohort that used LMP to determine GA do not significantly differ from those based on obstetrician report. The Cook's distance (vertical axis) is plotted for each sample (horizontal axis) to determine the overall contribution of each sample to the regression model.



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## **Chapter 5**

### **Summary and Conclusions**

Prenatal exposure to environmental stressors has been associated with the development of adverse neonatal and later-life conditions. It has been proposed that the associated outcomes result from a “programming” effect initiated by these prenatal exposures. An epigenetic candidate for these programming effects is differential methylation of cytosines in CpG dinucleotides. The objective of this study was to examine whether DNA methylation of a genome-wide set of CpGs from umbilical cord blood DNA associates with either child outcomes or prenatal environmental exposures in a prospectively-followed cohort of women receiving treatment for psychiatric illnesses. We hypothesized that each of the prenatal exposures and child outcomes would associate with DNA methylation both in genes previously reported with those associations and novel genes.

We began this study using the HumanMethylation27 BeadChip (Illumina) to ascertain whether DNA methylation from neonates whose mothers were being treated for psychiatric illness during pregnancy associated with maternal psychiatric diagnosis or depressive symptoms. Two previous candidate genes studies reported associations between maternal depressive symptoms and differential DNA methylation in the glucocorticoid receptor (*NR3C1*) and serotonin transporter (*SLC6A4*), two genes involved in stress-related responses. However, this study showed no associations between neonatal DNA methylation of any CpG locus interrogated and measures of maternal psychiatric diagnosis or depressive symptoms during pregnancy. Our study had >80% power to detect group differences explaining >14.5% of variation in methylation at a single CpG at a conservative Bonferroni significance level. At a significance level of  $p=.05$ , we had 90-99% power to replicate of the previous findings of the *NR3C1* and

*SLC6A4* genes. We observed no evidence to suggest that maternal depressive symptoms or anti-depressant medication use during pregnancy associate with differences in methylation at either *NR3C1* or *SLC6A4*, though we could not replicate the exact CpG loci previously reported. It is worth noting here that both prior “positive” claims of association between differences in methylation at candidate genes and maternal depression or medication use were focused candidate gene studies in which modest p-values were interpreted as significant. In addition, the absolute magnitudes of the claimed differences were quite small. It is tempting to speculate that as genome-wide approaches to DNA methylation analysis continue to develop, single-locus findings will be subject to the same high degree of skepticism to which single-SNP genetic associations are subject in the context of genome-wide association results.

We next examined whether there was an association between antidepressant exposure and neonatal DNA methylation in the same cohort. Previous studies have reported adverse outcomes that were associated with antidepressant exposure, but studies examining the associations between antidepressant exposure and neonatal DNA methylation have yielded negative results. In this study, antidepressants were examined in two categories: “serotonergic” (i.e., medications that act predominantly by altering serotonergic neurotransmission, usually by antagonizing the presynaptic serotonin transporter) and “non-serotonergic” (i.e., medications thought to act by influencing norepinephrine or dopamine more than serotonin). While exposure to these two categories individually showed no association with DNA methylation, a combined analysis of both categories revealed differential methylation of CpG sites in 2 genes: tumor necrosis factor receptor subfamily 21 (*TNFRSF21*) and cholinergic receptor,

nicotinic, alpha 2 (*CHRNA2*). It is noteworthy that though these two sites reached the significance threshold for false discovery rate, they did not reach significance for the more conservative Bonferroni criteria. While these two findings are of note and warrant replication and follow-up studies, our results generally did not support the hypothesis that neonatal DNA methylation would associate with antidepressant exposure.

