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Genome-wide DNA methylation in neonates exposed to maternal depression, anxiety, or SSRI medication during pregnancy

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Abbreviations: BMI, body mass index; BWH, Brigham and Women's Hospital; CI, confidence interval; DMR, differentially methylated region; FDR, false discovery rate; GO, gene ontology; SE, standard error; SES, socioeconomic status, SSRI, selective serotonin reuptake inhibitor

Despite the high prevalence of depression, anxiety, and use of antidepressant medications during pregnancy, there is much uncertainty around the impact of high levels of distress or antidepressant medications on the developing fetus. These intrauterine exposures may lead to epigenetic alterations to the DNA during this vulnerable time of fetal development, which may have important lifetime health consequences. In this study we investigated patterns of genome-wide DNA methylation using the Illumina Infinium Human Methylation450 BeadChip in the umbilical cord blood of neonates exposed to non-medicated maternal depression or anxiety (n = 13), or selective serotonin reuptake inhibitors (SSRIs) during pregnancy (n = 22), relative to unexposed neonates (n = 23). We identified 42 CpG sites with significantly different DNA methylation levels in neonates exposed to non-medicated depression or anxiety relative to controls. CpG site methylation was not significantly different in neonates exposed to SSRIs relative to the controls, after adjusting for multiple comparisons. In neonates exposed either to non-medicated maternal depression or SSRIs, the vast majority of CpG sites displayed lower DNA methylation relative to the controls, but differences were very small. A gene ontology analysis suggests significant clustering of the top genes associated with non-medicated maternal depression/anxiety, related to regulation of transcription, translation, and cell division processes (e.g., negative regulation of translation in response to oxidative stress, regulation of mRNA export from the nucleus, regulation of stem cell division). While the functional consequences of these findings are yet to be determined, these small DNA methylation differences may suggest a possible role for epigenetic processes in the development of neonates exposed to non-medicated maternal depression/anxiety.

Introduction

Depression and anxiety disorders are common in pregnant women, with an estimated prevalence of 7–18% for depressive,[1] and 8.5% for generalized anxiety disorders[2] during pregnancy. The impact of maternal depression and anxiety on the developing fetus is yet unknown, and remains a critical question in the current debate over treatment of depression during pregnancy. Symptoms of depression or anxiety during pregnancy have been associated with higher rates of adverse birth outcomes, including preterm births,[3] low birth weight babies,[4] and postnatal growth delays.[5] Children of depressed or anxious mothers are also more likely to develop depression and anxiety in adulthood.[6]

To reduce depressive symptoms during pregnancy, up to 8% of women in the US are prescribed antidepressant medications during pregnancy.[7,8] However, exposure to antidepressants during pregnancy has also been associated with poor birth outcomes, including lower birth weight,[8,9] lower Apgar scores,[10] or even withdrawal symptoms from antidepressants after birth[11] (though not all studies report significant adverse birth outcomes[12]). Much debate surrounds the direct effects of these medications on the developing fetus, though it is clear that many antidepressant medications cross the placenta,[13] including the most commonly prescribed medication during pregnancy: selective serotonin reuptake inhibitors (SSRIs). Currently, there is no consensus on the best course of treatment for depression or anxiety during pregnancy, as so much remains unknown about the biological mechanisms through which mental health problems and/or their associated medications may affect the fetus.

Epigenetic mechanisms may serve as one of the key pathways through which exposure to maternal depression, anxiety, or antidepressants may have long-term health consequences for

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Table 1. Characteristics of 58 Mother-Child Dyads from the Harvard Epigenetic Birth Cohort

	Exposed to non-medicated depression/ anxiety (n = 13)	Exposed to SSRIs (n = 22)	Controls (n = 23)
Maternal age in years, mean (SD)	29.6 (7.0)	34.5 (4.1)	32.7 (6.0)
Race, n (%)			
Black/Hispanic/ Other	3 (23.1)	1 (4.5)	3 (13.0)
White	10 (76.9)	21 (95.5)	20 (87.0)
Family-level SES, n (%)			
High	7 (53.9)	16 (72.7)	15 (65.2)
Low	4 (30.8)	5 (22.7)	6 (26.1)
Missing	2 (15.4)	1 (4.5)	2 (8.7%)
Smoking, n (%)	0 (0)	2 (9.1)	2 (8.7)
BMI, mean kg/m^2 (SD)	24.4 (5.4)	26.6 (5.5)	25.16 (2.9)
Neonate gender, n (%)			
Female	7 (53.9)	9 (40.9)	10 (43.5)
Gestational age in weeks, mean (SD)	39.7 (0.9)	39.3 (0.9)	39.3 (1.0)
Birth weight, gm, mean (SD)	3785.9 (487.7)	3562.1 (423.2)	3654.6 (445.2)

