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DNA methylation provides insight into intergenerational risk for preterm birth in African Americans

Sasha E Parets[1,2], Karen N Conneely[1,3], Varun Kilaru[2], Ramkumar Menon[4], and Alicia K Smith[1,2,*]

[1]Genetics and Molecular Biology Program; Emory University; Atlanta, GA USA; [2]Department of Psychiatry & Behavioral Sciences; Emory University School of Medicine; Atlanta, GA USA; [3]Department of Human Genetics; Emory University School of Medicine; Atlanta, GA USA; [4]Department of Obstetrics & Gynecology; Division of Maternal-Fetal Medicine Perinatal Research; The University of Texas Medical Branch at Galveston; Galveston, TX USA

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Abbreviations: DOHaD, developmental origin of health and disease; FDR, false discovery rate; GA, gestational age; PTB, preterm birth; SES, socioeconomic status.

African Americans are at increased risk for spontaneous preterm birth (PTB). Though PTB is heritable, genetic studies have not identified variants that account for its intergenerational risk, prompting the hypothesis that epigenetic factors may also contribute. The objective of this study was to evaluate DNA methylation from maternal leukocytes to identify patterns specific to PTB and its intergenerational risk. DNA from peripheral leukocytes from African American women that delivered preterm (24–34 weeks; N = 16) or at term (39–41 weeks; N = 24) was assessed for DNA methylation using the HumanMethylation450 BeadChip. In maternal samples, 17,829 CpG sites associated with PTB, but no CpG site remained associated after correction for multiple comparisons. Examination of paired maternal-fetal samples identified 5,171 CpG sites in which methylation of maternal samples correlated with methylation of her respective fetus (FDR < 0.05). These correlated sites were enriched for association with PTB in maternal leukocytes. The majority of correlated CpG sites could be attributed to one or more genetic variants. They were also significantly more likely to be in genes involved in metabolic, cardiovascular, and immune pathways, suggesting a role for genetic and environmental contributions to PTB risk and chronic disease. The results of this study may provide insight into the factors underlying intergenerational risk for PTB and its consequences.

Introduction

Though the overall rate of preterm birth has slightly decreased in recent years, African Americans have more than 1.5 times the risk of spontaneous preterm birth (PTB; <37 weeks gestation) and more than twice the risk of early PTB (<32 weeks) when compared to Caucasians.[1-3] Studies to date have identified numerous maternal risk factors for PTB,[1,4-7] such as low socioeconomic status (SES), but less than half of the increased risk in African Americans is explained by SES and other known risk factors.[8-10]

Personal and family history of PTB are the greatest risk factors for PTB, and studies estimate its heritability at 17–30%.[11-15 16,17] However, genetic studies have not identified variants that account for this intergenerational risk,[1,11,18-21] prompting the hypothesis that epigenetic factors may also contribute to PTB.[17,22-24] Few studies have evaluated the epigenetics of PTB, and those that have focus mostly on those born preterm.[25-28] Other studies focus on the short-term and long-term consequences of PTB for the neonate,[1,29-32] in part, because of interest in the developmental origin of health and disease (DOHaD) hypothesis.[32,33]

We recently evaluated DNA methylation in leukocytes from African American umbilical cord blood samples and identified thousands of DNA methylation differences between preterm and term fetuses.[25] These DNA methylation differences may underlie some of the risks associated with being born preterm, though longitudinal studies will be more informative for determining whether methylation differences observed at birth have long-term consequences or whether they simply reflect developmental differences. Cruickshank and colleagues performed one such study in 12 PTB cases and 12 matched controls.[28] They evaluated DNA methylation from blood at birth and at 18 years and observed substantial overlap with the PTB-associated CpGs we reported at birth. DNA methylation differences observed at birth were no longer associated with PTB status at 18 years for the majority of CpG sites examined, but they report 10 CpGs that continue to differ in methylation at both time points, suggesting the potential for a long-term epigenetic signature of PTB.

*Correspondence to: Alicia K Smith; Email: alicia.smith@emory.edu
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No study has examined genome-wide DNA methylation in leukocytes of women who deliver preterm. However, studies have recently begun documenting the long-term implications of delivering preterm for maternal health. Women who deliver preterm are at increased risk to develop cardiovascular and other chronic disorders as they age.[34-42] For example, a series of studies demonstrate that mothers who deliver very preterm are at subsequent risk for Type 2 diabetes.[38,43] The first, conducted primarily in Caucasian women from the Nurses' Health Study II, reports that women who deliver very early preterm are more likely to be diagnosed with Type 2 diabetes in the decade following pregnancy.[43] A second investigation of ~30,000 women from the Black Women's Health Study also reports that early preterm birth associates with a higher risk for developing Type 2 diabetes even after correcting for age at first birth, family history of diabetes, education, personal history of preterm birth, and body mass index.[38] They then demonstrated that the increased diabetes risk was independent of gestational diabetes history, consistent with other studies.[39,40]

