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Maternal vitamin D, DNA methylation at imprint regulatory regions, and offspring weight at birth, 1 year, and 3 years

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Abstract

Background/Objective—Vitamin D deficiency during pregnancy is associated with poor birth outcomes in some studies, but few have examined weight beyond birth. Additionally, little is known about how vitamin D influences DNA methylation of regulatory regions known to be involved in growth, as possible mediators to weight status in offspring.

Subjects/Methods—We conducted linear regressions to assess maternal plasma 25-hydroxyvitamin D (25(OH)D) by quartile and birth weight for gestational age *z*-score, 1-year weight-for-length *z*-score, and 3-year body mass index (BMI) *z*-score among 476 mother/infant dyads from a prospective cohort. We assessed maternal 25(OH)D and infant DNA methylation at 9 differentially methylated regions (DMRs) of genomically imprinted genes with known functions in fetal growth, including *H19*, *IGF2*, *MEG3*, *MEG3-IG*, *MEST*, *NNAT*, *PEG3*, *PLAGL1*, and *SGCE/PEG10*.

Results—Mean (standard deviation, SD) maternal 25(OH)D was 41.1 (14.2) nmol/L at a mean (SD) of 13.2 (5.5) weeks gestation. After adjustment for potential confounders, the first (Q1) and second (Q2) quartiles of 25(OH)D, compared to the fourth (Q4), were associated with lower birth

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Conflicts of Interests

The authors declare no conflicts of interest.

weight for gestational age *z*-scores (−0.43 units; CI −0.79, −0.07; p=0.02 for Q1 and −0.56 units; CI −0.89, −0.23; p=0.001 for Q2). Q1 compared to Q4 was associated with higher 1-year weight-for-length *z*-scores (0.78 units; 0.08, 1.54; p=0.04) and higher 3-year BMI *z*-scores (0.83 units; 0.11, 0.93; p=0.02). We did not observe associations between maternal 25(OH)D and methylation for any of the 9 DMRs after correcting for multiple testing.

Conclusions—Reduced maternal 25(OH)D was associated with lower birth weight for gestational age *z*-scores but higher 1-year weight-for-length and 3-year BMI *z*-scores in offspring. However, 25(OH)D does not appear to be operating through the regulatory sequences of the genomically imprinted genes we examined.

Introduction

Insufficient vitamin D levels in pregnancy have been associated with poor birth outcomes such as low birth weight and small for gestational age (SGA) (1–5), although associations are not consistent across studies. In studies in the United States (US) and the Netherlands, insufficient 25-hydroxyvitamin D (25(OH)D) levels in pregnancy were associated with an increased risk of SGA (1, 4). However, two studies observed this association among white women only (1, 6). Another study found this association among both black and white women, but the association was more pronounced for black women (7). This prior research suggests a link between lower cirulating 25(OH)D and adverse birth outcomes related to weight status, but few studies have examined associations between maternal 25(OH)D and offspring weight status beyond birth.

Three previous studies in the United Kingdom (UK) and Spain examined 25(OH)D in pregnancy and offspring weight in infancy and early childhood. One found that lower vitamin D status in pregnancy was associated with reduced fat mass in offspring at birth, but greater fat mass when children were 4 and 6 years of age (8). In a similar study, 25(OH)D3 deficiency in pregnancy was associated with an increased risk of overweight in offspring at age 1 but not age 4 years (9). A third study examined the combined effects of early modifiable risk factors on obesity at ages 4 and 6 years, including maternal obesity prior to pregnancy, excess weight gain and smoking during pregnancy, breastfeeding, and low vitamin D status in pregnancy (10). The researchers found that having a higher number of risk factors, including low vitamin D, increased the odds of overweight or obesity in children.

