Maternal vitamin D predominates over genetic factors in determining neonatal circulating vitamin D concentrations

Boris Novakovic, John C Galati, Anna Chen, Ruth Morley, Jeffrey M Craig, and Richard Saffery

ABSTRACT

Background: There are multiple potential regulators of neonatal vitamin D status of environmental, genetic, and epigenetic origins. The relation between these factors and circulating neonatal vitamin D has yet to be fully characterized.

Objective: The aim of this study was to examine the relative contribution of genetic factors, maternal circulating 25-hydroxyvitamin D [25(OH)D] concentrations, and the placental methylation level of the gene that encodes the primary catabolic enzyme of active vitamin D [25(OH)D-24-hydroxylase encoded by CYP24A1] to neonatal 25(OH)D concentrations.

Design: We used the classical twin study design to determine the genetic contribution to neonatal 25(OH)D. A total of 86 twin pairs (32 monozygotic and 54 dizygotic twin pairs) were included in this study. Serum 25(OH)D was measured by using a 25(OH)D kit. CYP24A1 promoter DNA methylation was measured by means of matrix-assisted laser desorption time-of-flight mass spectrometry.

Results: Maternal and neonatal 25(OH)D showed a strong association ($R^2 = 0.19$). Monozygotic and dizygotic within-pair serum 25(OH)D correlations were similar ($R^2 = 0.71$ and 0.67, respectively), which suggested no genetic effect. Placental CYP24A1 methylation did not show an association with maternal or neonatal 25(OH)D concentrations.

Conclusions: Our results suggest that maternal circulating 25(OH)D is the most significant regulator of neonatal circulating 25(OH)D concentrations, with underlying genetic factors playing a limited role. The placental methylation of the CYP24A1 promoter appears subject to a genetic influence, although no evidence of a relation between the methylation level of this gene and circulating maternal or neonatal 25(OH)D was apparent. Am J Clin Nutr 2012; 96:188–95.

INTRODUCTION

Vitamin D is a pleiotropic secosteroid hormone with important roles in the regulation of calcium homeostasis, cell proliferation, cell cycle control, and immune function (1). Vitamin D is also thought to play important roles during pregnancy, such as in the facilitation of the transport of several nutrients across the placenta, including calcium, and the modulation of the maternal immune response (2–4). Deficiency during pregnancy has been implicated as a risk factor of adverse outcomes including pre-eclampsia (5–7). Given these potentially important roles in the maintenance of health, interest in determining the factors that regulate circulating vitamin D concentrations has intensified in recent years.

Humans primarily acquire vitamin D precursors from diet and exposure to sunlight, and the relative contribution of these 2 factors is dependent on season and latitude (8). The vitamin D precursor (vitamin D$_3$) is hydroxylated to the major circulating form 25-hydroxyvitamin D [25(OH)D]$^\alpha$ in the liver by 25-hydroxylase. 25(OH)D is further hydroxylated in the kidney to the hormonal (active) form 1,25-dihydroxyvitamin D [1,25(OH)$_2$D$_3$] by 25(OH)D-1z-hydroxylase (encoded by CYP27B1) (9). The catabolism of both 25(OH)D and 1,25(OH)$_2$D$_3$ into less-active metabolites is carried out by 25(OH)D-24-hydroxylase (encoded by CYP24A1). 1,25(OH)$_2$D$_3$ regulates its own metabolism via a feedback loop, such that elevated concentrations induce the expression of CYP24A1 with concomitant downregulation of CYP27B1. This process results in a reduction of both 25(OH)D and 1,25(OH)$_2$D$_3$ (10).

During pregnancy, this process becomes uncoupled, resulting in elevated maternal concentrations of circulating 1,25(OH)$_2$D$_3$ (11). We have previously speculated that this process involves the placenta-specific methylation of the CYP24A1 gene (12) that directly attenuates the 1,25(OH)$_2$D$_3$ responsiveness of this gene in placental cells (13), potentially as a mechanism to maximize the transfer of 25(OH)D to the fetal circulation. Maternal 25(OH)D concentrations are the only source of vitamin D early in pregnancy and have been shown to correlate with neonatal concentrations at birth (14–16). Despite this, the potential role of
the placenta in the regulation of the transfer of 25(OH)D from maternal to fetal circulation remains unclear.