The mechanism underlying the relationship between PTB and the development of chronic disorders later in life is not yet clear, but some suggest that inflammation or immune dysregulation may increase risk for PTB and other chronic disorders.[38,44] DNA methylation patterns regulate the functional properties of immune cells[45,46] and associate with inflammatory markers,[47] chronic disorders,[48-50] and PTB.[27,51] We hypothesize that DNA methylation patterns may reveal genes whose regulation is unique to women who deliver preterm or provide insight into its intergenerational risk

Results

The cohort is comprised of African American women that deliver early preterm [gestational age (GA) range: 24.1–34.0 weeks] and at term (39.0–40.9 weeks). As expected, the groups differed by GA and birthweight, but did not differ significantly by any other demographic or clinical factor (**Table 1**).

Table 1. Demographics table for maternal samples

Phenotype	PTB (N=16) Mean ± SD	TB (N=24) Mean ± SD	*P*-value*
Maternal Age	24.69 ± 4.7	24.13 ± 6.2	NS
Weeks Gestation	30.2 ± 3.6	39.9 ± 0.4	<0.0001
Birthweight, grams	1555.8 ± 717.9	3309.7 ± 353.7	<0.0001
Birthweight percentile	38.9 ± 30.6	46.2 ± 26.7	NS
Parity	1.2 ± 1.4	1.4 ± 1.7	NS
BMI	29.93 ± 8.4	26.46 ± 5.9	NS
Household Income			
<15 K	(10) 66.7%	(11) 45.8%	NS
15–30 K	(2) 13.3%	(7) 29.2%	NS
>30 K	(3) 20%	(6) 25%	NS
Smoking	(4) 25%	(5) 21%	NS
Married	(5) 33.3%	(5) 20.8%	NS
Employed	(1) 7.1%	(6) 42.9%	NS
Chorioamnionitis	(9) 56%	NA	NS
Granulocytes	74.7 ± 9.2	69.6 ± 7.8	NS
Monocytes	9.8 ± 3.7	10.7 ± 3.2	NS

*NS indicates that the *P*-value is non-significant ($P > 0.05$).

Association between maternal DNA methylation and PTB

First, we examined the association between DNA methylation at each CpG site and PTB. Overall, 17,829 CpG sites associated with PTB ($1.83 \times 10^{-6} < P < 0.05$). Among the CpG sites with the strongest association were 2 (cg22486214, cg16980736) in regulatory associated protein of MTOR (*RPTOR*; $2.20 \times 10^{-5} < P < 1.03 \times 10^{-4}$). However, no CpG site remained associated after correction for multiple testing (FDR < 0.05; **Table S1**). We have previously reported DNA methylation differences that associate with GA in leukocytes from umbilical cord blood of fetuses born to this cohort of women.[25] CpG sites that associate with PTB in maternal leukocytes are more likely to associate with GA in fetuses when compared to those that do not associate with PTB (5.2% vs. 3.5%; $P < 2.2 \times 10^{-16}$), suggesting that there may be epigenetic factors shared between mothers who deliver preterm and their fetuses.

Correlation between maternal and fetal methylation

We next evaluated the relationship between maternal and fetal DNA methylation across the genome. We identified 5,171 CpG sites in which maternal methylation associated with fetal methylation (FDR < 0.05; **Fig. 1**; **Table S2**), 98.8% of which occurred in the same direction. These correlated CpG sites were consistent with the "heritable" CpG sites recently reported by McRae and colleagues[52] (Result S1). To empirically assess whether these differences are greater than expected by chance, we repeated the analysis comparing methylation from each mother to methylation from an unrelated fetus that was matched for PTB status and sex. Only 35 CpG sites associated between unrelated pairs (FDR < 0.05), suggesting that the high degree of correlation observed between a mother and her fetus is substantially greater than expected by chance.