Mechanistic insights linking vitamin D sufficiency during gestation and offspring weight are lacking, although epigenetic changes, including DNA methylation, have been proposed (11–13). Maternal micronutrient intake may lead to epigenetic changes relevant to birth outcomes, but there are few studies with data available to support this hypothesis (14, 15). Some studies have evaluated one-carbon cycle nutrients in relation to birth weight—two reported correlations between maternal vitamin B12 concentrations with infant global methylation levels (16) and *IGF2* promoter methylation measured in umbilical cord blood (17). DNA methylation at regulatory sequences of genomically imprinted genes has also been linked to maternal folic acid intake (18, 19) and circulating folate (20). An association between choline intake in early pregnancy and lower umbilical cord blood methylation

levels among male infants has also been reported (11). Maternal 25(OH)D in mid-pregnancy has been associated with higher DNA methylation levels of regulatory sequences of the transducin-like enhancer of split 1 gene (*TLE1*) (21). Other studies have examined the relationship between 25(OH)D and DNA methylation on other health outcomes, and they suggest that 25(OH)D may have a distinct role in shaping the epigenome (21–24).

In this study, we examined relationships between maternal circulating plasma concentrations of 25(OH)D in early to mid-pregnancy and offspring birth weight for gestational age *z*-score, 1-year weight-for-length *z*-score, and 3-year body mass index (BMI) *z*-score in a racially and ethnically diverse sample of women in the US. We further sought to determine whether 25(OH)D levels were associated with altered DNA methylation among the best characterized domains associated with the imprinted genes *H19*, *IGF2*, *MEG3*, *MEG3-IG*, *MEST*, *NNAT*, *PEG3*, *PLAGL1*, and *SGCE/PEG10*, which have critical functions in regulating growth and development (20, 25–27). Methylation at the regulatory regions of these genes is known to be vulnerable to the environment, including micronutrient levels (20, 28), and these early life changes in methylation are persistent throughout the life course (29). We hypothesized that 25(OH)D deficiency would be associated with lower birth weight and higher offspring weight. In exploratory analyses, we sought to determine whether this potential relationship was mediated, in part, by alterations of DNA methylation of important growth regulatory genes.

Materials and Methods

Study subjects

Study subjects were participants in the Newborn Epigenetics Study (NEST), an ongoing birth cohort in the southeastern US, designed to assess early exposures associated with epigenetic and environmental factors that may contribute to later chronic disease. The NEST study enrolled pregnant women from one of 3 prenatal clinics in Durham, North Carolina. A primary objective of the study was to explore the relationship between epigenetic changes during pregnancy and birth outcomes. To participate, women were required to be pregnant (no gestational age criterion applied), be at least 18 years of age, speak English or Spanish, and intend to use one of the two obstetric facilities for the index pregnancy to ensure continued access to medical records (30). Detailed recruitment and retention protocols for the NEST study have been published elsewhere (25, 26, 30). After obtaining written informed consent from each participant, we collected demographic information, medical history, and pregnancy outcomes via electronic medical record review, interviews, and questionnaires. Maternal blood samples were collected in pregnancy and used to assess plasma 25(OH)D. Birth outcomes, including infant birth weight, were recorded at the time of delivery and abstracted from the medical record. The Institutional Review Board of Duke University Medical Center approved this study and all clinical investigations were conducted according to the principles expressed in the Declaration of Helsinki.

Of the 1,700 pregnant women enrolled between July 2009 and December 2011, 292 withdrew due to either death of the child before birth (n= 120), death of the infant shortly after birth (n= 3), refusing further participation (n= 66), or giving birth at a hospital outside of our geographic area (n=103). Of the 1304 (76.7%) remaining women, we measured

25(OH)D in the first 530 who agreed to provide blood samples. We excluded 54 women with missing data on birth weight, leaving a total sample of 476 women and their offspring for this analysis. We compared the 476 women included in our analysis to the 828 women in the larger NEST sample but not included in our analysis and found that included women had higher income levels (43.7% vs. 55.1% had incomes <$25 000 per year, $p<0.01$) and the sample included fewer black women (38.5% vs. 41.3%, $p<0.01$). We adjusted for these variables in statistical analyses. Our sample also had infants with higher birth weights (3 301.5g vs. 3 219.3g, $p<0.01$), infants with longer gestation lengths (39.1 vs. 38.7 weeks, $p<0.01$), and more female infants (51% vs. 47%, $p=0.05$). The two groups did not differ with respect to pre-pregnancy BMI (27.4 vs. 27.6, $p=0.40$), marital/partner status (64.1% vs. 62.8% were married/partnered, $p=0.36$), smoking during pregnancy (16.6% vs. 13.9%, $p=0.22$), and gestational age at blood draw (13.2 vs. 12.9 weeks, $p=0.40$). We also compared the 476 women included in our analysis to the 292 women who withdrew and found that women in our sample had lower education levels (45.2% vs. 55.9% had an education of some college or more, $p<0.01$) and the sample included more women who had had one or more previous births (64.1% vs. 55.5%, $p<0.01$).