BMI, body mass index (pre-pregnancy); SD, standard deviation; SES, socioeconomic status.

the child. The epigenome is particularly vulnerable to environmental stressors in early stages of pregnancy, when embryonic cells are rapidly dividing and epigenetic marks are being erased and reset.[14] Throughout pregnancy, exposure to maternal depression or anxiety can lead to increased secretion of stress hormones such as cortisol or serotonin, in both the mother and the fetus. These hormones may lead to alteration of DNA methylation patterns in fetal genes involved in the function of the hypothalamic pituitary axis or other stress-response systems. Ultimately, these DNA methylation changes may alter gene expression patterns that predispose offspring of distressed mothers to develop affective disorders in adulthood.[14] In rodent models, exposure to prenatal stressors has been linked to altered DNA methylation. For example, increased DNA methylation was found at the promoter of the cortisol catalyzing gene, *HSD11B2*, in the placenta and the fetal cortex of rats whose mothers were exposed to chronic restraint during pregnancy.[15] Increased DNA methylation was also found at the promoter of the glucocorticoid receptor in the hippocampus of rats exposed to poor maternal care during the first postnatal week. This DNA methylation change was associated with differences in stress response among the offspring.[16]

Exposure to SSRIs during fetal development may also affect DNA methylation patterns in neonates. Animal models have reported altered serotonin levels and stress-response feedback systems of the brain with SSRI exposure.[17,18] One study suggested that exposure to the SSRI fluoxetine (Prozac) in adult rats induced expression of epigenetic regulatory factors (e.g., methyl CpG-binding-protein-1 and -2), which led to repressed transcription of downstream neuronal genes.[19] The epigenetic effects in humans of prenatal exposure to high levels of depression, anxiety, or SSRIs remain largely unknown.

While some studies have investigated DNA methylation levels in adults with depression (e.g., serotonin transporter gene,[20] BDNF[21]), few studies have investigated DNA methylation patterns in children exposed to maternal depression during pregnancy, and these have primarily focused on a few specific candidate genes. For example, one study investigated epigenetic effects of maternal depressive symptoms and serotonin reuptake inhibitor use on the glucocorticoid receptor gene and the repetitive element *LINE1* in cord blood of the neonate, but did not find strong evidence for altered DNA methylation at either locus.[22] A similar study found some evidence for an association between maternal depressed mood and DNA methylation at the serotonin transporter gene (*SLC6A4)* in the cord blood of neonates.[23]

To our knowledge, genome-wide DNA methylation patterns related to exposure to maternal depression in the developing fetus have only been considered in one study.[24] This study examined DNA methylation in the neonatal cord blood of the children of 201 women, all with lifetime diagnosis of a mood disorder, 43% with prenatal major depressive episode, and 75% on antidepressant medications. They did not find any significant DNA methylation changes in neonatal cord blood resulting from either maternal depressive symptoms or psychiatric diagnosis, using the 27K Illumina Infinium Methylation BeadChip. Very small DNA methylation differences (~2-3%) at a few genes were associated with antidepressant treatment.[24] More epigenomic analyses are warranted to improve our understanding of the associations between depression, anxiety, antidepressants, and DNA methylation.

In the current study, we investigate if different patterns of genome-wide DNA methylation can be detected in the umbilical cord blood of neonates exposed to maternal depression, anxiety, or a commonly used antidepressant medication, SSRIs, during pregnancy, relative to healthy controls. We hypothesized that exposure to these maternal mental health problems and to SSRIs during pregnancy may both alter DNA methylation patterns at birth relative to the control samples.

Results

The distribution of the characteristics of the 58 mother-child dyads from the Harvard Epigenetic Birth Cohort included in this study by exposure to non-medicated maternal depression/anxiety or SSRIs is shown in **Table 1**. Mothers who were classified with non-medicated depression/anxiety were significantly younger than those using SSRIs, and birth weight was substantially lower among neonates exposed to SSRIs relative to those exposed to non-medicated depression/anxiety and controls. The total sample was primarily comprised of White participants (88%), and the majority was classified as having high family socioeconomic status (SES) (65.5%). The mean maternal age was 32.6 y, mean

gestational age 39.4 wk, mean birth weight was 3655.2 g, and the mean pre-pregnancy maternal body mass index (BMI) was 25.4 kg/m^2.