There is a wide range of variation in methylation levels across the 5,171 correlated CpG sites (**Fig. 2A**), that is consistent with distribution of variation in all CpG sites, assessed on the array (**Fig. 2B**). Correlated CpG sites were more likely to occur in regions of low CpG density (i.e., shelves and open seas) and less likely occur in regulatory regions near the transcription start site (i.e., promoters, 1st exon, and 5′UTRs) when compared to uncorrelated CpG sites ($1.5 \times 10^{-6} < P < 2.2 \times 10^{-16}$; **Table 2**). They were also more likely to be located in genes involved in metabolic (i.e., Type 1 and Type 2 diabetes mellitus), cardiovascular (i.e., viral myocarditis, arrhythmogenic right ventricular cardiomyopathy), and immune (i.e., graft vs. host disease, allograft rejection) pathways (**Table 3**). The majority of correlated CpG sites (3,857; 74.6%) could be attributed to one or more genetic variants, defined as a SNP that either overlapped with the CpG probe sequence (1,393; 26.9%) or associated with the CpG as a methylation quantitative trait locus (meQTL; 2,464; 47.7%). We also found that CpG sites that correlated between a mother and her fetus were enriched for meQTLs at increasingly stringent significance levels (**Fig. 3**), consistent with

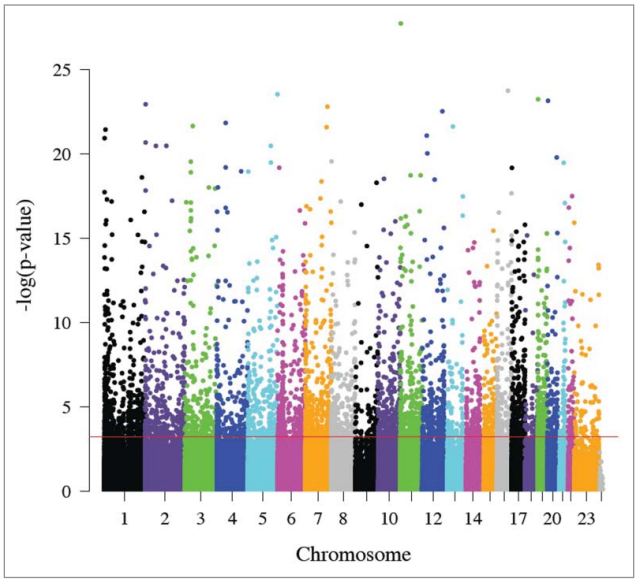

Figure 1. Manhattan plot of the relationship between maternal and fetal DNA methylation. The x-axis represents the position of each CpG site by chromosome. The y-axis represents the negative $\log_{10}$ of the *P*-value for the association between maternal and fetal methylation for each CpG site. The red line indicates experiment-wide significance based on a false discovery rate of 5% such that the 5,171 CpG sites above this line are significantly correlated in leukocytes from a mother and her fetus.

the results of McRae et al., who reported that sequence variation accounts for the majority of heritable DNA methylation patterns.[52] The remaining 1,314 CpG sites (25.4%) could not be attributed to genetic factors and may reflect the shared environment. Both classes of CpG sites support the pathways identified in the combined analysis when evaluated individually (**Table 3**). Evaluation of the 200 genes containing CpG sites whose methylation levels are both predictive of cord blood methylation and associate with PTB in maternal samples also reveal enrichment for genes in the Type 2 diabetes mellitus pathway (KEGG:04930; $P = 0.015$).

Correlated CpG sites were more likely than uncorrelated sites to associate with PTB in maternal samples (**Table 2**; OR = 1.7; $P < 2.2 \times 10^{-16}$), but there was no difference in the rates of CpG sites influenced by genetic versus non-genetic factors among those associated with PTB ($P = 0.41$). Thus, we re-examined the relationship between maternal and fetal DNA methylation separately in PTB and term birth pairs. There were 79 CpG sites that correlated (FDR < 0.05) in the PTB pairs, 57 (72.2%) of which were unique to the PTB samples. PTB-specific sites were enriched in genes involved in vascular smooth muscle contraction (KEGG:04270; $P = 0.037$). Consistent with the results of the combined analysis, the majority (70.2%) of the correlated CpG sites specific to PTB were attributable to genetic variation.

Correlation Between Maternal and Fetal Gene Expression

We next examined whether genes containing correlated CpG sites have correlated gene expression levels. Correlated CpG sites (N = 5,171) were located in 3,297 expression probes, representing 2,282 unique genes and 1,015 transcript variants. Maternal expression of 738 transcripts (22.3%; $2.3 \times 10^{-14} < P < 0.05$) predicted fetal expression, 357 (10.8%) of which remained associated after correction for multiple tests (FDR < 0.05; **Table S3**). For example, methylation of CpG sites in MHC class I polypeptide-related sequence B (*MICB*) associate with PTB in maternal leukocytes and correlate with methylation in fetal samples (**Figs. 4A–4B**). Maternal *MICB* expression also predicts fetal *MICB* expression ($P = 1.09 \times 10^{-3}$; **Fig. 4C**). Interestingly, levels of the protein encoded by *MICB* fall prior to parturition.[53] Pathway analysis of genes with correlated expression levels (FDR < 0.05) were consistent with the results of those performed with correlated CpG sites (data not shown).