Maternal blood vitamin D

Trained phlebotomists collected blood samples from women using a 10-ml lavender top Vacutainer tube (Becton-Dickinson, No. 366643) containing K2EDTA from which whole blood, plasma and buffy coat were obtained and stored in aliquots at −80°C prior to assay. The tubes were made of polyethylene terephthalate. Following removal of a 1-ml aliquot of whole blood from the Vacutainer tube, the remaining sample was centrifuged using standard protocols to separate plasma from the red cells and buffy coat. For the measurement of plasma 25(OH)D concentrations, we employed the commercially available immunodiagnostic system (IDS Fountain Hills AZ) enzyme immunoassay (cat AC57F1) (Craft Technologies, Inc, Wilson, NC, USA). This kit uses a 96-well format and is a competitive enzyme immunoassay; the 96 well microplate is coated with a sheep anti-human 25(OH)D antibody. The kit provides calibrants to create the standard curve and two controls. Values for the standard curve range from 0 to 364 nmol/L. We used twenty-five microliters of plasma to perform, in duplicate, the immunoassay for 98.8% of samples; the manufacturer's instructions were followed and %CVs of duplicates for all samples was ≤13%. In all assays, 4 quality control plasmas were included: one vitamin D deficient and one vitamin D sufficient sample provided by the kit, a CTI in-house vitamin D deficient plasma sample, and the plasma samples provided by the study. All controls were within expected ranges. The colorimetric reaction was read at 450 nm using a Molecular Devices Versamax Tunable microplate reader. We analyzed data using Softmax Pro version 3.1. We were not able to apply established cut-points from previous studies (7, 31) because most of the women in our sample were deemed deficient, based on these criteria, and very few women had levels of 25(OH)D that would be categorized as sufficient. Thus, we examined 25(OH)D in quartiles rather than categorizing by cut-points.

DNA methylation

Trained phlebotomists collected infant blood samples from umbilical cord blood at delivery using EDTA-treated acutainer tubes. The samples were centrifuged using standard protocols

to separate plasma, red cells and buffy coat. An aliquot of the buffy coat was used for DNA extraction (Qiagen, Valencia, CA, USA). All samples were stored at −80°C prior to use. DNA was extracted using Puregene reagents (Qiagen, Germantown, MD) and quantified using a Nanodrop 1000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA). We treated 800ng of maternal and infant genomic DNA with sodium bisulfite using the Zymo EZ DNA Methylation kit (Zymo Research, Irvine, CA, USA) to convert unmethylated cytosines to uracils, leaving methylated cytosines unchanged. We performed pyrosequencing using a Pyromark Q96 MD pyrosequencer (Qiagen) to measure DNA methylation at the 9 imprint regulatory regions. We measured 9 differentially methylated regions (DMRs), including *H19*, *IGF2*, *MEG3*, *MEG3-IG*, *MEST*, *NNAT*, *PEG3*, *PLAGL1*, and *SGCE/PEG10*. Pyrosequencing assay design, genomic coordinates, assay conditions and assay validation have been described previously (32, 33).

We determined percent methylation for each CpG cytosine using Pyro Q-CpG Software (Qiagen, Venlo, Limburg, The Netherlands). The DMRs in which DNA methylation was measured include two regions regulating the *IGF2* and *H19* imprinted domains (one upstream of the imprinted *IGF2* promoters and one upstream of the *H19* locus at the imprint center), two regions regulating the *MEG3/DLK1* imprinted domain (the imprint center intergenic to *DLK1* and *MEG3* and the DMR within the *MEG3* promoter region), the *PEG3* promoter region, the *MEST* promoter region, the *PEG10* promoter region from which *SGCE* is also transcribed in the opposite direction, the *NNAT* promoter, and the *PLAGL1* promoter.