Site-by-site regressions

The robust standard error (SE) regression models revealed 42 CpG sites (out of 453 857 tested) in which DNA methylation levels were significantly different (false discovery rate [FDR]-adjusted $P < 0.1$) in those exposed to non-medicated depression/anxiety, relative to controls (**Table 2**; **Table S1**), and no CpG sites significantly different in neonates exposed to SSRIs, after adjustment for SES, maternal BMI, maternal age, and chip (for batch effects). The vast majority of the significant CpG sites had lower DNA methylation in neonates exposed to non-medicated maternal depression/anxiety (33/42, 78.6%), relative to controls. Using a more conservative estimate of significance, we identified 10 sites significant at the stricter FDR-adjusted level of $P \leq 0.05$, and none in those exposed to SSRIs (**Table 2**; **Table S1**). For these ten most significant sites, effect sizes ranged from 9% lower to 3.6% higher level of DNA methylation, relative to controls. Among the 42 significant (FDR-adjusted $P < 0.1$) sites associated with non-medicated depression, the majority were located in CpG islands (24/42, 57.1%), 2/42 (4.8%) in shelves, 6/42 (14.3%) in shores, and the remaining 10 (23.8%) were in regions classified as others/open sea. The proportion of significant sites located in CpG islands (57.1%) relative to the proportion of sites in CpG islands in the full set of sites tested in the array (31.5%) was significantly different ($P < 0.001$).

Only one gene contained two CpG sites significantly associated with non-medicated maternal depression/anxiety (cg11846236, Beta = –0.08, 95% Confidence Interval [CI]: –0.10, –0.06; and cg17913386, Beta = –0.09, 95% CI: –0.11, –0.06). These sites were both located in the 1st exon of *Col7a1,* a gene associated with collagen production. One of these sites, cg17913386, was also marginally associated with SSRI use (unadjusted $P < 10^{-5}$). Though differences in DNA methylation in either group of neonates relative to controls for these sites were relatively small (ranging from 6–9%), these were among the largest differences identified in the study.

Regional cluster analyses

In order to place the individual CpG sites into a broader genomic context, we also analyzed regions surrounding the most significant individual sites by examining clusters of adjacent loci within 1kb of each other. None of the significant CpG sites that were identified in site-by-site analyses to be associated with non-medicated maternal depression/anxiety had significant surrounding regions. Even when examining the 1kb region that included the two sites in *Col7a1* that were both found to be significant in the site-by-site analyses, the average methylation of the cluster was not significantly associated with non-medicated maternal depression/anxiety. When examining all 1kb clusters in the array, no cluster with >1 CpG site was significantly associated with non-medicated maternal depression/anxiety or with SSRI use. Only one cluster located in *TMEM120b* was found to be marginally significantly associated with non-medicated maternal depression/anxiety (β = –0.01, 95% CI: –0.02, –0.01; $P = 0.06$). This cluster contained 2 CpG sites, neither of which was significant in the site-by-site analysis.

Table 2. Summary of site-by-site robust SE regressions

	Number of significant CpG sites (FDR-adjusted $P < 0.05$)	Number of significant CpG sites (FDR-adjusted $P \leq 0.1$)
Non-medicated maternal depression/anxiety	10	42
Higher DNA methylation[A]	2 (20%)	9 (21.4%)
Lower DNA methylation[A]	8 (80%)	33 (78.6%)
SSRI Exposed	0	0
Higher DNA methylation[A]	0	0
Lower DNA methylation[A]	0	0

Regressions adjusted for age of the mother, body mass index (BMI), family SES, and chip. [A]Higher or lower DNA methylation is in reference to the control group.

Gene ontology (GO) results

A total of 39 biological process terms were significantly enriched among the 100 sites most significantly associated with exposure to non-medicated maternal depression/anxiety at $P < 0.01$. Many of these terms were related to regulation of transcription, translation, and metabolic processes, such as negative regulation of translation in response to oxidative stress, regulation of mRNA export from the nucleus, and regulation of stem cell division (**Table S2**).

Candidate gene-specific analyses

In analysis of 10 a priori selected candidate genes that have been previously associated with depression, stress, or epigenetic regulation, we identified very few significant sites, after Bonferroni correction for the number of probes within each gene (**Table S3**). Specifically, significant associations with exposure to non-medicated maternal depression/anxiety were identified at one site in *NFKB2*, and at a marginally significant site within each of *FKBP5*, *NR3C1*, and *CRHR1.* Significant associations with SSRI exposure were also found at the same CpG site in *NFKB2,* as well as one site in *SLC6A4*. Among the 2 tested epigenetic regulator genes, no sites were significantly associated with non-medicated depression/anxiety, and a marginally significant association with SSRI exposure was found at one site in *DNMT3a* (**Table S3**).