Discussion

In this study, no CpG site of large effect size was associated with PTB in African American women, though thousands of CpG sites were nominally significant. Only 5.2% of the CpG sites that were associated with GA in the leukocytes of umbilical cord blood of fetuses born preterm also associated with PTB in maternal samples, suggesting that the majority of CpG sites associated with GA in fetuses may reflect developmental differences. Nevertheless, our study does provide insight into correlated methylation and expression patterns in maternal-fetal pairs and suggests they are enriched in biological pathways implicated in PTB and chronic disease risk.

One of the biggest risk factors for PTB is a prior history or a family history. We identified 5,171 CpG sites in which maternal methylation predicts fetal methylation. The vast majority of correlated CpG sites (98.8%) occurred in the same direction, consistent with a high degree of genetic and environmental similarity in these pairs. These correlated CpG sites were enriched in areas of low CpG density, regions of high inter-individual variation that are more likely to associate with environmental factors and complex diseases.[54] Though this study did not specifically evaluate sequence variation, methylation of almost 75% of correlated CpG sites could be attributed to genetic variation, such as a SNP or meQTL. The results of this study were consistent with those reported in a large multigenerational cohort of Caucasians,[52] which determined that heritable CpG sites were primarily under genetic influence. African Americans and Caucasians have distinct patterns of genetic-epigenetic correlation[55] that may contribute to the increased risk for PTB and other disorders more common in African Americans.

Correlated CpG sites were also enriched among genes whose expression levels were correlated in maternal-fetal pairs, providing a potential mechanism linking correlated methylation in women who deliver preterm to biological differences. For example, *MICB* is part of the MHC class I chain and is induced by cellular stress to initiate an immune response.[53] Activation of inflammatory pathways has been implicated in the timing of parturition specifically in PTB,[11] and we observed lower methylation of CpGs in *MICB* in PTB as well as correlated methylation