Offspring weight

Main outcomes of interest were offspring birth weight for gestational age *z*-score, 1-year weight-for-length *z*-score, and 3-year BMI *z*-score. Secondarily and in more exploratory analyses, we examined birth weight, birth length, and birth weight-for-length z-score as outcomes. We abstracted information on infant birth weight in grams and length in centimeters from the medical record. We calculated birth weight for gestational age *z*-score using international reference data put forth by Intergrowth-21st Newborn Birth Weight Standards and Z Scores (34). We obtained clinician-measured weight and length data from medical records for the 1-year outcome and calculated 1-year weight-for-length *z*-scores using World Health Organization reference data (35). Trained NEST research assistants measured height and weight at age 3 years using a calibrated stadiometer (Shorr Productions, Olney, MD) and scale (Seca model 881; Seca Corporation, Havover, MD). We calculated 3-year BMI *z*-scores using Centers for Disease Control and Prevention reference data (36).

Other measures

Through maternal report by questionnaire and interview, we collected data on race/ethnicity (black, white, other race, Hispanic/Latina), age (18–20, 21–27, 28–34, and ≥35 years), smoking status (no smoking during pregnancy, smoking during pregnancy), education (<high school degree, high school degree, ≥some college), and household income (< $25,000, $25,000–49,999, ≥$50,000 per year). We extracted data on maternal parity (primiparous, multiparous), maternal pre-pregnancy BMI (weight in kg/height in m^2), and infant sex from the medical record. Pre-pregnancy weight was either self-reported by women

at the first prenatal appointment, or was based on weight at that time of the appointment if women were <18 weeks gestation. Gestation length was calculated based on the date of the last menstrual period or from the second trimester ultrasound if the two estimates differed by >10 days. We categorized season of pregnancy blood draw as winter (December, January, February), spring (March, April, May), summer (June, July, August), or fall (September, October, November).

Statistical analysis

To examine the relationship between 25(OH)D in quartiles and our weight outcomes of interest we fit separate linear regression models. We adjusted for covariates of *a priori* interest, including maternal race/ethnicity, age, smoking status, education, pre-pregnancy BMI, household income, and season of blood draw. We assessed possible non-linear trends between 25(OH)D and our weight outcomes. The Akaike Information Criterion (AICs) between the continuous model and the quartile model for 25(OH)D were essentially non-distinguishable and the linear models did not show a substantially better fit. However, at least one prior study suggested a possible U-shaped relationship between 25(OH)D and birth weight (1). Thus, based on a combination of this previous information and model fit, we modeled 25(OH)D in quartiles for all analyses. Next, we fit separate linear regression models to estimate the relationship between 25(OH)D in quartiles and each individual DMR, adjusting for the same covariates as the first step in exploring the possibility for a potential mediation analysis. We conducted all analyses using SAS version 9.4 (SAS Institute, Cary, North Carolina, US) and a significance level (α) of <0.05.

Results

Among 476 women and infants included in analyses, 183 (38.5%) identified as non-Hispanic black, 128 (26.9%) as non-Hispanic white, 45 (9.5%) identified as other or multiple race, and 120 (25.2%) as Hispanic/Latina (Table 1). Just over half of women (54.6%) had a high school diploma or less, 43.7% had household incomes <$25,000 per year, and 64.1% were married or living with a partner. The mean (standard deviation, SD) gestational age at blood draw was 13.2 (5.5) weeks; 321 (67.4%) in the first trimester, 145 (30.5%) in the second trimester, and 10 (2.1%) in the third trimester. For season of blood draw, 110 (23.1%) occurred in winter, 140 (29.4%) in spring, 139 (29.2%) in summer, and 87 (18.3) in fall. The mean (SD) level of 25(OH)D was 41.1 (14.2) nmol/L for all women in the sample. The median value for 25(OH)D was 40.82 nmol/L (interquartile range of 19.23 nmol/L). The range of 25(OH)D values was 12.28 nmol/L to ≤31.18 nmol/L for quartile 1, >31.18 nmol/L to ≤40.82 nmol/L for quartile 2, >40.82 nmol/L to ≤50.41 nmol/L for quartile 3, and >50.41 nmol/L to ≤92.75 nmol/L for quartile 4. A Kolmogorov–Smirnov test indicated no significant departure from normality for 25(OH)D (p=0.08). The mean (SD) offspring birth weight for gestational age *z*-score was 0.4 (1.0), 1-year weight-for-length *z*-score was 0.7 (1.9), and 3-year BMI *z*-score was 0.2 (1.5). Some maternal covariates identified *a priori* were significant predictors of the offspring weight outcomes, including maternal race/ethnicity, smoking during pregnancy, pre-pregnancy BMI (birth weight for gestational age *z*-score analysis only), and maternal age (birth weight for gestational age *z*-score analysis only).