Pyroverification results

In order to verify some of the significant CpG sites from the genome-wide analyses, we selected site cg17913386, one of the significant sites (FDR-adjusted *P* value < 0.05) within the *Col7a1* gene, and 5 surrounding CpG sites for pyrosequencing. The gene was selected for verification as it contained two significant CpG sites, one of which had the largest difference in DNA methylation between those exposed to non-medicated depression and the controls in the microarray. Pyrosequencing confirmed the significant association at this locus, as well as across all 4 tested surrounding sites. Specifically, a significantly lower DNA methylation level was found at each of the 6 sites (all $P < 0.005$), and on average across all sites (*P* value < 0.001), in those exposed to non-medicated maternal depression/anxiety (mean = 6.58%) or SSRIs (mean = 5.70%), relative to controls (mean = 8.61%). Regression analyses for each CpG site, adjusting for SES, maternal BMI,

and maternal age, revealed significantly lower methylation level for those exposed to non-medicated depression/anxiety (all $P < 0.005$) and for those exposed to SSRIs (all $P \leq 0.05$), relative to controls. Regression results for the average DNA methylation across all 6 sites showed a similar pattern as was found for each individual site, for those exposed to non-medicated depression/anxiety (Beta = –3.3, SE = 0.87, $P < 0.001$) and for those exposed to SSRIs (Beta = –1.89, SE = 0.71, $P = 0.01$).

Discussion

In this study we present a comprehensive genome-wide analysis of DNA methylation in cord blood of neonates born to mothers reporting non-medicated depression or anxiety during pregnancy or mothers using SSRIs during pregnancy. We observed 42 CpG sites with DNA methylation levels significantly associated (FDR-adjusted P value < 0.1) with non-medicated maternal depression/anxiety but no sites significantly associated with SSRI use, after adjusting for extensive multiple testing. Our gene ontology analysis highlighted a number of biological pathways enriched for genes related to the regulation of transcription and translation of DNA (**Table S2**). However, we note that the majority of the significant sites had very small DNA methylation differences between groups, and the regions neighboring the significant sites did not differ significantly in DNA methylation from unexposed neonates. Thus, despite many DNA methylation differences observed between groups, these effects are small and should be replicated in larger studies to eliminate chance findings, and also to boost power to detect somewhat small effects.

Our study utilized the most recently recommended analytical techniques for normalizing and analyzing DNA methylation data from genome-wide microarrays, e.g., removing non-specific probes, adjusting for probe-type bias using a β-mixture quantile normalization technique, and analyzing CpG sites individually and in regional clusters. In synthesizing findings across these analytical approaches, we note that each analysis provided unique insights. For example, most of the significant genes identified in the site-by-site regressions were not captured by the regional analyses. The lack of regional significance for the individually significant CpG sites indicates that no surrounding sites reached significance, or alternatively that the microarray did not include any neighboring sites within 1kb of the significant sites. On the other hand, for the one marginally significant region that contained two CpG sites, neither site was identified as significant by the individual site-by-site analyses. These examples highlight the benefit of combining both approaches, which allowed for identification of significant individual sites and regions, and helped place some individual sites into a regional context. Even when the region surrounding an individual site is not significant, it may be useful to further investigate the significant individual sites as some studies of DNA methylation, particularly with cancer phenotypes, have found that DNA methylation of only one CpG site can alter gene expression.[25,26]

One surprising result was the identification of significant DNA methylation differences in two CpG sites within *Col7a1*. This gene encodes the α chain of type XII collagen, which serves as an anchoring fibril between the epithelia and the stroma. Mutations in this gene have been linked with dystrophic epidermolysis bullosa, a blistering skin condition.[27] To our knowledge, no prior studies have investigated DNA methylation in this gene or linked it with depression or other psychological experiences.

The significant sites we identified to be associated with non-medicated maternal depression/anxiety were not identified in the only prior study to examine epigenome-wide effects of this exposure in neonatal cord blood.[24] In contrast, the prior study identified two CpG sites associated with SSRI use, one located in a tumor necrosis factor receptor subfamily 21 (*TNFRSF21*) and the other in a cholinergic receptor, nicotinic, alpha2 (*CHRNA2*). In their study, both of these sites had very small differences between groups (1–3%).[24] DNA methylation at neither of these sites differed from controls in our study. These different results could stem from different assessment criteria used to evaluate depression and anxiety between studies, different types of regression analyses, or may indicate that the two sites identified in the prior study were false-positive results. Additionally, the majority (36/42) of the new sites we identified could not have been identified in the prior study, as they were not included on the older 27K Illumina platform.