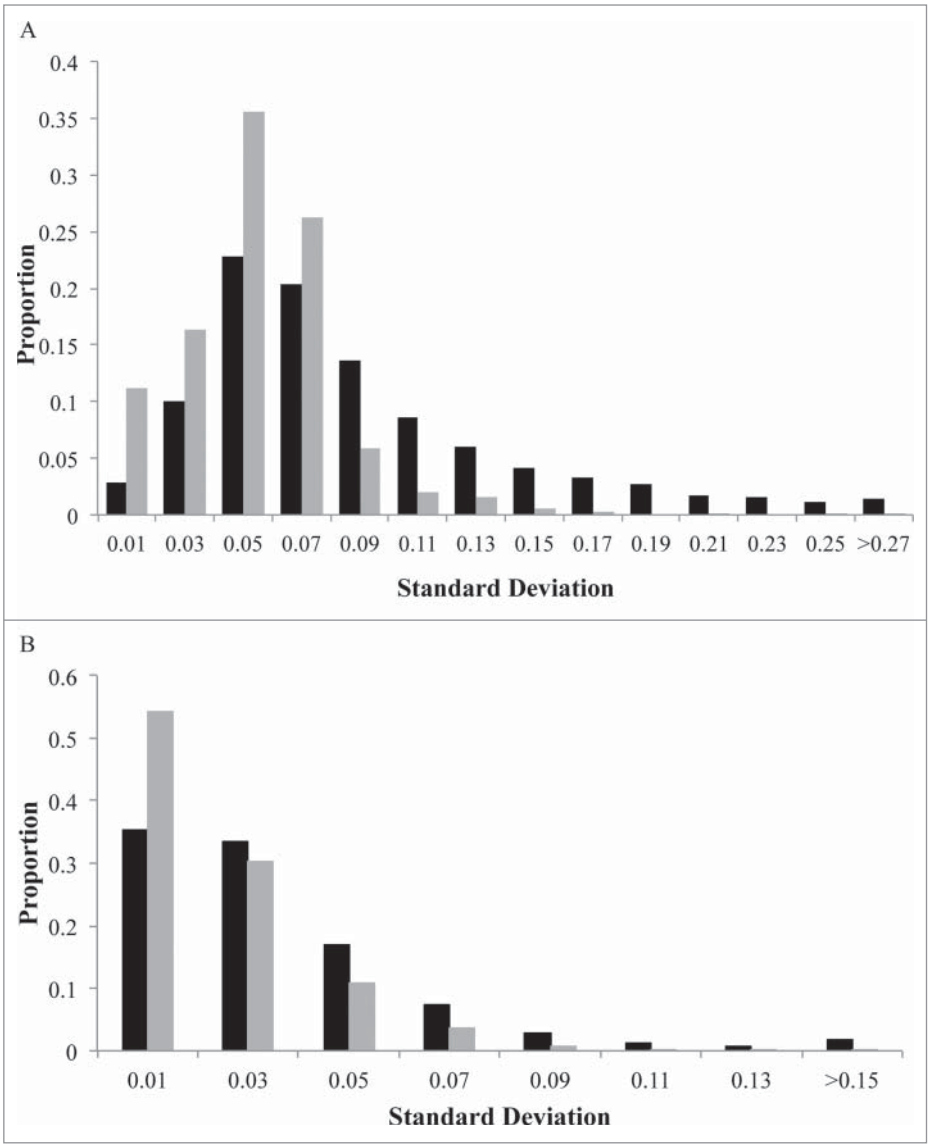

Figure 2. Distribution of maternal DNA methylation for CpG sites. The x-axis represents the standard deviation (SD) of each CpG site's methylation (β) values in maternal leukocytes. The y-axis indicates the proportion of CpG sites in bins determined by SD. Black represents CpG sites that may be attributed to genetic variation while gray represents CpG sites that cannot be attributed to genetic variation. Graph (A) depicts the distribution of correlated CpG sites (n = 5,171). Graph (B) depicts the distribution of all CpG sites (n = 479,808).

and expression patterns in maternal-fetal pairs. These results suggest a complex relationship between sequence variation, DNA methylation, and gene expression that should be considered in future studies of PTB.

Correlated CpG sites were enriched in genes involved in chronic disorders that are common in African Americans,[1,34-36,56-59] suggesting an epigenetic link between PTB and metabolic, cardiovascular, and immune dysregulation across generations. Though we made every effort to limit inclusion of clinical factors that could influence these results, such as gestational diabetes or preeclampsia, the implications of these findings are difficult to interpret. However, the results suggest that chronic disorders diagnosed subsequent to preterm delivery may not be limited to those indicated by gestational diabetes or hypertension. On the contrary, spontaneous PTB with unknown etiology may also increase maternal risk for chronic disorders during her lifetime. In general, African American women have higher levels of inflammation when compared to Caucasian women, and chronic inflammation has been presented as a potential mechanism through which disparities in the rates of PTB and other chronic conditions occur.[11,17,24,38,44,57,60] For example, Liu and colleagues examined DNA methylation of 8 imprinted genes in umbilical cord blood samples for association with PTB and infection status.[26] Though they did not find any association with PTB, they reported that pleiomorphic adenoma gene-like 1 (*PLAGL1*) DNA methylation associates with chorioamnionitis. Consistent with these results, correlated CpG sites exclusive to PTB pairs were also identified in genes involved in immune regulation. Other environmental factors, such as high BMI, psychosocial stress, smoking, and infection also increase inflammation and PTB risk. It will be important for future studies to consider these factors, as DNA methylation may mediate these relationships. For example, in an animal model, Yao and colleagues demonstrated that prenatal stress results in neuroendocrine and metabolic differences as well as shorter gestational length in subsequent generations. They linked their findings to epigenetic regulation of the placenta and other key tissues, providing a potential mechanism for inter-generational transmission of stress.[61]

This study has a number of strengths and limitations. The primary limitation is the sample size, which is, in part, due to the fact that we restricted the design to only African Americans with spontaneous PTB prior to 34 weeks gestation and uncomplicated controls. However, our cases span different categories of PTB based on GA, extremely preterm, very preterm, and moderate preterm, based on the World Health Organization classification, and it would have been ideal if there was sufficient power to examine each group individually. In this study, we did not identify any individual CpG sites that associated with PTB in maternal samples. Thus, it is reasonable to conclude that there are no CpG sites of large effect, though evaluations of larger cohorts may reveal associations of more subtle effect. However, our study was well powered to detect CpG sites whose methylation and expression levels correlated in

Table 2. Enrichment for correlation analysis of maternal and fetal blood

	Correlated	Not Correlated	*P*-value
CpG Islands	17.1%	31.3%	$< 2.2 \times 10^{-16}$
Shores	21.9%	23.2%	1.5×10^{-6}
Shelves	12.1%	9.7%	9.0×10^{-9}
Open Sea	49.0%	35.7%	$< 2.2 \times 10^{-16}$
Promoter	17.3%	25.2%	$< 2.2 \times 10^{-16}$
5′UTR	6.0%	8.9%	9.9×10^{-13}
1st Exon	2.4%	4.7%	8.9×10^{-15}
Gene Body	37.0%	33.2%	7.2×10^{-9}
3′UTR	4.9%	3.6%	1.2×10^{-6}
Intragenic	32.4%	24.3%	$< 2.2 \times 10^{-16}$
PTB	6.5%	3.8%	$<2.2 \times 10^{-16}$

maternal-fetal pairs. In time, these results may provide a foundation through which individual epigenetic patterns can be used in early pregnancy to predict risk for preterm delivery.