Previous twin studies have provided compelling evidence for a role of genetic factors in the determination of circulating 25(OH)D concentrations in adults. Such studies have been based on the comparison of genetically identical monozygotic twins with dizygotic pairs that share, on average, 50% of their genetic variation; the greater the difference in concordance within monozygotic pairs compared with dizygotic pairs, the greater the genetic effect. Adult monozygotic twins show a higher concordance for serum 25(OH)D concentrations than do dizygotic twins in winter months (17, 18) but not in summer when sunlight exposure is higher. However, the opposite effect has also been reported (19). Furthermore, recent genome-wide association studies have associated single nucleotide polymorphisms in CYP2R1 and CYP24A1 genes with 25(OH)D concentrations in adults (20, 21), which suggested a direct effect of genetic variation on circulating concentrations. Equivalent data are not available for neonates or children.

Thus there are multiple potential regulators of neonatal 25(OH)D status of environmental, genetic, and epigenetic origin. In this study, we have used a twin study design to examine the contribution of genetic factors, circulating maternal 25(OH)D, and placental DNA methylation status of the CYP24A1 gene to neonatal 25(OH)D concentrations.

SUBJECTS AND METHODS

Sample collection

Women who were pregnant with twins were recruited from 3 major Melbourne hospitals as part of the Peri/post-natal Epigenetic Twins Study (PETS) (22). Sample collection from twins at the time of delivery was carried out with appropriate human ethics clearances from the Royal Women’s Hospital (06/21), Mercy Hospital for Women (R06/30), and Monash Medical Centre (06117C). Of 251 women recruited, 106 maternal serum samples (maternal and both twins) were available in 86 instances (32 monozygotic and 54 dizygotic twin pairs). Matched serum samples from twins were available in 86 instances (32 monozygotic and 54 dizygotic), and placental tissue was available for 68 of these twin pairs. Zygosity was determined with a 12-marker microsatellite test (23) by using DNA from cord and/or buccal samples (22). The features of the study population are described in Table 1.

Serum 25(OH)D measurement

Serum circulating vitamin D [25(OH)D] concentrations were measured by using the LIAISON 25-OH Vitamin D Total kit (DiaSorin) at the Monash Medical Centre Pathology Department. This methodology has recently been validated as an accurate approach for the measurement of circulating 25(OH)D in serum because, unlike the HPLC tandem mass spectrometry method, it does not detect 3-epi-25(OH)D3, which was recently identified in the majority of human serum samples (24). The interassay CV was 8.2% at 38 nmol/L. The laboratory routinely participates in the Royal College of Pathologists of Australasia & Australasian Association of Clinical Biochemistry Quality Assurance Program for 25(OH)D measurement. Over the duration of this study, for a target value set by using an isotopic dilution tandem mass spectrometry of 43 nmol/L (specimens 35–10, 36–07, and 36–12), the laboratory achieved values that ranged from 47 to 49 nmol/L. For a target value of 118 nmol/L (specimens 35–11, 36–02, and 36–11), values from 108 to 112 nmol/L were achieved.

DNA extraction and bisulphite conversion

DNA was extracted by using the standard phenol-chloroform method, and bisulphite was converted by using a Methyl Easy bisulphite modification kit (Human Genetic Signatures) according to the manufacturer’s instructions, as previously described (25). All samples within individual twin pairs were processed in parallel to reduce the potential variation caused by the conversion process.

Polymerase chain reaction amplification and methylation analysis

The DNA methylation level at the CYP24A1 promoter region was quantified by using the Sequenom MassARRAY EpiTYPER platform (Sequenom Inc) as previously described (25, 26). Primer pairs for amplification were designed by using the SEQUENOM EpiDesigner Web tool (http://www.epidesigner.com) to target the same region previously covered in Novakovic et al (13). See Figure S1 under “Supplemental data” in the online issue for an illustration of analyzable cytosine-phosphate-guanine dinucleotide (CpG) sites within the CYP24A1 assay. The CYP24A1 promoter region was targeted by using the following primers: forward 5’ aggaagagagTGTTTAGTAGTTGGTTAGTTGGTTGGG 3’ and reverse 5’ cagataatgactactatagggagaagct- AACTAACCCCTTTACTCCTTTCC 3’.