One of the key questions of this study was to determine if exposure to SSRIs had a unique impact on the epigenome of the developing child relative to exposure to non-medicated maternal depression/anxiety. The medication itself (regardless of the underlying indication) may alter DNA methylation patterns, or the medication may simply serve as an indicator of more severe underlying psychopathology, which actually drives altered DNA methylation patterns. Finally, the medication may reduce the depression which is influencing DNA methylation and thereby attenuate apparent effects of exposure to depression. In a study that has not randomized individuals to take medication or not, it can be difficult to distinguish between these alternatives. Moreover, this question is further complicated by the fact that, while medication can reduce symptoms of depression, it is not always effective. Given that no CpG sites in this study were associated with SSRI exposure, but 10 were highly significantly (P_adj < 0.05) associated with exposure to non-medicated maternal depression/anxiety, these results suggest that we are able to distinguish between the two exposures, and that the medication may have less of an impact on DNA methylation of the developing fetus than the underlying pathology.

One noteworthy finding of our study was that the majority of significant sites identified across analyses had consistently lower DNA methylation in neonates exposed to SSRIs or non-medicated depression/anxiety compared with unexposed neonates. Typically, low DNA methylation in the promoter region of a gene is associated with upregulation of gene expression.[28] However, the biological function of DNA methylation varies greatly across different genomic contexts.[29] In the current study, DNA methylation levels appear consistently lower in those exposed to non-medicated depression/anxiety and in those exposed to SSRIs relative to controls, regardless of the gene or genomic location of the site. The one exception is the gene with

the largest β coefficient among those with FDR-adjusted *P* values < 0.1 (*AKAP11*), which had a higher DNA methylation (20% methylation difference) in those exposed to non-medicated maternal depression/anxiety. Prior studies of early life adversity and genome-wide DNA methylation patterns have found both increased and decreased DNA methylation in response to early life stressors.[30,31] While these studies assessed different early life adverse exposures across different tissues, the generally mixed evidence of both increased and decreased DNA methylation suggests complex and diverse epigenetic processes, for which the full functional meaning remains to be determined.

A few of the a priori selected candidate genes contained one CpG site that was strongly associated with non-medicated maternal depression/anxiety or with SSRI medication, after Bonferroni correction for the many sites tested within each gene. The biological significance of these associations remains uncertain, because these significant sites were distributed throughout different genomic regions with varying regulatory roles (e.g., gene body, 5′UTR), only one site per gene was found to be associated (out of dozens or in some cases 100s of tested sites), and because the effect sizes were all very small (all < 1%). In prior studies of these candidate genes, both *NR3C1* and *SLC6A4* have demonstrated altered methylation in cord blood of children exposed to depression or maternal mood disorders during pregnancy,[22,23] as well as in DNA from placenta.[32] In these studies, very small DNA methylation differences were found with marginal significance at a few specific CpG sites in each gene (e.g., CpGs 6 and CpG 9 in *SLC6A4*,[23] and CpGs 1, 2, and 3 in *NR3C1*[22] for cord blood, CpG 2 in *NR3C1* in placenta[32]). Our study did not identify significant associations at the same sites as these prior studies but did discover altered DNA methylation at novel sites in these genes (**Table S3**). This lack of replication was in part due to the fact that the two significant sites in *SLC6A4*, CpG 6 and 9, were not included on our microarray, though CpG1, CpG4, and CpG5 were included and not found to be significant in either study. For *NR3C1*, significant associations were previously found with CpGs 1, 2, and 3, with CpG 3 being associated both with maternal depressive symptoms and cortisol levels in infants, and located within a potential NGF1-A consensus binding site, along with CpG4.[22] In our analysis, only CpGs 1, 4, 5, and 8 were included in the microarray, and no differences in methylation were found at these sites.

A recent study investigated depressed mood in mothers and DNA methylation at nine differentially methylated regions (DMR) regulating imprinted genes in the cord blood of 922 neonates.[33] Their study found 2.4% higher DNA methylation at the MEG3 DMR in those exposed vs. unexposed to severe maternal depressed mood during pregnancy, and similar to our study, found no associations with SSRI use. While 5 CpG sites within MEG3 in our study had significant unadjusted *P* values (< 0.05), they did not retain significance after Bonferroni adjustment for the 54 sites tested.