This is the first epigenetic study of maternal-fetal pairs for PTB and the first study of heritable CpG sites in African Americans, an understudied population with an increased risk of PTB. The results of this study support a complex genetic and environmental relationship underlying the intergenerational risk for PTB and are consistent with the hypothesis that pregnancy complications, including spontaneous PTB, may be an early indicator of future risk for mothers as well as their fetuses. Future studies should prospectively examine women who are at high risk for PTB throughout pregnancy and beyond.

Methods

Nashville Birth Cohort

All subjects were recruited at Centennial Women's Hospital and the Perinatal Research Center in Nashville, TN beginning in 2003 as part of the Nashville Birth Cohort that was established to examine biological risk factors that distinguish spontaneous preterm from term labor. Pregnant women were enrolled at the time of admission for labor at preterm or term after obtaining informed consent. Maternal demographic and clinical data (race, socioeconomic education, household income, marital status, cigarette smoking) were recorded from medical records or by interviews during the consenting process. Demographic and clinical data specific to the fetus were collected from clinical records. Gestational age (GA) was determined by maternal reporting of the last menstrual period and corroboration by ultrasound dating. Birth weight percentile was based on GA in accordance with the United States national reference.[62] Race was identified by self-reporting that traced back to 3 generations from maternal and paternal side of the fetus. Only African Americans of non-Hispanic ethnicity were included in this study.

Subjects were included in this study if they had contractions (rate of 2 contractions/10 min) leading to delivery either at preterm or at term. Cases delivered preterm with intact membranes between $24^{1/7}$ weeks and $34^{0/7}$ weeks. Controls delivered ($>39^{0/7}$ weeks) with spontaneous term labor and delivery and no current or history of pregnancy related complications including preterm birth and preterm or pre-labor rupture of

Table 3. Pathway analysis of CpG sites in genes that correlate in maternal-fetal pairs

	KEGG ID	Enrichment *P*-value Correlated N=2639	Enrichment *P*-value Genetic N=1985	Enrichment *P*-value Non-genetic N=831
Metabolic				
Type 1 diabetes mellitus	04940	0.006	0.018	NS
Fructose and mannose metabolism	00051	0.028	0.043	NS
Ether lipid metabolism	00565	0.034	0.017	NS
Type 2 diabetes mellitus	04930	0.038		NS
Glycolysis/Gluconeogenesis	00010	0.045	NS	NS
Cardiovascular				
Arrhythmogenic right ventricular cardiomyopathy	05412	0.004	0.031	0.012
Hypertrophic cardiomyopathy	05410	0.013	NS	0.022
Dilated cardiomyopathy	05414	0.015		0.033
Viral myocarditis	05416	0.020	0.041	NS
Immune				
Natural killer cell mediated cytotoxicity	04650	0.013	0.018	NS
Fc gamma R-mediated phagocytosis	04666	0.021	0.021	NS
Graft-vs.-host disease	05332	0.026	0.011	NS
Allograft rejection	05330	0.040	NS	NS
Other				
Tight Junction	04530	9.8×10^{-4}	0.001	NS
Endocytosis	04144	0.001	0.006	0.036
Focal adhesion	04510	0.012	0.011	0.034
Cell adhesion molecules	04514	0.021	0.030	NS
ECM-receptor interaction	04512	NS	NS	0.020
Regulation of actin cytoskeleton	04810	NS	NS	0.028

The enrichment *P*-value indicates whether genes with correlated CpG sites are more likely to occur in the indicated biological pathway. The group of correlated CpGs is also stratified by whether or not they can be attributed to genetic variation. NS indicates that the *P*-value is not significant.