Statistical analysis

The association of placental CYP24A1 DNA methylation with maternal circulating 25(OH)D was assessed by using linear regression with robust SEs used to account for the potential

<table>
<thead>
<tr>
<th>Characteristics of the study population</th>
<th>Monozygotic pairs</th>
<th>Dizygotic pairs</th>
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<tr>
<td>Chorionicity</td>
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<tr>
<td>Dichorionic</td>
<td>9</td>
<td>54</td>
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<tr>
<td>Monochorionic</td>
<td>23</td>
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<tr>
<td>Sex</td>
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<tr>
<td>F/F</td>
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<td>14</td>
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<tr>
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<tr>
<td>M/F</td>
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<tr>
<td>GA (wk)</td>
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<td></td>
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<tr>
<td>Median ± SD</td>
<td>36 ± 1.79</td>
<td>37 ± 1.06</td>
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<tr>
<td>Minimum–maximum</td>
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<td>33–38</td>
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<tr>
<td>BW (kg)</td>
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<tr>
<td>Median ± SD</td>
<td>2.44 ± 0.41</td>
<td>2.69 ± 0.39</td>
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<tr>
<td>Minimum–maximum</td>
<td>1.25–3.18</td>
<td>1.44–3.95</td>
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<tr>
<td>BW discordance (kg)</td>
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<tr>
<td>Average ± SD</td>
<td>0.29 ± 0.21</td>
<td>0.34 ± 0.29</td>
</tr>
<tr>
<td>Minimum–maximum</td>
<td>0.03–0.88</td>
<td>0.01–1.10</td>
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* A total of 172 newborn twins (86 pairs) were included in the study, which consisted of 32 monozygotic and 54 dizygotic twin pairs. BW, body weight; GA, gestational age.
within-baby and within-twin-pair correlation of methylation levels. Methylation values at each CpG unit and maternal circulating 25(OH)D concentrations were normalized before analysis by subtracting the mean and dividing by the sample SD of each variable. Therefore, the linear regression coefficient estimated the increase in methylation SDs across the assay for each 1-SD increase in maternal 25(OH)D. These linear regressions were performed with Stata 11 (2009; StataCorp). All other analyses were performed with the R software package version 2.13.1 (R Development Core Team; http://cran.r-project.org/), including intraclass correlation (ICC) analysis and the generation of figures.

RESULTS

Circulating maternal 25(OH)D at 28 wk gestation predicted neonatal 25(OH)D concentrations

As part of the PETS (22), we collected maternal peripheral blood and cord blood samples and placental biopsies from a subset of 251 twin and mother trios. Maternal circulating 25(OH)D concentrations ranged from 13 to 102 nmol/L, whereas neonatal circulating 25(OH)D concentrations ranged from 12.9 to 138 nmol/L. Within-pair differences in cord blood ranged from 0.1 to 53 nmol/L, whereas the biggest difference between maternal and corresponding neonatal 25(OH)D concentrations was 109 nmol/L (Table 2). In agreement with previous studies (14–16), we showed a clear correlation between maternal and neonatal 25(OH)D concentrations ($R^2 = 0.19$), with a 0.5-SD increase in average cord 25(OH)D for each 1-SD increase in maternal 25(OH)D ($P < 0.001$, Student’s $t$ test) (Figure 1). A trend toward higher circulating 25(OH)D in newborns relative to their mothers was also observed, especially in the lower range of maternal concentrations. To examine this effect further, the 86 twin pairs were separated into 4 groups (quartiles) on the basis of maternal circulating 25(OH)D concentrations [quartile 1: 13–46 nmol/L; quartile 2: 48–58 nmol/L; quartile 3: 58–67 nmol/L; and quartile 4: 68–102 nmol/L] and neonatal circulating 25(OH)D concentrations plotted next to maternal 25(OH)D concentrations for each quartile (Figure 2). At low maternal concentrations (lowest quartile), neonatal concentrations were generally higher than those of mothers ($P < 0.001$; Student’s $t$ test), whereas at higher maternal concentrations, this association was not apparent.