Limitations and strengths

A number of limitations need be considered when interpreting our results. The sample size of our study was relatively small for detecting small effects in a genome-wide analysis, though it was comparable to similar studies using whole genome approaches.[24,30] Second, because our sample was cross-sectional, we are not able to determine the direction of causality between depression/anxiety and DNA methylation levels in the neonates. Reverse causation is not a significant concern, however, as DNA methylation levels in the cord blood of the children are not likely to affect depression/anxiety symptoms in the mother. Third, our study shares a common limitation among studies of antidepressant use during pregnancy, which is the difficulty of separating the severity of depression/anxiety from the effects of medication on the fetus. Because women who take SSRIs during pregnancy usually experience more severe depression than the untreated group,[8] any differences found between the SSRI-treated and non-treated depressed groups may be due to differences in severity of depression, rather than medication. In this study, data on severity or duration of depression/anxiety were not available, and we were therefore unable to distinguish these effects. Furthermore, since our measure of depression/anxiety was based on chart review of labor and delivery forms, we did not have information on the date of initiation of depression/anxiety, or whether medication was prescribed following the report of symptoms noted by the obstetrician during labor and delivery. We recognize that measures of diagnosed depression may underestimate the prevalence of depression in the population since typically only the more severe cases come to clinical attention.[34] However, this underestimate would make it more difficult to see differences between our groups, thereby biasing results toward the null (or making our estimates more conservative). Fourth, we made substantive efforts to eliminate Type I errors by conducting very conservative multiple comparison tests. However, when examining over 450 000 sites, these efforts are often overly restrictive. Finally, we note that this study examined DNA methylation in the buffy coat of cord blood, which contains a heterogeneous mixture of white blood cell types. If exposure of neonates to maternal depression/anxiety or SSRIs caused a shift in cell populations due to increased inflammation, the change in cord blood methylation may be purely reflecting variation in cell types. While this may not be the variation of interest, it may be a component of the total association between maternal depression/anxiety or SSRIs and methylation and does not violate our internal validity. Mediation by shifts in cell population does not appear to have a large impact on our results, given that none of the methylation differences we identified were located in genes related to immune system function or inflammation. The use of peripheral white blood cells also limits inferences regarding pathways involving the brain. However, a number of studies have in fact recently found the same relative patterns of inter-individual variability in methylation levels across brain and blood, despite large tissue-specific differences within individuals.[35] Furthermore, stress hormones, such as cortisol, and SSRI medications can cross the placental barrier and have been detected in cord blood,[36,37] and thus these cells may be directly affected by these exposures.

Despite these limitations, our study is the first to use a genome-wide array including 450,000 CpG sites to detect DNA methylation differences throughout the genome in cord blood of neonates born to mothers experiencing depression/anxiety or

exposed to SSRIs during pregnancy. Regardless of whether or not the small differences are involved in causal biological pathways affecting later life health, the large number of differences indicates that epigenetic changes are detectable in an easily accessible cord blood sample. These findings suggest that epigenetic profiles may be sensitive to these early adverse exposures.

Materials and Methods

Study population

Participants in this study were selected from 1941 mother-child dyads in the Harvard Epigenetic Birth Cohort, collected between 2007 and 2009 at the Brigham and Women's Hospital (BWH) in Boston, MA. Details of the data and biospecimen collection have been described elsewhere.[38] In brief, pregnant women were asked to complete a 2-page questionnaire and asked permission to abstract information from their pregnancy charts and to collect samples from umbilical cord and placenta after detachment for research purposes. The questionnaire elicited information about race and ethnicity, height, age, smoking habits, and alcohol consumption, among other pregnancy attributes and behaviors. The study protocol for the sample collection and data analysis was approved by the BWH and Harvard School of Public Health Institutional Review Boards.

We considered cord blood samples among all participants from the birth cohort where a report of depression or anxiety during pregnancy was recorded by a physician in the obstetrical medical records, based on a report of symptoms (n = 64). Among these participants, we excluded any with pregnancy complications or illnesses (e.g., preterm birth (<39 wk), preeclampsia, gestational diabetes), any who reported drinking alcohol during the pregnancy, twin births, use of artificial reproduction, or any medications during pregnancy other than SSRIs. Of the remaining 37 mother-neonate dyads evaluated, 24 neonates had mothers who reported taking SSRIs, and 13 neonates had no reported maternal use of medication. For each neonate born to a mother who reported SSRI use, we identified a control participant as a healthy neonate born to a non-medicated mother with no report of depression during pregnancy, loosely matched on gender of offspring, maternal age (+/– 2 y), maternal BMI, and mother's self-identified race. Within the same set of 24 controls that were matched to neonates exposed to SSRIs, 13 were also matched to neonates who reported non-medicated depression or anxiety during pregnancy on the same criteria as listed above. After removing one control neonate with extremely outlying DNA methylation values and one SSRI-exposed neonate with unclear sex determination, the remaining population for study consisted of 13 neonates exposed to non-medicated maternal depression or anxiety, 22 exposed to SSRIs, and 23 healthy controls.

