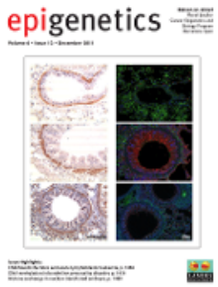

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Neonatal DNA methylation patterns associate with gestational age

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Key words: genome-wide DNA methylation, gestational age, arginine vasopressin and oxytocin

Abbreviations: GA, gestational age; FDR, false discovery rate; LMP, last menstrual period; WMHP, Women's Mental Health Program; CANDLE, Conditions Affecting Neurocognitive Development and Learning in Early Childhood

Risk for adverse neonatal outcome increases with declining gestational age (GA), and changes in DNA methylation may contribute to the relationship between GA and adverse health outcomes in offspring. To test this hypothesis, we evaluated the association between GA and more than 27,000 CpG sites in neonatal DNA extracted from umbilical cord blood from two prospectively-characterized cohorts: (1) a discovery cohort consisting of 259 neonates from women with a history of neuropsychiatric disorders and (2) a replication cohort consisting of 194 neonates of uncomplicated mothers. GA was determined by obstetrician report and maternal last menstrual period. The associations between proportion of DNA methylated and GA were evaluated by fitting a separate linear mixed effects model for each CpG site, adjusting for relevant covariates including neonatal sex, race, parity, birth weight percentile and chip effects. CpG sites in 39 genes were associated with GA (false discovery rate <0.05) in the discovery cohort. The same CpG sites in 25 of these genes replicated in the replication cohort, with each association replicating in the same direction. Notably, these CpG sites were located in genes previously implicated in labor and delivery (e.g., *AVP, OXT, CRHBP* and *ESR1*) or that may influence the risk for adverse health outcomes later in life (e.g., *DUOX2*, *TMEM176A* and *CASP8*). All associations were independent of method of delivery or induction of labor. These results suggest neonatal DNA methylation varies with GA even in term deliveries. The potential contribution of these changes to clinically significant postnatal outcomes warrants further investigation.

Introduction

Most deliveries in the US occur 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,[1] recent evidence also demonstrates an increased risk for adverse neonatal outcomes, particularly respiratory complications, with declining gestational age.[2,3] 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 with those delivered at 40 weeks.[4] The importance of gestational age in determining neonatal outcome has been repeatedly emphasized and refined as reflected by the stringent American College of Obstetrics and Gynecology guidelines for elective induction.[5] Many reports suggest that a reduction in the rate of elective deliveries prior to 39 weeks reduces neonatal morbidity and mortality.[6,7]

While the mechanisms responsible for associations between GA, birth weight and related parameters 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.[8] For example, evidence suggests that maternal nutrition can have dramatic effects on neonatal outcomes by altering offspring DNA methylation.[9]

To date most studies of neonatal DNA methylation have focused on premature or small for gestational age neonates.[10-12] Genome-wide DNA methylation in healthy, full-term neonates has not yet been examined. Therefore, despite reports of DNA methylation varying as adults age,[13-16] 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.

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Table 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)	<0.0001
Parity		0.9 (1.0)		1.1 (1.2)	NS
Sex					
Male	128 (49.0)		101 (52.1)		0.635
Female	131 (51.0)		93 (47.9)		
Child race					
Caucasian	232 (89.6)		84 (43.3)		<0.0001
African American	27 (10.4)		110 (56.7)		
GA, weeks*					
<37	18 (6.9)		12 (6.1)		NS
37–41	240 (92.7)	38.8 (1.2)	182 (93.8)	39.0 (1.3)	
>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)		0.0088

*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.