TABLE 2
Maternal and neonatal circulating 25(OH)D characteristics of monozygotic and dizygotic births

<table>
<thead>
<tr>
<th></th>
<th>Monozygotic maternal</th>
<th>Monozygotic neonatal</th>
<th>Dizygotic maternal</th>
<th>Dizygotic neonatal</th>
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<tbody>
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<td>Circulating 25(OH)D (nmol/L)</td>
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<tr>
<td>Median ± SD</td>
<td>55.50 ± 15.18</td>
<td>60.70 ± 17.18</td>
<td>58 ± 19.86</td>
<td>60.90 ± 24.04</td>
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1 25(OH)D, 25-hydroxyvitamin D.
Minimal evidence of genetic and mode-of-placentation variation in the regulation of neonatal 25(OH)D concentrations

The demonstrated relation between maternal and neonatal circulating 25(OH)D was reflected in strong correlations within twin pairs ($R^2 = 0.68$). When separated by zygosity [monozygotic ($n = 32$ pairs) and dizygotic ($n = 54$)], within-pair correlations remained high with little variation between classes ($R^2 = 0.71$ and $0.67$, respectively) (Figure 3). Similarly, the calculation of ICCs for monozygotic (0.91) and dizygotic (0.90) twins revealed no increase in the variation of vitamin D concentrations in association with increased genetic variation, although monozygotic twins as a group were slightly more similar than their dizygotic counterparts. Thus, any genetic effect in the monozygotic twins was likely to be minimal relative to the effect of maternal circulating 25(OH)D concentrations.

To examine a potential effect of chorionicity on vitamin D concentrations within pairs, correlations were calculated for monozygotic monochorionic (shared placenta; $n = 23$ pairs) and dichorionic (separate placenta; $n = 9$) separately. Monozygotic and dichorionic twins showed a similar within-pair correlation, with a slightly lower correlation ($R^2 = 0.68$) shown in monochorionic twins than in dichorionic pairs ($R^2 = 0.77$) (see Figure S2 under “Supplemental data” in the online issue). This result suggested that chorionicity may be of minimal importance in the determination of neonatal 25(OH)D status, but this possibility requires additional investigation in a larger sample size.

Placental 25(OH)D-24-hydroxylase gene (CYP24A1) methylation levels were regulated by genetic factors

We previously demonstrated intermediate concentrations (average ± SD: 56.5 ± 10.5%) of placenta-specific CYP24A1 methylation (13). In the current study, we analyzed DNA methylation levels in placenta tissue of 86 twin pairs for whom cord blood 25(OH)D concentrations were also available. The mean methylation level in twin placental tissue was 0.38 (38%; see Figure S1 under “Supplemental data” in the online issue). Individual CpG units within the assay showed variations in mean $\beta$ methylation that ranged from 0.26 (CpG_7.8) to 0.52 (CpG_5) (see Figure S1 under “Supplemental data” in the online issue). The within-pair correlation of the CYP24A1 assay mean methylation for 87 twin pairs (36 monozygotic and 51 dizygotic) was $R^2 = 0.46$ for monozygotic pairs and 0.14 for dizygotic pairs (Figure 4).

Similarly, ICC measurements were higher in monozygotic than in dizygotic pairs (0.70 and 0.48, respectively). Individual CpG sites within the assay showed variation in the degree of correlation that ranged from 0.031 (CpG_24) to 0.467 (CpG_17) (see Table S1 under “Supplemental data” in the online issue).

The ICC measure tests the degree of similarity within pairs, relative to the overall variation within the given group (monozygotic or dizygotic twins). An alternative, nonrelative measure is the absolute methylation discordance, which was also calculated for each CpG unit (see Figure S3 under “Supplemental data” in the online issue). The vast majority of absolute within-pair $\beta$-value discordances ($\Delta\beta$) were <0.2 in both monozygotic and dizygotic twin pairs. The median $\Delta\beta$ was lower in monozygotic than in dizygotic pairs in 7 of the 9 CpG units examined, with the exceptions of CpG_2.3.4 and CpG_20.21, and also when $\Delta\beta$ was calculated from methylation averaged (for each infant) over the entire assay ($P = 0.02$).