Measures of non-medicated depression/anxiety and SSRIs

Participants were classified as having been exposed to prenatal depression or anxiety if a report of depression and/or anxiety during pregnancy was explicitly noted by their obstetricians in labor and delivery forms. Detailed information about date of onset, severity, or duration of the depression or anxiety was not available. Among those classified with depression or anxiety, three participants were classified as having experienced anxiety, five as having experienced depression (1 attempted suicide), and 5 had experienced both anxiety and depression. Due to small sample sizes, these were all grouped together for a total of 13 classified as depressed/anxious. Information on depression-related medication throughout the pregnancy or at the time of the delivery was abstracted from medical charts. Only neonates exposed to the following antidepressant medications were included in the SSRI-exposed group: Zoloft (48%, 11/23), Prozac (26%, 6/23), Celexa (17%, 4/23), and Paxil (9%, 2/23).

Covariates

Data on maternal age at delivery, height, pre-pregnancy weight, and sex of the neonate were abstracted from the labor and delivery medical records, and if missing, were supplemented by the self-reported questionnaires. Maternal pre-pregnancy BMI was calculated as weight in kilograms divided by the square of height in meters (kg/m^2). Parental occupations were abstracted from pregnancy charts, and each parent's occupation was classified into one of five job levels, based on the level of education, experience, and training necessary to perform the occupation, defined by the Occupational Information Network (O*NET, http://www.onetonline.org/). A variable of family SES was calculated based on the sum of the job level scores of mother's and father's occupations, when father was present (ranging from lowest [1] to highest [5]). When father was absent, mother's score was used. Family SES was classified as high if the sum of the parents' scores was greater than or equal to 5 or low if the sum was less than 5.

Sample collection, preparation, and genome-wide DNA methylation assays

Umbilical cord blood was collected from the base of the cord in an EDTA tube, processed immediately, and then stored at –80 °C. DNA was isolated from the buffy coat layer of the cord blood using the QIAamp DNA Mini Kit (Qiagen, 51306).

For genome-wide analysis, the Illumina Infinium Human Methylation450 BeadChip was used to interrogate DNA methylation at 485,755 CpG sites, spanning 99% of RefSeq genes. This Beadchip includes probe types of two different chemistries: (1) Type I probes, in which two different probe types interrogate each CpG site, one which targets methylated DNA and one which targets unmethylated DNA, and (2) Type II probes which bind to the nucleotide just before the target site, and create a single base extension of G or A complementary to the methylated C or unmethylated T.

For each sample, 1 μg of genomic DNA was processed at the USC Epigenome Center, as previously described.[39] In brief, after randomly ordering the samples across the chips, genomic DNA was bisulfite converted using the EZ DNA methylation Kit (Zymo Research, D5006), whole genome amplified, fragmented, and hybridized to BeadChip arrays. Following DNA extension with biotin-labeled dNTPs, each array was stained with antibodies and scanned to detect Cy3 labeled probes on the green channel, and Cy5 labeled probes on the red channel. Quality control samples (Zymo Research, D5014) of known DNA methylation levels were included across the chips, including two each of 0%,

100%, and 50% (created by mixing the 0% and 100% samples) samples, as well as duplicates of one participant's cord blood samples. The quality control samples were distributed such that one sample of known DNA methylation level was included on each chip. See supplemental methods for details on quality control measures of the microarray data and normalization techniques.

Statistical analyses overview

We conducted a series of analyses on the genome wide microarray data, with each technique designed to capture potentially distinct patterns of DNA methylation. The first analysis consisted of site-by-site regressions, which analyzed each CpG site individually. Second, we conducted regional analyses, designed to capture an average pattern of DNA methylation among neighboring sites, in order to place the individual CpG site findings into a regional context. The site-by-site and regional methods were anticipated to generally capture overlapping sets of genes, though unique genes may also be identified by each analysis. For these analyses, significance was determined following multiple testing corrections for all the CpG sites (for site-by-site analyses) or regions (for regional analyses) in the array. Third, a GO analysis was performed on the top 100 significant sites identified by the site-by-site analyses to determine if significant sites clustered in functional biological pathways. Finally, we also conducted a candidate gene analysis, designed to analyze a priori selected genes related to stress, depression, or epigenetic regulation in prior studies. For these analyses, Bonferroni corrections for all assayed CpG sites within each selected gene were used. We anticipated some of the CpG sites in the candidate genes might be significantly associated with exposure to maternal depression/anxiety or SSRIs even if they were not identified in the more strictly adjusted genome-wide analyses. Finally, pyrosequencing was used as an independent and more precise technique to confirm one of the significant findings from the microarray.