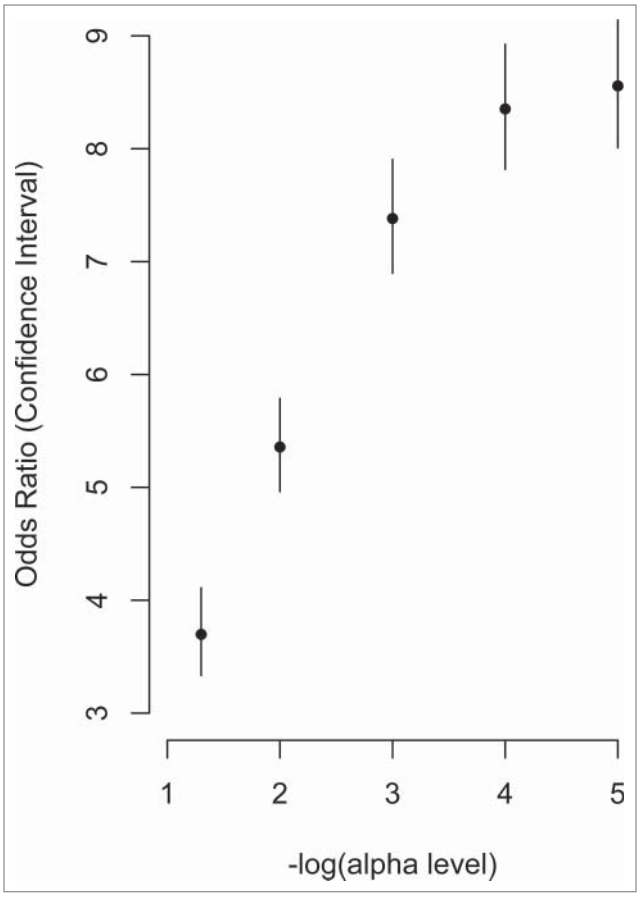

Figure 3. Correlated CpG sites and their enrichment in meQTLs. The x-axis represents increasing statistical significance of genotype-methylation associations (cis-meQTLs). The $-\log_{10}$ is used to represent the statistical threshold (α level) used to define meQTLs (i.e., 1 is equivalent to $P < 0.1$, and 2 is equivalent to $P < 0.01$, etc.). The y-axis represents the odds ratios comparing the probability that a correlated CpG site associates with a genotype (i.e., is an meQTL) with greater likelihood than an uncorrelated CpG site. The vertical lines represent the confidence interval for each odds ratio.

the membranes (pPROM). In addition, controls were excluded if they had any surgical procedures during pregnancy, were treated for preterm labor, or were treated for suspected intra-amniotic infection. Subjects who had multiple gestations, pre-eclampsia, placenta-previa, fetal anomalies, and/or medical or surgical complications during pregnancy were excluded from the study. This study was conducted in accordance with the Helsinki Declaration of 1975.

Biological Sample Collection and DNA Extraction

Maternal peripheral blood samples were collected in EDTA tubes at time of admission for labor. Blood samples were centrifuged at 3,000 rpm to separate plasma, and buffy coats were aliquoted and stored at −80°C. DNA was extracted using the Autopure automated system (Gentra Systems, Minneapolis, MN).

DNA Methylation Analysis

For each subject, >485,000 CpG sites across the genome were interrogated using the HumanMethylation450 BeadChip (Illumina, San Diego, CA).[63,64] Briefly, 1 μg of DNA from maternal leukocytes was converted with sodium bisulfite, amplified, fragmented, and hybridized on the BeadChip according to the manufacturer's instructions. CpGassoc was used to perform quality control and calculate β-values.[65] Data points with probe detection *P*-values >0.001 were set to missing, and CpG sites with missing data for >10% of samples were excluded from analysis; 479,808 CpG sites passed the above criteria. Samples with probe detection call rates <90% and those with an average intensity value of either <50% of the experiment-wide sample mean or <2,000 arbitrary units (AU) were excluded from further analysis. One sample of female DNA from a stable lymphoblast cell line (Coriell) was included on each BeadChip as a technical control throughout the experiment and assessed for reproducibility using the Pearson correlation coefficient, to ensure that Pearson correlation coefficient >0.99 for all pairwise comparisons of technical replicates. For each individual sample and CpG site,