Results

Two distinct cohorts were utilized in this study. The discovery and replication cohorts were similar in terms of the distributions of GA at delivery, birth weight percentile and parity (**Table 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 < 0.05; $1.3 \times 10^{-7} < p < 9.3 \times 10^{-5}$; **Table 2 and Figs. S1 and S2**). Of these, 29 sites (70.7%) showed a decrease in methylation levels with increasing GA, while the remaining 12 (29.3%) showed an increase. Of the three neonates whose GA was estimated based on the mother's last menstrual period, one showed mild evidence of being an influential point in regression models for five of the 41 CpG sites. However, exclusion of this individual from the analysis had a negligible impact on the reported results for the 41 sites ($1.4 \times 10^{-7} < p < 9.7 \times 10^{-5}$).

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 3**). Of the 12 biological processes implicated, several were specifically involved in pregnancy and development-related genes, while the rest were involved in more general cellular processes including transcriptional regulation, signal transduction and cell adhesion. When we performed a similar analysis on identically sized gene sets generated from ten permuted data sets, an average of 4.1 independent biological processes (range = 0–10) were implicated.

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 ($1.6 \times 10^{-6} \leq p < 0.05$; **Table 2**). Among these, we observed differential methylation of genes enriched in signal transduction and response to estradiol stimulus (**Table 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 < 0.05) for the 41 CpG sites differentially methylated in the discovery cohort. Also, there were no nominally significant differences ($p < 0.05$) 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.[17] 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*.[18,19] Peripherally, AVP regulates renal water excretion and hemostasis, in part through regulating von Willebrand factor (vWF) activity,[20] and we observed differential methylation of CpG sites in glycoprotein IX (*GP9*), a component of the vWF receptor.[21]

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

Probe	Associated gene	Discovery t-statistic	Discovery p value	Replication t-statistic	Replication p value
Decreased methylation in both cohorts					
cg16536918	*AVP*	-5.39	1.9×10^{-7}	-3.81	9.4×10^{-5}
cg06051311	*TRIM15*	-5.08	7.9×10^{-7}	-4.81	1.6×10^{-6}
cg21842274	*CRHBP*	-4.95	1.5×10^{-6}	-2.36	9.7×10^{-3}
cg20994801	*PIK3CD*	-4.57	8.3×10^{-6}	-1.80	0.037
cg14409083	*EMP1*	-4.52	1.0×10^{-5}	-2.36	9.6×10^{-3}
cg25551168	*AVP*	-4.52	1.0×10^{-5}	-4.22	2.0×10^{-5}
cg13813391	*CMTM2*	-4.41	1.6×10^{-5}	-2.22	0.014
cg26267561	*OXT*	-4.35	2.1×10^{-5}	-2.38	9.1×10^{-3}
cg20291222	*CAPS2*	-4.33	2.3×10^{-5}	-2.12	0.018
cg14423778	*MBNL1*	-4.28	2.9×10^{-5}	-1.81	0.036
cg22417398	*SCYL1*	-4.26	3.0×10^{-5}	-1.91	0.029
cg01143454	*C20orf141*	-4.22	3.6×10^{-5}	-4.31	1.3×10^{-5}
cg09244244	*KIAA0372*	-4.21	3.8×10^{-5}	-3.09	1.2×10^{-3}
cg15561986	*POMT2*	-4.20	4.0×10^{-5}	-2.15	0.017
cg09523691	*ATG12*	-4.10	5.7×10^{-5}	-1.89	0.030
cg10652277	*SLC30A9*	-4.03	7.7×10^{-5}	-2.25	0.013
cg26799474	*CASP8*	-4.01	8.2×10^{-5}	-3.45	3.46×10^{-4}
cg00411097	*MGC9712*	-4.01	8.2×10^{-5}	-2.78	3.1×10^{-3}
cg16301617	*TMC6*	-4.00	8.6×10^{-5}	-2.16	0.016
Decreased methylation in discovery cohort only					
cg20337106	*C6orf139*	-5.18	4.9×10^{-7}	-1.4	0.081
cg04536922	*FAM13A1*	-4.41	1.6×10^{-5}	-1.07	0.14
cg22854223	*CD82*	-4.42	1.6×10^{-5}	-1.54	0.063
cg16545105	*CRHBP*	-4.33	2.2×10^{-5}	-0.66	0.25
cg05664072	*PER2*	-4.33	2.3×10^{-5}	-0.57	0.29
cg17530977	*GLI3*	-4.30	2.6×10^{-5}	0.88	0.19
cg13393195	*IQCD*	-4.23	3.5×10^{-5}	-0.089	0.47
cg08996521	*CISH*	-4.12	5.3×10^{-5}	-0.38	0.35
cg03395898	*TGFB3*	-4.06	6.8×10^{-5}	-1.27	0.10
cg14011734	*TATDN2*	-3.98	9.3×10^{-5}	-0.02	0.49
Increased methylation in both cohorts					
cg11540997	*DUOX2*	5.46	1.3×10^{-7}	2.78	3.0×10^{-3}
cg16098726	*GP9*	5.21	4.5×10^{-7}	2.67	4.2×10^{-3}
cg05294455	*MYL4*	5.16	5.4×10^{-7}	4.03	4.2×10^{-5}
cg27210390	*TOM1L1*	5.02	1.1×10^{-6}	3.45	3.5×10^{-4}
cg26385222	*TMEM176A*	4.86	2.3×10^{-6}	3.29	6.1×10^{-4}
cg15626350	*ESR1*	4.24	3.2×10^{-5}	2.36	9.8×10^{-3}
cg00594952	*RIMS3*	4.06	6.8×10^{-5}	3.58	2.2×10^{-4}
Increased methylation in discovery cohort only					
cg03098721	*TTLL7*	5.26	3.4×10^{-7}	1.06	0.15
cg01919208	*LAMB2*	4.19	4.1×10^{-5}	-0.25	0.40
cg12188416	*TP73L*	4.13	5.2×10^{-5}	0.64	0.26
cg12564962	*DSCR6*	4.06	7.0×10^{-5}	0.23	0.41
cg05726109	*GP1BB*	4.02	8.0×10^{-5}	1.21	0.11