Site-by-site regression analyses

Each CpG site was separately tested for association with exposure to non-medicated maternal depression/anxiety or SSRIs with a multivariate linear regression using robust standard errors (SE). We used robust SE regression, as the majority of DNA methylation values did not follow a normal distribution, and the regression coefficient remains easily interpretable, e.g., the β coefficient represents the difference in percent DNA methylation between each group relative to the control group. DNA methylation level at each site was regressed against dummy variables for exposure to non-medicated maternal depression/anxiety and exposure to SSRIs, relative to the reference group of controls (unexposed to medication or depression/anxiety), as well as covariates for age of the mother, BMI, and family SES. These covariates were included as potential confounders, because they may be associated with depression,[40,41] but also may be independently linked with altered DNA methylation in the neonates at various genes.[31,42,43] Models were also tested with sex of the neonate included as a main effect and in interaction with maternal depression status, but these variables did not significantly improve model fit, and given our small sample size, were excluded from final models. As samples are processed in batches of 12/chip, all models also included a covariate for batch effects by chip. *P* values were adjusted for genome-wide significance using False Discovery Rate (FDR) adjustment.

For verification of microarray results, pyrosequencing of bisulfite treated DNA was used to quantitatively assay DNA methylation levels at one of the CpG sites identified as significant in these analyses. Details of the bisulfite treatment and pyrosequencing methods can be found in supplemental methods.

Regional analysis

A regional analysis was also performed, averaging DNA methylation levels across clusters of CpG loci, in order to explore coordinated regional DNA methylation. Clusters were defined by contiguous sites on the same chromosome with less than 1kb between adjacent loci, resulting in 157 537 clusters. Average DNA methylation in each cluster was modeled as a function of depression exposure, adjusting for the covariates included in the site-specific analysis, utilizing robust standard errors for inference. All *P* values were adjusted for multiple testing using FDR adjustment. All genome-wide analyses were conducted using R v2.15.1.

Gene ontology

Functional enrichment of significant genes was evaluated with a GO analysis using a conditional hypergeometric test, conditioning on parent GO terms. The GO analyses were tested separately using biological process terms for the 100 genes with smallest *P* values associated with non-medicated maternal depression/anxiety. Enrichment was assessed relative to all the genes interrogated by the 450k array, which covers 99% of the human genome. Terms with P values < 0.01 were considered significant.

Candidate genes

We also investigated 10 a priori chosen candidate genes that have previously been related to depression, stress, or to epigenetic regulation. These genes include the glucocorticoid receptor gene (*NR3C1),* in which differential DNA methylation was found in the hippocampus of rat pups exposed to variation in maternal care,[16] and in humans who have experienced a history of child abuse;[44] the FK506 binding protein 5 gene (*FKBP5),* which is an important regulator of the glucocorticoid receptor complex, and demethylation of this gene has been associated with experience of childhood trauma;[45] the brain-derived neurotrophic factor gene (*BDNF*), which has been linked with increased DNA methylation in the brains of suicide victims;[46] the serotonin transporter gene (*SLC6A4)* which has an important role in brain development, and DNA methylation patterns of this gene in white blood cells have been associated with brain 5-HT synthesis;[47] the genes for corticotrophin releasing hormone receptors (*CRHR1* and *CRHR2*)[48] which are important mediators of stress, and have been observed to influence development of adult depression in conjunction with experiences of child abuse; and lastly nuclear factor kappa-B subunits 1 and 2 (*NFKB1* and *NFKB2)*, two genes involved in transcription regulation of pro-inflammatory cytokines and implicated in studies of psychosocial stress.[49] We also examined DNA methylation at all CpG sites located within two different epigenetic regulator genes, including DNA methyltransferases (*DNMT1* and *DNMT3a),* which are responsible for transfer of methyl groups to DNA. All CpG sites associated with

each candidate gene were evaluated individually using the multivariate regression described for the site-specific analysis. *P* values were adjusted for multiple testing using a Bonferroni correction for the number of CpG sites tested in each gene.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/epigenetics/article/28853

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