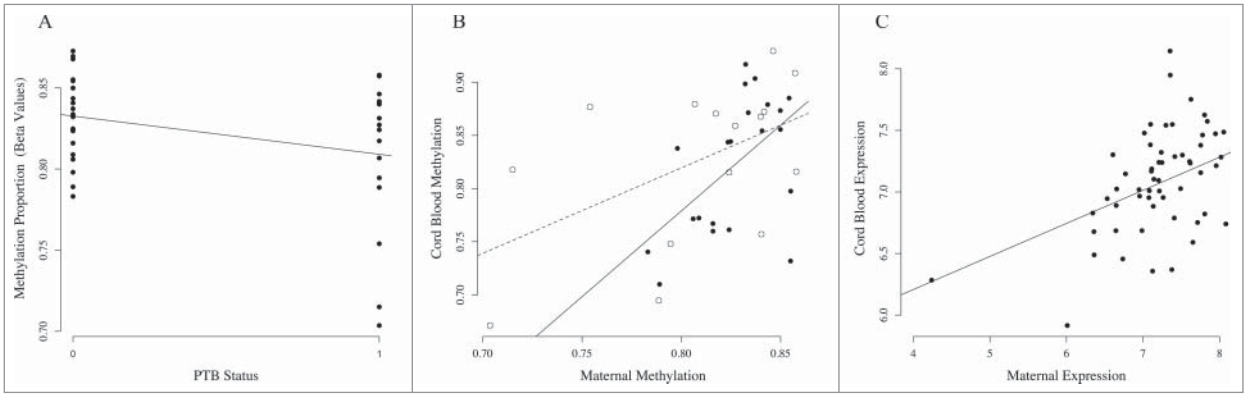

Figure 4. Association of *MICB* methylation and expression in PTB and TB pairs. (**A**) Association between cg06284756 in maternal leukocytes and PTB. The x-axis represents PTB status where 0 denotes term birth and 1 denotes PTB. The y-axis represents the methylation levels (β values) for cg06284756. (**B**) Correlation between maternal methylation (x-axis) and fetal methylation (y-axis) for cg06284756. Open circles represent maternal-fetal pairs that are preterm and closed circles represent pairs that are term. The dashed line represents correlation in PTB pairs, and the solid line represents correlation in term birth (TB) pairs. (**C**) Correlation between maternal leukocyte *MICB* expression (x-axis) and fetal leukocyte *MICB* expression (y-axis).

the signals from methylated (M) and unmethylated (U) bead types were used to calculate a β value as $\beta = M/(U+M)$.

Statistical Analysis

MethLAB was used to test for association with PTB via linear regressions that modeled β-values as the outcome and PTB as the independent variable, adjusting for maternal age, cell composition and positional effects of the array as covariates.[66] Cell type proportions were estimated using publically available data (GSE36069) as a reference panel for applying the method described by Houseman and colleagues[67,68] to our data. We examined the association between methylation of each CpG site and potential confounding factors including: birth weight percentile, gravidity, parity, infection, and smoking. In a univariate analysis, these factors did not associate with methylation of any CpG site after correction for multiple testing; thus, they were not included as covariates in the final model. Logit transformation of the β-values (i.e., M-values) did not substantially alter the results, so analyses of untransformed β are presented to ease biological interpretation and to make comparisons to our previous study.[25] For all genome-wide analyses, the False Discovery Rate (FDR) was controlled at 5% using Storey's q-value.[69] For all replication analyses, we set the significance threshold at a nominal $p < 0.05$.

To evaluate the relationship between DNA methylation in maternal and fetal (leukocytes from umbilical cord blood) samples, linear regressions compared β-values for each maternal sample to those of her fetus for each CpG site while accounting for positional effects on the array and cellular proportions. To compare the observed correlations to what would be observed if maternal and fetal methylation were completely independent, we repeated the analysis comparing each mother to an unrelated fetus that was matched for case status, positional effects, and fetal sex. We also performed an exploratory analysis that evaluated the relationship between methylation in preterm and term pairs separately.

The location of each CpG site was determined using the Illumina array annotation for the HumanMethylation450 BeadChip based on build 37 of the human genome. Chi-square tests were used to compare the number of correlated CpG sites that did or did not occur in a particular gene region (e.g., promoter, 5′UTR, body, 1st exon, 3′UTR, or intragenic regions) to the sites not associated with PTB in that gene region. We performed similar tests of enrichment for regions characterized by CpG density (islands, shores, shelves, and open seas). DAVID was used to evaluate whether groups of CpG sites, were in genes enriched for any specific biological pathways and focused specifically on KEGG pathways.[70,71]

To determine if genetic variation influenced DNA methylation, methylation quantitative trait loci (meQTL) were identified by applying the approach described previously[55] to the methylation data from African American subjects in the Grady Trauma Project.[47,72-74] Briefly, the relationship between the proportion of methylation at each CpG site and each SNP within 50 kb of that site was examined via linear regression, where methylation was modeled as a linear function of the number of reference alleles (0, 1, or 2). CpG sites were excluded from the meQTL analysis if the probe sequence contained a SNP with a minor allele frequency greater than 1% in any population, as identified from the 1,000 genomes project (TGP). [75] In total, 98,741 CpG sites had a TGP SNP within its probe sequence, and an additional 74,712 were meQTLs in an African American cohort (FDR < 0.05). We then plotted the odds ratio of whether correlated CpG sites were enriched in meQTLs at varying significance levels.

Gene expression

Correlation of gene expression was assessed using publicly available data from maternal-fetal pairs (GSE27272).[76] Expression of total RNA in umbilical cord blood and maternal peripheral blood was evaluated using the HumanRef-8v3.0 BeadChip (Illumina). The data was extracted using Illumina's BeadStudio Software v3 and then quantile normalized using Lumi.[77] For each gene containing a correlated CpG site (**Table S2**), linear regressions were used to compare expression of each maternal sample to that of her fetus to evaluate whether genes with correlated CpG sites also have correlated expression levels.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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