After adjustment for potential confounders, the first (Q1) and second (Q2) quartiles of 25(OH)D, compared to the fourth (Q4) as reference, were associated with lower birth weight for gestational age *z*-scores (−0.43 units; −0.79, −0.07; p=0.02 for Q1 and −0.56 units; −0.89, −0.23; p=0.001 for Q2) (Table 2). When we examined weight outcomes at 1 and 3 years, Q1 of 25(OH)D only (compared to Q4) was associated with higher 1-year weight-for-length *z*-scores (0.78 units; 0.08, 1.54; p=0.04) and higher 3-year BMI *z*-scores (0.83 units; 0.11, 0.93; p=0.02). In exploratory analyses, we observed a relationship between 25(OH)D and birth weight but not birth length. For birth weight, Q1 and Q2 of 25(OH)D (compared to Q4) were associated with lower birth weights (−233.16 grams; −449.97, −17.34; p=0.03 for Q1 and −283.63 grams; −482.70, −84.56; p=0.005 for Q2). We did not observe an association between 25(OH)D and birth length (data not shown). For birth weight-for-length *z*-score, Q1 and Q2 of 25(OH)D (compared to Q4) were associated with lower birth weight-for-length *z*-scores (−0.53 units; −0.94, −0.11; p=0.01 for Q1 and −0.63 units; −1.00, −0.25; p=0.001 for Q2).

Mean (SD) methylation levels/fractions for each DMR were as follows: 47.8 (3.8) for *H19*, 51.7 (4.4) for *IGF2*, 72.5 (5.6) for *MEG3*, 49.8 (3.1) for *MEG3-IG*, 43.6 (1.5) for *MEST*, 55.3 (2.2) for *NNAT*, 36.3 (3.6) for *PEG3*, 57.7 (6.6) for *PLAGL1*, and 45.7 (5.6) for *SGCE/PEG10*. We did not observe associations between maternal 25(OH)D and methylation for any of the 9 DMRs examined after applying a conservative bonferroni correction for multiple testing, as associated p-values were not <0.005 (Table 3). Thus, we did not pursue a mediation analysis further.

Discussion

We undertook an analysis to evaluate if lower 25(OH)D was associated with birth weight and postnatal growth. We found that reduced levels of maternal 25(OH)D were associated with lower birth weight for gestational age *z*-scores but higher 1-year weight-for-length *z*-scores and higher 3-year BMI *z*-scores. This finding supports previous studies linking vitamin D and fetal growth (1–5). While we cannot exclude the possibility that the association between 25(OH)D and birth weight acts through epigenetic mechanisms, we found no relationship between 25(OH)D and infant DNA methylation at the examined imprinted gene DMRs. This suggests that it is unlikely that 25(OH)D is acting through these DMRs to influence offspring weight.

Our findings are somewhat similar to those of a study conducted by Bodnar et al. (1). In that study, lower vitamin D was associated with SGA for white but not black women living in the northern US (1). Similarly and also in the northern US, Gernand and colleagues (6) found that lower 25(OH)D was associated with an increased risk of SGA among white but not black women. Our NEST sample size precluded stratified analyses by race, but we observed associations between 23(OH)D and offspring weight in a relatively mixed sample of black and white women. Leffelaar et al. (4) observed an association between maternal 25-hydroxyvitamin D (25(OH)D) deficiency in early pregancy and risk of SGA in a sample of predominately white women from the Netherlands (4). Additionally, Burris and colleagues (7) observed an increased risk of SGA for both black and white women with lower levels of 25(OH)D in pregnancy, but noted a higher risk for black women.

