MATERNAL VITAMIN D STATUS RELATED TO TRIACYLGLYCEROL IN EARLY PREGNANCY AND SUBSEQUENT RISK FOR ADVERSE PREGNANCY OUTCOMES

By

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ABSTRACT

Research has suggested roles of vitamin D in health beyond its action on calcium homeostasis and bone health. Recent studies revealed a high proportion of pregnant women having low vitamin D status. This may lead to increased risks for preeclampsia, gestational diabetes (GDM), and cesarean delivery. Findings on the effects of vitamin D on these adverse pregnancy outcomes have been inconsistent. To our knowledge, no studies have examined maternal vitamin D status with circulating triacylglycerol (TAG) concentrations.

This study was conducted to assess the effects of maternal vitamin D status on subsequent risk for pregnancy complications and to determine the effectiveness of 25-hydroxyvitamin D [25(OH)D] to TAG ratio to indicate vitamin D status. We measured the plasma 25(OH)D and TAG concentrations of 299 pregnant women in their 8^{th} to 20^{th} week of gestation, and examined the association between 25(OH)D concentrations, 25(OH)D/TAG ratios and the risk of preeclampsia, GDM, and cesarean delivery.

Of the 299 subjects, five developed preeclampsia, 15 developed GDM, and 89 delivered their infants by cesarean section. Women diagnosed with preeclampsia or GDM had significantly lower 25(OH)D/TAG ratios than women without these complications. Women with 25(OH)D concentrations and 25(OH)D/TAG below medians had increased odds of preeclampsia (OR, 4.05; 95% CI, 0.45-36.71 and OR, 4.05; 95% CI, 0.45-36.71 respectively) and GDM (OR, 2.12; 95% CI, 0.71-6.38 and OR, 2.96, 95% CI, 0.92-9.55 respectively). These results were not statistically significant because of the small number of affected women, but the association between 25(OH)D/TAG and GDM risk was close to significant ($P = 0.07$). Women with 25(OH)D concentrations and 25(OH)D/TAG below medians also had reduced odds for cesarean delivery (OR, 0.54; 95% CI, 0.32-0.89 and OR, 0.74; 95% CI, 0.45-1.22 respectively), but only the

association between 25(OH)D concentration and risk of cesarean delivery was statistically significant.

This study suggested increased preeclampsia and GDM risks in women with low vitamin D status. Few cases of these events compromised the statistical significance of results. The increased risk of cesarean delivery in women with higher vitamin D status shown has to be reevaluated because reasons for cesarean delivery were not included in the analysis.

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Chapter 1

INTRODUCTION

Studies has shown vitamin D to be an important nutrient for skeletal growth and bone health (1). It helps to maintain calcium homeostasis by increasing intestinal absorption and renal reabsorption of calcium and phosphorus, regulating parathyroid secretion, and regulating bone calcium mobilization (2). Vitamin D deficiency results in rickets in children and osteomalacia in adults (1). The presence of vitamin D receptors in the pancreas, T lymphocytes and other tissues suggests that vitamin D may have other beneficial effects on health besides bone health, such as immunomodulation, cell differentiation, and muscle strengthening (2, 3).

Several studies (4-7) examined the vitamin D status of pregnant women in the US since 2007. They showed poor vitamin D status of US women during early pregnancy. Studies found an inverse association between maternal vitamin D status during pregnancy and risk for preeclampsia (8-11), gestational diabetes (12-14), and cesarean section (15). There are relatively few studies done, and their findings are inconsistent. Additional studies are needed to support the relationship between maternal vitamin D status and these pregnancy outcomes.

Maternal circulating triacylglycerol (TAG) increases dramatically with weeks of gestation (16). The assessment of maternal vitamin D status may not be accurate without taking the significant increase in circulating TAG throughout pregnancy into consideration. This may be the reason why the findings of vitamin D studies during pregnancy are inconsistent. Therefore, studies to examine the effect of circulating 25(OH)D in relation to circulating TAG on pregnancy outcomes are necessary.

Statement of Purpose

The purpose of this study was to determine the vitamin D status of a cohort of pregnant women in Kansas City metropolitan area, to assess its relationship with adverse pregnancy outcomes, and to examine if plasma 25(OH)D concentrations relative to circulating TAG concentrations will be a better indicator of maternal vitamin D status.

Research Questions

The primary research question is: Should plasma 25(OH)D concentration be adjusted for circulating triacylglycerol concentration in assessing maternal vitamin D status?