Placental CYP24A1 methylation levels were not correlated with maternal or neonatal 25(OH)D

Maternal 25(OH)D concentrations were not correlated with absolute CYP24A1 methylation ($R^2 = 0.01$) (Figure 5) or CYP24A1 methylation discordance ($R^2 = 1 \times 10^{-3}$; see Figure S4 under “Supplemental data” in the online issue). These results failed to reveal a relation between maternal 25(OH)D concentrations and CYP24A1 methylation, and it is unlikely that small differences in CYP24A1 methylation contribute to maternal 25(OH)D concentrations in healthy human pregnancies. Furthermore, we showed no evidence of an association between neonatal 25(OH)D concentrations and mean concentrations of CYP24A1 promoter methylation ($R^2 = 1 \times 10^{-6}$; see Figure S5 under “Supplemental data” in the online issue) or specific methylation levels at individual CpG sites. The possibility that CYP24A1 methylation variation may be associated with a variation in neonatal 25(OH)D concentrations was also investigated, and no evidence of an association was shown ($R^2 = 0.02$; see Figure S6 under “Supplemental data” in the online issue). However, a weak association between methylation and 25(OH)D discordance was observed for one CpG site (CpG_18) in the
absence of correction for multiple testing or an assessment of the correlation between methylation states at individual CpG sites (Table 3).

DISCUSSION

Of the 251 twin and mother trios recruited in the PETS cohort (22), we selected trios for whom maternal and fetal serum was available and placental tissue was collected, which reduced the number to 106 twin and mother trios. Serum samples from both twins were available for 86 trios within this group from 32 monozygotic and 54 dizygotic pairs. Although there is substantial controversy as to concentrations of 25(OH)D that could be described as deficient on the basis of criteria of vitamin D adequacy of ≥50 nmol/L as defined in the recent Institute of Medicine report (8), 31% of 106 mothers in this study were likely to be vitamin D deficient. In addition to the well-documented link between vitamin D deficiency and rickets, there are emerging data, albeit far from conclusive, that link vitamin D deficiency to several complex disorders (1, 27, 28). Despite this, the current
Institute of Medicine guidelines warn against the concept that more is better by highlighting that there is no evidence that higher concentrations confer greater health benefits (8).

The mean circulating maternal 25(OH)D concentration in all mothers was 56.5 nmol/L, which was similar to a previously reported mean of 48 nmol/L in a South Australian singleton birth cohort (16). Maternal circulating 25(OH)D (measured at 28 wk gestation) correlated with neonatal 25(OH)D concentrations \( (R^2 = 0.19) \) as previously reported (14, 16). These findings suggested that vitamin D homeostasis in twin pregnancies does not differ significantly from that in singleton pregnancies. Interestingly, we observed that neonatal 25(OH)D concentrations were generally higher than maternal concentrations (slope: \( y = 0.52x + 32.86 \); Figure 1), which was also evident in the analysis by Thomas et al (16), which showed a slope of \( y = 0.42x + 44.25 \). This effect was stronger in cases in which maternal 25(OH)D concentrations were very low (Figure 2), with higher neonatal than maternal concentrations in the lowest maternal quartile (quartile 1) \( (P = 0.02) \). This result suggested that, although maternal concentrations are critical in the determination of neonatal circulating 25(OH)D, the fetus also has some capacity for self-regulation.

To determine the contribution of underlying genetic factors to neonatal 25(OH)D concentrations, twin pairs were separated into monozygotic and dizygotic groups. The 2 groups did not differ materially in gestational age, birth weight, or circulating concentrations of 25(OH)D (Tables 1 and 2). In our study, monozygotic and dizygotic twins showed similar circulating 25(OH)D correlations \( (R^2 = 0.70 \text{ and } 0.67, \text{ respectively}) \), which suggested that the genetic contribution to neonatal 25(OH)D concentrations during in utero development is minimal relative to the shared environmental factor of maternal 25(OH)D concentrations. In addition, no evidence of a sex-specific or gestational effect of neonatal 25(OH)D concentrations was observed. Other demographic data were not tested.