However, there were some differences on how the studies categorized vitamin D. Gernand et al. used the Endocrine Society's definition of sufficiency at >75 nmol/L (6, 37). Bodnar et al. quantified their 25(OH)D exposure using cut-points and defined sufficiency as >80 nmol/L (1). In our study, 9 women had 25(OH)D values above 75 nmol/L and two had values above 80 nmol/L, therefore few women would be categorized as having sufficient 25(OH)D levels using these cut-points. Using the cut-points in the Burris study (7), as noted above 9 women in our study had 25(OH)D values above 75 nmol/L, 91 had values between 50–74 nmol/L, 259 had values between 25 and 49 nmol/L, and 50 had values less than 25 nmol/L. The mean 25(OH)D value in our sample was 41.1, which falls within the deficient range using the Endocrine's society's cut-points. We did not have enough women who fell within the sufficient category to dichotomize our exposure variable. Using the Bodnar cut-points described above, two studies of non-Hispanic white and non-Hispanic black pregnant women in the Southern US found 41% and 48% to be deficient with an additional 37% and 41% categorized as insufficient, respectively (38, 39). Another study of pregnant women in the Northeastern US found 83% of black and 47% of white women to be either deficient or insufficient in 25(OH)D using the same cut-points (31). However, cut-points for defining deficiency and insufficiency have yet to be adopted widely. While levels of 25(OH)D in our sample were on the lower end of published reference data, these 25(OH)D values are consistent with previous studies assessing levels in pregnant black women (21, 31, 38–41).

We also found that lower levels of 25(OH)D were associated with higher offspring weight at 1 and 3 years. These results are consistent with findings from two prior studies examining maternal vitamin D in pregnancy and obesity in children with longer follow-up periods at ages 4 and 6 years (8, 10). One study found that lower vitamin D status in pregnancy was associated with reduced fat mass in offspring at birth, but greater fat mass when children were 4 and 6 years of age (8). Conversely, a previous study observed associations between 25(OH)D and risk of overweight when offspring were 1 year but not 4 years of age (9). Another study examined multiple risk factors of obesity at ages 4 and 6 years, including low vitamin D status in pregnancy, and observed increased odds of overweight or obesity in children (10). Although mechanisms by which vitamin D deficiency increases obesity risk are an active topic of investigation, *in vitro* studies have shown that vitamin D inhibits expression of uncoupling protein 2 (UCP2) in adipose tissue and differentiation of pre-adipocytes (42). Thus, based on our findings and these prior studies, insufficient maternal vitamin D status in pregnancy could play a role in the development of childhood obesity.

We did not observe associations between 25(OH)D and methylation at the imprinted gene DMRs examined after correcting for multiple testing, despite their known relationship with fetal growth. Given our sample size, the relatively small number of imprinted DMRs examined, and small effect sizes expected in DMR methylation variation in otherwise healthy populations, we cannot exclude the possibility that insufficient statistical power may have contributed to our inability to detect differences. However, our data suggest a u-shaped relationship between 25(OH)D and the *MEST* DMR. Located on chromosome 7, the *MEST* regulatory DMR is integrally involved in growth and development (27, 43–45). While the exact biological function of the proteins encoded by genes in this imprinted domain are an active topic of investigation (46), there is some evidence that *MEST* may be involved in metabolic pathways that affect growth and maintenance of mesodermal cells through its

hydrolase activity (44, 45). *MEST* is monoallelically expressed from the paternal allele in a wide variety of tissues during prenatal and postnatal development and has been associated with growth retardation (47, 48). In our previous work, we found that high maternal stress during pregnancy was associated with alterations in infant DNA methylation at the *MEST* DMR (27), suggesting that this DMR is highly susceptible to adverse exposures in utero. Additionally, in infants born to women with gestational diabetes mellitus compared to infants of mothers without diabetes, decreased methylation levels at the *MEST* DMR were found in umbilical cord blood and placental tissue (43). Given the non-significant associations between 25(OH)D and the DMRs examined in our analyses, regulatory regions other than those evaluated here, or other epigenetic mechanisms, may link maternal vitamin D and fetal growth and should be explored.