The secondary research questions are:

1) What is the prevalence of vitamin D deficiency (25(OH)D <50 nmol/L) and the prevalence of vitamin D insufficiency (25(OH)D 50-75 nmol/L) in a Kansas City cohort of pregnant women?

2) Does the maternal vitamin D status during pregnancy relate to adverse pregnancy outcomes?

Chapter 2

REVIEW OF LITERATURE

Vitamin D Metabolism

Two major forms of vitamin D exist. Ergocalciferol (vitamin D_2) is a 28-carbon derivative of ergosterol synthesized by yeasts and fungi, and cholecalciferol (vitamin D_3) is a 27-carbon derivative of cholesterol synthesized in animal and human skin after exposure to ultraviolet B irradiation (1, 17, 18). The term "vitamin D" will be used to represent both vitamin D_2 and vitamin D_3 in the rest of this thesis, unless otherwise specified.

After the ingestion of vitamin D, it is absorbed within the small intestine. Vitamin D is subsequently incorporated into the chylomicrons by the enterocytes, and enters the lymphatic system (19). The endogenously produced vitamin D_3 in the skin enters the bloodstream and is bound either tightly to vitamin D binding protein (DBP) or loosely to albumin or lipoproteins (18, 20). When vitamin D reaches the liver, it is hydroxylated by hepatic 25-hydroxylase to form 25(OH)D, which is the major circulating and storage form of vitamin D metabolites, and the major indicator used to measure vitamin D status. In the kidney or certain extrarenal tissues, such as placenta (21), 25(OH)D can be hydroxylated by 1α-hydroxylase (1-α-OHase) to form 1,25-dihydroxyvitamin D ($1,25(OH)_2D$), the active form of vitamin D (19). Both 25(OH)D and $1,25(OH)_2D$ can be hydroxylated by renal or extrarenal 24-hydroxylase to form more polar metabolites for excretion (19, 22).

Mechanism of Action

Circulating $1,25(OH)_2D$ regulates over numerous genes (18, 23). It acts through binding to a nuclear receptor, the vitamin D receptor (VDR). The VDR in turn forms a heterodimer with the retinoid X receptor and binds to the promoter region of the target gene. The subsequent binding

of co-activators or co-repressors to the heterodimer will induce or repress the transcription of the gene (17). VDR may also be associated with the plasma membrane, and its binding to $1,25(OH)_2D$ activates second messenger system(s) and results in rapid non-genomic responses (24). Vitamin D performs a variety of physiological functions primarily through these mechanisms.

Challenges in Vitamin D Studies

There is no consensus on the definition of vitamin D deficiency, insufficiency, and sufficiency in terms of circulating 25(OH)D concentration. Different combinations of cut points have been used to define vitamin D status by different investigators. Commonly used cut points include 25 nmol/L (13, 25, 26), 37.5 nmol/L (6, 9, 15, 27), 50 nmol/L (4, 5, 8, 11, 25-27), 75 nmol/L (4, 9, 11, 12), and 80 nmol/L (5, 6, 8, 26).

The accuracy of the measurement of 25(OH)D concentrations varies with the assay methods and the technicians. The interlaboratory variability in 25(OH)D measurement is so substantial that an individual considered to be sufficient in vitamin D in one laboratory may appear to have vitamin D insufficiency in another laboratory (28). Also, many assay methods underestimate vitamin D_2 concentrations (29-31). These factors make interpreting and comparing the results of vitamin D studies challenging.

Vitamin D in Pregnancy

Maternal circulating $1,25(OH)_2D$ concentrations increase from pre-pregnancy values starting from the first trimester, and can double or triple the pre-pregnancy values in the second and the third trimester (32). This increase in maternal $1,25(OH)_2D$ concentrations may help the fetus to accrete about 30 g of calcium for development during pregnancy (33) and may be important for normal pregnancy. Therefore, vitamin D requirement is increased during pregnancy.