Table 3. Co-occurring gene ontology results for the 41 experiment-wide significant genes in the discovery cohort

Annotations	GO term	Corrected p value*	Genes
Inner ear development	GO:0048839	2.78×10^{-5}	*GLI3*, *TGFB3*, ***DUOX2***
Female pregnancy	GO:0007565	5.66×10^{-4}	*TGFB3*, ***CRHBP***, ***OXT***
Response to estradiol stimulus	GO:0032355	5.86×10^{-4}	***ESR1***, ***OXT***, ***CASP8***
Heart development	GO:0007507	0.0022	***CASP8***, *GLI3*, ***OXT***
In utero embryonic development	GO:0001701	0.0027	*TGFB3*, *GLI3*, ***MBNL1***
Blood coagulation and platelet activation	GO:0007596 and GO:0030168	0.0057	*TGFB3*, *GP1BB*, ***GP9***
Signal transduction	GO:0007165	0.0056	***ESR1***, *FAM13A1*, ***CRHBP***, ***AVP***, ***PIK3CD***, ***OXT***
DNA-dependent transcription	GO:0006351	0.0076	*GLI3*, *TP73L*, ***ESR1***, ***SLC30A9***
Positive regulation of transcription	GO:0045944	0.011	*TGFB3*, *GLI3*, *TP73L*
Negative regulation of transcription from RNA polymerase II promoter	GO:0000122	0.013	*GLI3*, *TP73L*, *PER2*
Positive regulation of transcription from RNA polymerase II	GO:0045944	0.022	*TGFB3*, *GLI3*, *TP73L*
Cell adhesion	GO:0007155	0.033	*GP1BB*, ***GP9***, *LAMB2*

Gene names in bold associated with GA in the replication cohort. *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.