In summary, the analyses of neonatal DNA methylation and maternal depression and treatment were generally negative, with the exceptions discussed above. As stated previously, these results suggest that possible associations between maternal depression and treatment and their respective adverse conditions later in life could be due to genetic variants that have yet to be characterized. Additionally, such outcomes could be facilitated by other epigenetic mechanisms such as histone modifications that, although they interact with DNA methylation, are nevertheless not influencing differential methylation significantly in umbilical cord blood. It could also be the case that DNA methylation at genes or CpG loci not interrogated by the HumanMethylation27 BeadChip used in this study are the vehicles for these methylation “programming” marks. This conjecture will require a more exhaustive examination of DNA methylation by another chip or method in order to be adequately tested. Finally, though our analyses were well powered to detect effects of similar size to those previously reported, differential DNA methylation with effects smaller than this threshold could theoretically exert a biologically-significant influence on gene regulation. Alternatively, effects could be larger in other relevant tissues or cell-types. Finally, small differences in methylation could increase as methylation continues to diverge after delivery to levels that later become biologically significant. In the future, this study could be duplicated using

animal models, where prenatal exposures to maternal depression-like symptoms and antidepressants could be separated, removing a perpetual complication in comparable human studies.

Finally, we tested whether there were any associations between DNA methylation and estimated gestational age. Previous studies, both genome-wide and candidate gene, have consistently reported that age associates with differential methylation. In our analysis, 41 CpG sites in 39 genes associated with gestational age. Data from an independent, community cohort of neonates replicated associations between differential methylation and gestational age in 26 of these original CpG sites from 25 genes. It is unlikely that such a high rate of replication is due to chance. Many of the genes exhibiting gestational-age-associated differential methylation are known to be involved in pregnancy and delivery, notably *AVP*, *OXT*, *CRHBP* and *ESRI*. Other genes from this group have known involvements in adverse outcomes later in life (e.g. *DUOX2*, *TMEM176A* and *CASP8*), though the children from this cohort have not been assessed for such outcomes.

Our studies provide novel insights into the associations between a genome-wide set of CpG loci and perinatal influences using umbilical cord blood DNA in a prospectively-followed cohort. A clinically-important area for future investigation is to determine whether the same genes we examined in largely term cohorts also show differential methylation in pre-term infants. The methylation trends seen in this study suggest that methylation at the implicated genes varies proportionally to gestational age, and suggest a possible continuation of increased or decreased methylation in earlier gestational ages that could contribute to phenotypes associated with prenatal births. An

effort to correlate the methylation patterns seen in our gestational age analysis with maternal methylation patterns could also reveal potential readily-accessible future biomarkers for predicting gestational ages, or health outcomes associated with variation in gestational age. A parallel study in a preterm cohort could likewise produce possible biomarkers for predicting preterm births, which would be a valuable clinical tool. These data will also be of use in future prospective studies that assess the methylation patterns of genes reported here in an attempt to discern any epigenetic “programming” effects associated with prenatal stress and exposures. For example, these genes associated with gestational age suggest specific directions for follow-up studies of children in this and other prospective cohorts with regard to health conditions known to associate with gestational age, such as immunological and behavioral complications. If available, this line of research could also include post-mortem brain and other tissue samples from individuals with accessible birth records. Likewise, animal models could provide a more practical way to contribute to the characterization of future impacts of relative gestational age potentially mediated through DNA methylation at these genes.

The results of this study provide novel insights into the dynamics of neonatal DNA methylation in a genome-wide set of CpG loci from umbilical cord blood DNA, providing the largest snapshot of the influence of prenatal exposures on neonatal DNA methylation patterns to date. While the two genes found to be associated with any antidepressant exposure should be examined further, the bulk of our results concerning the influence of maternal psychiatric illness and treatment on neonatal DNA methylation were negative. This suggests that any causal influence these exposures have on later life conditions could be mediated through as yet undiscovered genetic variants or other

epigenetic mechanisms. Concerning the first scenario, it remains possible that these genetic variants underlie or are otherwise correlated with the maternal psychiatric illness because of the difficulty of disentangling the occurrence of depressive diagnosis and symptoms with antidepressant exposure (as discussed previously). Our analysis of DNA methylation and gestational age extend studies of the relationship between age and methylation to the *in utero* period. As mentioned previously, the genes found to associate with gestational age should be examined further with regards to both preterm deliveries and postnatal health outcomes. Additionally, the entire set of 27,578 loci examined here could be used in conjunction with corresponding methylation data in older cohorts to study the progression of methylation patterns across the human lifespan.