Studies have shown a majority of pregnant women in the US have suboptimal vitamin D status, even with a high rate of prenatal vitamin consumption. Ginde et al. (4) selected 928 pregnant women and 5,173 non-pregnant women from the NHANES database between 2001 and 2006. They determined the prevalence of vitamin D insufficiency, defined as serum 25-hydroxyvitamin D (25(OH)D) <50 nmol/L or <75 nmol/L, among pregnant women and non-pregnant women. This study reported 42% and 78% of non-pregnant women had serum 25(OH)D concentrations less than 50 nmol/L and 75 nmol/L respectively, while 46% and 83% of pregnant women in their first trimesters had serum 25(OH)D concentrations less than 50 nmol/L and 75 nmol/L respectively. A study conducted in South Carolina found 41% of pregnant women were deficient in vitamin D (25(OH)D <50 nmol/L) and another 41% of pregnant women were insufficient in vitamin D (25(OH)D 50-80 nmol/L) during early pregnancy (5). Another Pennsylvania study found 62% of Caucasian pregnant women and 96% of African American pregnant women were deficient or insufficient (25(OH)D <80 nmol/L) in vitamin D during early pregnancy (6). A small study examined the prevalence of vitamin D deficiency and insufficiency in 80 pregnant African American adolescents and revealed that 52% and 36% of African American adolescents were low (25(OH)D <50 nmol/L) in vitamin D during the second and third trimester respectively (27).

This suboptimal maternal vitamin D status may predispose their infants to adverse conditions including rickets because maternal and infant serum 25(OH)D concentrations are highly correlated (34, 35). Hollis et al. (36) reported neonatal 25(OH)D concentrations were about half of maternal 25(OH)D concentrations. Vitamin D status in pregnant women warrants increased attention.

Vitamin D and Preeclampsia

Preeclampsia occurs in about 3% of pregnancies in the United States (37). It is usually diagnosed after 20 weeks of gestation. A characteristic of preeclampsia is hypertension—systolic blood pressure >140 mm Hg or diastolic blood pressure >90 mm Hg in women normotensive before 20 weeks of gestation—with some degree of proteinuria (38). Risk factors for preeclampsia include previous history of preeclampsia, preexisting diabetes and multifetal pregnancy (38). Preeclampsia is associated with a number of complications, such as preterm delivery and fetal growth restriction (39).

The pathogenesis of preeclampsia is still unclear, but it seems to be related to abnormal placentation, reduced placental perfusion, and abnormal maternal inflammatory response (39). An imbalance of proimflammatory Th1-type cytokines and anti-inflammatory Th2-type cytokines was found in preeclamptic women, with a higher Th1 to Th2 ratio (40). Placentas of preeclamptic women were also found to have significantly lower vascular endothelial growth factor (VEGF) (18) and lower 1-α-OHase expression and activity, compared to the placentas from uncomplicated pregnancies (41).

Studies support the idea that lower vitamin D status may play a role in the development of preeclampsia. The active form of the vitamin [1,25$(OH)_2$D] was proposed to be important for normal placentation, angiogenesis, and immunological tolerance for normal implantation. For example, 1, 25$(OH)_2$D is anti-inflammatory by down-regulating the expression of Th1-type cytokines and up-regulating Th2-type cytokines (42, 43). Also, it enhances the expression of HOXA10, an important gene for implantation (44), and up-regulates VEGF for angiogenesis (45). The vitamin D status of women at conception and during early pregnancy is likely a risk factor for preeclampsia.

Research studies found an inverse relationship between maternal vitamin D status during pregnancy and risk for preeclampsia. A nested case-control study reported significantly lower serum 25(OH)D concentrations at less than 22 weeks of gestation in 55 women who subsequently developed preeclampsia compared to 219 non-preeclamptic controls (9). Another nested case-control study of 43 cases and 198 controls reported women with serum 25(OH)D concentrations <50 nmol/L between the 15^{th} and 20^{th} week of gestation was associated with a 5-fold increased risk of developing severe preeclampsia, when compared to women with serum 25(OH)D concentrations >75 nmol/L (11). Robinson et al. (8) measured maternal plasma 25(OH)D concentrations of 50 women at the time of diagnosis of early-onset severe preeclampsia (EOSPE)—severe preeclampsia diagnosed before 34 weeks of gestation—and compared these with the concentrations of the 100 control pregnant women. They reported a 63% decreased odds of EOSPE with every 25-nmol/L increase in the plasma 25(OH)D concentrations of the EOSPE patients. Haugen et al. (10) measured the vitamin D intake of 23,423 women during pregnancy. They found women who developed preeclampsia had significantly lower supplemental vitamin D intake during pregnancy compared to women who were non-preeclamptic.

In contrast, Powe et al. (46) found no significant differences in maternal serum 25(OH)D concentrations during the first trimester between 39 preeclamptic subjects and 131 non-preeclamptic subjects. They concluded that maternal 25(OH)D concentrations in the first trimester were not associated with the risk of subsequent preeclmpsia.