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,[22] which is released from the placenta.[23] CRHBP decreases dramatically toward the end of the third trimester releasing CRH prior to the onset of parturition.[24]

We observed increased methylation of estrogen receptor 1 *(ESR1)*, which is largely increases prior to labor,[25,26] although ESR1 decreases in the lower uterine segment as labor progresses.[27] Estrogen in umbilical cord blood positively correlates with birth weight,[28] and neonates in both the discovery and replication cohorts were, on average, below the 50th percentile for gestational age-corrected birth weight. ESR1 is activated by solute carrier family 30, member 9 (*SLC30A9* a.k.a. *GAC63*),[29] 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*),[30] immune cell maturation (transmembrane protein 176A; *TMEM176A)*,[31] and apoptosis (caspase 8; *CASP8*).[32] 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;[33] (3) differences in the umbilical cord blood processing procedures between the cohorts (<2 h vs. <24 h) 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,[34,35] 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.[36] In the discovery cohort, we evaluated the correlation between GA determined by obstetrician report and LMP (r = 0.68) and found that the estimates differed by 3.6 d 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 with 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.[37] However, repetition of the analysis on permuted data sets confirmed that our analysis implicated a greater number of biological processes than expected under random chance. Despite the limitations stated here, 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.

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 to examine the perinatal course of illness, perinatal pharmacokinetic alterations, and impact of maternal stress on offspring as part of the Specialized Center of Research on Sex and Gender Factors (SCOR) or the Translational Research Center in Behavioral Sciences (TRCBS) through Emory's WMHP were screened for inclusion in the current study. Briefly, the study enrolled women with a personal or family history of psychiatric illness and/or epilepsy prior to 14 weeks gestation. 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 1**). The inclusion criteria were: (A) maternal age >17 y 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. Exclusion criteria were: (A) unstable maternal non-psychiatric medical illnesses requiring pharmacological treatment during pregnancy (e.g., asthma, autoimmune disorders); (B) abnormal maternal thyroid stimulating hormone or (C) maternal use of lithium, stimulants or migraine medications.

Replication cohort: Conditions Affecting Neurocognitive Development and 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 1**). Solicitation for inclusion occurs by advertising in local gynecological clinics, and 1,303 women and their infants have been enrolled in the study. Maternal selection criteria for this analysis included: (A) maternal age 18–40 y; (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%; $p < 0.001$), have received a high school diploma or beyond (65.9 vs. 39.8%; $p < 0.001$), and have incomes >200% of the poverty level (53.8 vs. 43.7%; $p < 0.01$) when compared with the overall CANDLE cohort. The neonates were also less likely to be African American (56.7 vs. 67.7%; $p < 0.01$) and had a higher gestational age on average (39.0 vs. 38.7 weeks, $p = 0.03$).

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 determine whether any of these three subjects substantially influenced the association between CpG sites and GA, where Cook's distance >0.0154 (4/n, where n = 259) was considered evidence of influence in a regression model. 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.

Biological sample collection and DNA extraction. WHMP (discovery cohort) umbilical cord blood samples were collected at birth, stored on ice, and processed within 2 h of delivery. Plasma was separated by centrifugation at 4°C, and the cellular fraction was frozen at -80°C until processing. DNA was extracted from the cellular fraction at the Emory Biomarker Service Center using a Qiagen Biorobot M48. For the CANDLE study (replication cohort), whole umbilical cord blood samples were stored at 4°C and processed within 24 h of delivery. DNA was isolated using a Maxwell 16 (Promega Corp.,) automated nucleic acids extractor.