Adult twin studies have previously shown that underlying genetics play a minimal role in the determination of circulating 25(OH)D concentrations during summer months when sunlight exposure is high and a greater role during the winter months when...
sunlight exposure (and, therefore, vitamin D availability) is low (17, 18). Our data supported a general capacity for the fetus to self-regulate 25(OH)D concentrations to some extent, but we showed no evidence that this was genetically regulated, which was most likely a result of the limited sample size. It would be interesting to reinvestigate the fetal capacity for self-regulation in the future by using a larger group of mothers pregnant with twins.

Two-thirds of monozygotic twins are monochorionic (share a single placenta) (29). Monozygotic monochorionic twin pairs have an increased risk of intrauterine growth restriction, fetal death, and twin-twin transfusion syndrome (29–32). Therefore, we tested the effect of sharing or having separate placentas (chorionicity) on neonatal 25(OH)D concentrations by separating monozygotic pairs into monochorionic (n = 23) and dichorionic (n = 9) groups. In our analysis, monochorionic and dichorionic pairs showed a similar concordance for 25(OH)D concentrations and, therefore, provided no evidence to suggest that the mode of placentation is a determinant of neonatal 25(OH)D concentrations. Although this result was not unexpected, because no differences were observed between monozygotic and dizygotic twin pairs, this conclusion was based on a small number of samples.

We previously reported placenta-specific, methylation-mediated downregulation of the CYP24A1 gene in the human placenta (13). Such methylation is only seen elsewhere in association with malignancy (33, 34). Several twin-based studies have supported a role of genetics in the determination of methylation status at specific loci, particularly imprinted regions (35, 36). We measured the contribution of genetic and environmental/stochastic factors to the CYP24A1 promoter methylation level to determine the likelihood that it could be involved in neonatal vitamin D homeostasis independently of the DNA sequence. Monozygotic twins generally showed a higher concordance for CYP24A1 methylation across the assay [measured as either ICC (monozygotic = 0.7; dizygotic = 0.48) or a correlation coefficient (monozygotic = 0.46; dizygotic = 0.13)]. Therefore, it seems highly likely that DNA methylation levels at this promoter region are under genetic control despite the variation in correlations at several individual CpG sites within the assay that were indicative of the influence of nonshared environments or random or stochastic events.

The placental DNA methylome has been investigated for >2 decades and revealed several unique properties (37–41). In recent years, several studies have examined the potential relation between placental DNA methylation patterns and specific maternal environments or fetal outcomes. Abnormal gene placental methylation patterns have been associated with growth restriction (42, 43) and early-onset preeclampsia (44, 45), whereas methylation at repetitive elements (46), WNT2 (47), and the glucocorticoid receptor gene (48) have been linked with birthweight.

We hypothesized that the methylation of the CYP24A1 promoter in the placenta may represent a pregnancy-specific mechanism to facilitate the transfer of 25(OH)D from maternal to neonatal circulations by preventing its degradation in the placenta (12, 13). The cumulative data we have produced do not support such a relation because there was no evidence of an association of CYP24A1 methylation with either neonatal or maternal 25(OH)D concentrations. However, we have only examined this relation at birth and not earlier in gestation when such a relation might be anticipated to be stronger. In addition, it is possible that the CYP24A1 regulation of vitamin D metabolism plays a role in placental function independent of any transport function. Additional studies are warranted to test these possibilities.

In conclusion, to our knowledge, this is the first study to examine the contribution of genetic and environmental/stochastic factors to neonatal 25(OH)D concentrations. Previous twin studies in adults have shown that the genetic contribution is more important during winter months when vitamin D availability is low. Our analysis revealed no differences in 25(OH)D concordance between monozygotic and dizygotic twins, which suggests that neonatal 25(OH)D concentrations are not influenced greatly by underlying genetic factors. However, the caveat to this finding was the relatively low sample size. Our results indicate that neonatal 25(OH)D concentrations are primarily controlled by maternal circulating concentrations with some evidence that underlyings genetics may play a greater role in neonatal vitamin D homeostasis in a situation of maternal vitamin D deficiency. The role of underlying genetics in situations of maternal vitamin D deficiency will require further investigation.

REFERENCES

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