Vitamin D and Gestational Diabetes Mellitus

GDM is diagnosed between 24 and 28 weeks of gestation, or earlier if a woman has increased risk for GDM. To screen for GDM, a 1 hour 50-g oral glucose tolerance test (OGTT) is

usually done. This is followed by a 3 hour 100-g OGTT if the 1 hour test yields a positive result. Two or more abnormal values in the 3 hour test lead to the diagnosis of GDM. Risk factors of GDM include increasing maternal age, obesity, previous history of GDM, family history of diabetes mellitus, previous delivery of an infant with macrosomia, and recurrent miscarriages (13). GDM increases the risk of fetal macrosomia, which may lead to a cesarean delivery, and the woman's risk of developing metabolic syndrome and type 2 diabetes (47).

In pregnant women with GDM, pancreatic β cells fail to increase insulin secretion in response to the reduced insulin sensitivity during pregnancy (47). Both VDR and 1-α-OHase are expressed in pancreatic islets (1). Vitamin D is also known to improve insulin sensitivity by enhancing the expression of insulin receptors (48). Vitamin D may reduce the risk of GDM by regulating insulin release and insulin sensitivity (49).

Soheilykhah et al. (14) compared the serum 25(OH)D concentrations in 54 pregnant women with GDM, 39 pregnant women with impaired glucose tolerance (IGT), and 111 pregnant women with normal glucose tolerance at 24-28 weeks of gestation. They reported a significantly lower vitamin D status in the GDM and the IGT groups ($P = 0.001$). A cross-sectional study involving 741 pregnant Iranian women showed that gestational diabetes was significantly more prevalent in women with 25(OH)D_3 <12.5 nmol/L than women with 25(OH)D_3 >34.9 nmol/L. The same study showed maternal serum 25(OH)D concentration is inversely associated with insulin resistance indicated by the HOMA index values, which is an insulin resistance index calculated from the fasting plasma glucose and the fasting plasma insulin concentrations (13). This association was confirmed in a cohort study involving 307 pregnant women (50).

In contrast, Farrant et al. (51) found no association between maternal serum 25(OH)D concentration during pregnancy and risk for gestational diabetes in a study of 559 Indian women.

Vitamin D and Cesarean Section

About 32% of births in the US are delivered by cesarean section (52). Low maternal vitamin D status during pregnancy may be a risk factor for primary cesarean delivery. Merewood et al. (15) measured vitamin D concentrations of 253 mothers after delivery. They reported that the risk for primary cesarean section in women with vitamin D concentrations <37.5 nmol/L was almost four times higher than women with higher vitamin D concentrations. They proposed maternal vitamin D status may be associated with risk for primary cesarean section through calcium's role in the initiation of labor, or by increasing preeclampsia risk. Studies revealed a significant increase in maternal serum calcium concentrations at the time of vaginal delivery (53), and suggested the role of serum calcium in smooth muscle function in labor (54). In contrast, Bowyer et al. (25) found no significant association between maternal 25(OH)D concentrations and mode of delivery in a study of 971 pregnant women.

Vitamin D and Triacylglycerol

A longitudinal study of 2159 subjects in Norway reported a significant inverse association between serum 25(OH)D and TAG in adults (55). This inverse association was also found in a cross-sectional study of 909 subjects in Finland (β = -0.17, P < 0.001) (56). However, other studies did not support this association (57, 58). To the best of our knowledge, no previous studies have examined both circulating 25(OH)D and TAG concentrations in pregnant women. During pregnancy, maternal circulating TAG increases dramatically with weeks of gestation due to increased mobilization of maternal fat stores to provide fatty acids for the fetus (16). This increased mobilization of maternal fat stores could influence the amount of 25(OH)D carried in the plasma because vitamin D is a fat-soluble vitamin and is stored in adipose tissue (2). Higher circulating vitamin D concentrations may not necessarily reflect better vitamin D status when

circulating TAG concentrations are taken into account. Plasma 25(OH)D concentration to TAG concentration ratio may be a better measure of vitamin D status during pregnancy and may help to explain the inconsistent results of vitamin D studies on pregnancy.

Chapter 3

METHODS

Overview

The purpose of this study was to determine the vitamin D status of a cohort of pregnant women in Kansas City metropolitan area, to assess its relationship with adverse pregnancy outcomes, and to examine if plasma 25(OH)D concentrations relative to circulating TAG concentrations would be a better indicator of vitamin D status.