There are some limitations to this study. First, the 476 NEST participants analyzed here are not entirely representative of the larger population. Women participating in the study attended one of three obstetric clinics in Durham, North Carolina that served a high percentage of low-income women. One clinic in particular cared for a large proportion of non-Hispanic white women with high-risk pregnancies. The demographic composition of our sample also included a higher representation of non-Hispanic black women than the local population. Thus, some of our results may be due, in part, to the make-up of the sample, which can limit generalizability, as non-Hispanic/Latina black women may have lower 25(OH)D levels and a higher prevalence of SGA infants (7, 31).

Next, while the majority of women in our sample had blood drawn in the first trimester (67%) with a mean gestational age of 13.2 weeks, a substantial number (31%) had blood drawn in the second trimester. A recent study found that 25(OH)D levels in late compared to early pregnancy were most associated with preterm birth in a racially and ethnically diverse sample of women from two large cohorts in the US (5). However, Farrant et al. (49) examined serum 25(OH)D concentrations in late pregnancy and found no associations with these birth outcomes among women in India. Similarly, Schneuer et al. (50) did not observe associations between first trimester 25(OH)D and SGA in a cohort of Australian women.

Finally, our immunoassay for vitamin D does exhibit minimal cross reactivity with several minor D2/D3 metabolites, which may result in negligible over estimation of total 25(OH)D levels. Of concern is the ability of analytical methodologies to discriminate between 25(OH)D and the C3 25(OH)D epimer. Recent studies suggest that most quantitative assessments of 25(OH)D are likely biased due to the presence of epimeric vitamin D metabolites. The clinical and biological relevance of epimeric vitamin D metabolites is under intensive evaluation and the necessity to distinguish epimeric from non-epimeric vitamin D forms in epidemiological studies like ours is currently being debated (48, 51). We did not differentiate individual contributions of vitamin D metabolites to 25(OH)D values. Moreover, we were not able to use NIST calibrants for our assay; NIST offers only alcohol-based calibrators for 25(OH)D, which do not work well with the EIA kits used in our study. However, previous studies have used this immunoassay (52–57) and any overestimation of 25(OH)D would apply uniformly to all women in the sample, such that there should not be any differential effect across groups.

A number of factors influence 25(OH)D status, including dietary intake of vitamin D-rich foods or single nutrient supplementation, solar ultraviolet light exposure, sunscreen and use of protective clothing to block the sun, and skin pigmentation (58). Many, but not all of these factors are modifiable through intervention. Previous intervention studies have aimed to increase dietary intake or supplementation of vitamin D in pregnancy with relatively high success (59–62). A recent meta-analysis identified 13 trials evaluating vitamin D supplementation, circulating 25(OH)D, and infant birth outcomes. Overall, supplementation increased birth weight and length but did not affect the incidence of SGA or low birth weight (62). The researchers recommended larger, better designed trials to more definitely describe these relationships to inform policy recommendations. While additional randomized studies are needed to evaluate increased dietary and supplemental vitamin D intake in pregnancy on infant outcomes such as birth weight (60–63), this study contributes to growing evidence suggesting that maternal vitamin D is important to fetal growth. Our findings support recent calls to increase 25(OH)D status through public health intervention (37, 64, 65).

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Table 1

Characteristics of Mother/infant dyads (n=476) in the Newborn Epigenetics Study

Maternal characteristics	**Mean**	**Standard Deviation**
Gestational age at blood draw, weeks	13.2	5.5
Pre-pregnancy body mass index, kg/m^2	27.4	7.0
	n	**Percent**
Parity		
Primiparous	171	35.9
Multiparous	305	64.1
Education		
≤High school degree	260	54.6
≥Some college or beyond	214	45.0
Annual household income, USD		
<$25,000	208	43.7
$25,000–49,999	53	11.3
≥$50,000	117	24.6
Age, years		
18–20	36	7.6
21–27	190	39.9
28–34	176	37.0
≥35	74	15.6
Marital status		
Never married	138	29.0
Married/partner	305	64.1
Other	28	5.9
Smoking status		
Prior to pregnancy	46	10.0
During pregnancy	79	16.6
Never smoker	341	71.6
Season of blood draw		