DNA methylation analysis. The HumanMethylation27 BeadChip (Illumina) was used to interrogate 27,578 independent CpG sites in 14,495 genes. Genomic DNA (1 μg discovery, 750 ng replication) was bisulfite-converted using a Zymo EZ DNA Methylation-Gold kit (Zymo Research) and analyzed using the HumanMethylation27 BeadChip procedure according

to the manufacturer's instructions (Illumina). For the discovery samples, a single female genomic DNA sample was run on each BeadChip as a technical control to assess chip quality and chip-to-chip variability in signal. Three samples with probe detection call rates <90% or with an average intensity value of either <50% of the experiment-wide sample mean or <2,000 arbitrary units (AU) were excluded from the analysis. Hierarchical clustering was performed on the β values of the remaining samples, and no outliers were evident based on global methylation patterns. Fluorescent hybridization signals representing relative levels of converted and unconverted CpGs were extracted from the Methylation Module of the BeadStudio software. For each sample, the signals from methylated (*M*) and unmethylated (*U*) bead types were used to determine a β value for each queried locus. Each β value is calculated as $\beta = M/(U + M)$ and is roughly the proportion of CpGs that are methylated at a particular locus for that sample.

Statistical analysis. *Discovery cohort.* Consistent with our previous study,[38] all analyses were based on linear models with log[β/(1-β)] as the outcome. Associations between log[β/(1-β)] and GA were evaluated continuously by fitting a separate linear mixed effects model for each of the 27,578 CpG sites with log[β/(1-β)] as the outcome and GA as the independent variable. Random effects for chip and batch were included in the model to allow for chip-to-chip differences in measurement of the proportion of DNA methylated. The models were also adjusted for neonatal sex, race, parity and maternal hypertension (chronic, pregnancy-induced, and/or preeclampsia) because they were independently associated with methylation patterns or GA. Finally, to account for the correlation between GA and birth weight, we adjusted for neonatal percentile of birth weight with respect to gender and gestational week based on a United States Natality survey.[39] In the discovery cohort, the correlation between percentile birth weight and GA is substantially less (r = 0.15) than the correlation between birth weight and GA (r = 0.49). 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. To account for 27,578 independent tests in the discovery cohort, the false discovery rate (FDR) was controlled at 0.05 using the method described by Storey et al.[40]

Gene Ontology analysis was conducted using GeneCodis 2.0[41,42] for the 39 genes that demonstrated evidence of differential methylation in the discovery cohort to evaluate if specific biological processes were enriched in the data set. 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. To account for potential limitations in this approach,[37] we also re-performed this analysis on ten identically sized gene sets generated by permutation. In each permutation, we randomly permuted GA and then re-analyzed all 27,578 CpG sites for association with GA as described above. We then selected the most significant genes from each permuted analysis to create ten gene sets of identical size to the original. For each of the ten permutation-based gene sets, we computed the number of independent biological processes with $p < 0.05$, and compared this to the number of processes with $p < 0.05$ in the original gene set.

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[β/(1-β)] 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 = 0.10 vs. r = 0.51). Because this was a replication study, we performed a one-sided test ($\alpha = 0.05$) for each CpG site to test for associations between GA and methylation in the same direction as observed in the discovery cohort.

Disclosure of Potential Conflicts of Interest

The following lifetime disclosures were reported. Dr. Smith has received research support from NIH, AFSP and Schering Plough Pharmaceuticals. Dr. Newport has received research support from Eli Lilly, GSK, Janssen, NIH, NARSAD and Wyeth, has served on speakers or advisory boards for Astra-Zeneca, Eli Lilly, GSK, Pfizer and Wyeth, and has received honoraria from Astra-Zeneca, Eli Lilly, GSK, Pfizer and Wyeth. Ms. Knight has received research support from NIH, NARSAD, Wyeth, BMS, Cyberonics, Eli Lilly, Forest, Janssen and Novartis. A family member is a GSK employee and holds GSK stock options. Dr. Stowe has received research support from NIH, GSK, Pfizer and Wyeth, has served on speakers or advisory boards for Pfizer, Eli Lilly, Wyeth, BMS and GSK, and has received honoraria from Eli Lilly, GSK, Pfizer and Wyeth. No other disclosures were reported.

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Note

Supplemental material can be found at:
www.landesbioscience.com/journals/epigenetics/article/18296

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