Maternal characteristics	**Mean**	**Standard Deviation**
Winter	110	23.1
Spring	140	29.4
Summer	139	29.2
Fall	87	18.3
Race/ethnicity		
Black	183	38.5
White	128	26.9
Other or multiple race	45	9.5
Hispanic/Latina	120	25.2
Infant characteristics	**Mean**	**Standard Deviation**
Gestational age, weeks	39.1	1.7
	n	**Percent**
Sex, female	243	51.1

Table 2

Adjusted[a] estimates and 95% confidence intervals (CI) of maternal 25(OH)D nmol/L by quartile and offspring weight in the Newborn Epigenetics Study

	Birth weight for gestational age *z*-score[b] (n=476)			1-year weight-for-length *z*-score[c] (n=211)			3-year body mass index *z*-score[d] (n=186)		
25(OH)D nmol/L	**Estimate**	**95% CI**	**P-value**	**Estimate**	**95% CI**	**P-value**	**Estimate**	**95% CI**	**P-value**
Quartile 1	−0.43	−0.79, −0.07	0.02	0.78	0.08, 1.54	0.04	0.83	0.11, 1.13	0.02
Quartile 2	−0.56	−0.89, −0.23	0.001	0.29	−0.86, 0.72	0.66	0.59	−0.07, 1.04	0.08
Quartile 3	−0.22	−0.51, 0.07	0.14	−0.09	−0.55, 0.61	0.54	−0.32	−0.92, 0.28	0.30
Quartile 4	Ref	--	--	Ref	--	--	Ref	--	--

[a] Adjusted for maternal race/ethnicity, age, smoking during pregnancy, education, pre-pregnancy body mass index, infant sex, household income, and season of blood draw.

[b] Birth weight for gestational age *z*-score calculated using Intergrowth-21st Newborn Birth Weight Standards and Z Scores reference data.

[c] 1-year weight-for-length *z*-score calculated using World Health Organization reference data.

[d] 3-year body mass index *z*-score calculated using Centers for Disease Control and Prevention reference data.

Table 3

Adjusted[a] estimates and 95% confidence intervals (CI) of maternal 25(OH)D nmol/L by quartile[b] and infant DNA methylation for 9 Differentially Methylated Regions (DMR) (n=476)

	Quartile 1			Quartile 2			Quartile 3		
DMR	**Estimate**	**CI**	**P-value[c]**	**Estimate**	**CI**	**P-value[c]**	**Estimate**	**CI**	**P- value[c]**
H19	−0.88	−2.34, 0.59	0.24	−0.95	−2.31, 0.41	0.17	−1.22	−2.43, −0.01	0.04
IGF2	−1.18	−2.84, 0.48	0.16	−0.68	−2.21, 0.84	0.38	−1.09	−2.43, 0.25	0.11
MEG3	0.77	−1.43, 2.96	0.49	0.86	−1.22, 2.93	0.42	−0.12	−1.98, 1.73	0.90
MEG3_ IG	−0.74	−2.04, 0.55	0.26	−0.26	−1.41, 0.90	0.66	−0.76	−1.77, 0.25	0.14
MEST	−1.72	−3.90, 0.47	0.12	−0.30	−2.27, 1.67	0.77	−2.07	−3.83, −0.31	0.02
NNAT	−0.12	−3.06, 2.82	0.94	−0.45	−3.21, 2.32	0.75	−2.26	−4.68, 0.17	0.07
PEG3	0.53	−0.77, 1.82	0.43	0.40	−0.80, 1.61	0.51	0.51	−0.56, 1.57	0.36
PLAGL1	−0.66	−3.22, 1.89	0.61	−0.33	−2.69, 2.02	0.78	−0.94	−3.05, 1.17	0.38
SGCE/ PEG10	−0.44	−2.52, 1.63	0.67	−0.87	−2.76, 1.02	0.36	−1.32	−3.01, 0.37	0.12

[a] Adjusted for maternal race/ethnicity, age, smoking during pregnancy, education, pre-pregnancy body mass index, infant sex, household income, and season of blood draw.

[b] Reference was Quartile 4 maternal 25(OH)D nmol/L.

[c] Significance level of <0.005 used with Bonferroni correction for multiple testing.