Sample

This study used a sample of subjects from the Kansas University DHA Outcomes Study (KUDOS), a double-blind, randomized clinical trial designed to determine whether increased prenatal docosahexaenoic acid (DHA) intake would improve pregnancy outcomes and the cognitive development of infants. The subjects were randomly assigned to one of two groups—one consumed capsules with DHA-oil and the other consumed capsules with ordinary food oil. Pregnant women aged 16-35 years, in their 8^{th} to 20^{th} week of gestation, with BMI <40, who agreed to return to the study center for delivery were included in the trial. Pregnant women who had multiple fetuses in the index pregnancy, serious illness, diabetes or gestational diabetes, elevated blood pressure at baseline, and were unable or unwilling to consume the study capsules until delivery were excluded. 350 pregnant women who met these criteria were enrolled in the KUDOS trial. This vitamin D study was based on the data of the 299 women who had information at delivery.

Setting

The subjects were recruited from April 2006 to November 2009 at University of Kansas Medical Center (KUMC) in Kansas City, Kansas, Truman Medical Center in Kansas City,

Missouri, and St. Luke's Hospital in Kansas City, Missouri where the women planned to deliver their infants. Women who responded to a broadcast e-mail at KUMC were also enrolled and they delivered at the hospitals mentioned and five additional area hospitals.

Ethics

The procedures and protocols of the KUDOS trial were approved by Human Subjects Committee at University of Kansas Medical Center (HSC#10186), and this vitamin D study is covered under the KUDOS protocol because it involves nutritional assessment from available blood samples.

Procedure

Informed consent was obtained from each subject. Non-fasting blood samples were drawn from the women at enrollment and were stored at -80°C after separating into plasma and red blood cell samples. Weight and blood pressure were measured, and other maternal characteristics (years of education, prenatal vitamin use, smoking status, etc.) were self-reported at enrollment. Maternal body mass index (BMI) was calculated from measured weight at enrollment and either measured or reported height. Race was self-reported as African American, Caucasian, Hispanic, or Other. Subjects within the "Other" category included Indian American, Hawaiian, and Korean. Due to the small number of subjects in that category (n = 3), they were combined with the Hispanic group for statistical analyses. Medical records were reviewed for information on preeclampsia, GDM, and the mode of delivery for the index pregnancy.

Plasma samples collected at enrollment were used for 25(OH)D and TAG assays. One of the 299 subjects lacked plasma sample at enrollment, and the postpartum plasma sample of this subject collected within two days of delivery was used instead.

Vitamin D deficiency, insufficiency and sufficiency were defined as plasma 25(OH)D below 50 nmol/L, 50-75 nmol/L and above 75 nmol/L respectively in this study. These cutoffs were chosen because they have been used in recently published studies (5, 8, 11, 12, 14). We expect that our results would be more comparable to the findings of recent studies by using these cutoffs. Also, 25(OH)D less than 50 nmol/L may be associated with increased risk of nonskeletal chronic diseases (59) and 25(OH)D concentrations above 75 nmol/L may be required to prevent secondary hyperparathyroidism (60).

For the purpose of this study, adverse pregnancy outcomes include the diagnosis of preeclampsia or GDM, or having a cesarean delivery in the index pregnancy. A subject was considered to have adverse pregnancy outcomes when at least one of these conditions occurred. In this study, 18 subjects did not have complete oral challenge test results, and no diagnosis of GDM could be made. They were thus excluded in the statistical analyses of GDM and adverse pregnancy outcomes.

Laboratory Assays

Plasma 25(OH)D concentration was analyzed by the Kansas Intellectual and Developmental Disabilities Research Center (K-IDDRC) laboratory using an enzyme-linked immunosorbent assay (ELISA) kit (Immundiagnostik AG, Bensheim, Germany). The assay could detect 25(OH)D concentrations as low as 3.2 nmol/L. The intra- and inter-assay coefficients of variation for the ELISA were both 7.0%. The ELISA recognizes 100% of 25(OH)D_3 and 67.8% of 25(OH)D_2. The 25(OH)D concentrations obtained from the ELISA and HPLC were highly correlated ($r = 0.943$) (61). The 25(OH)D assay procedure is described in Appendix A.

Plasma TAG analysis was performed using a triglyceride assay kit from Cayman Chemical Company, Michigan, USA. The assay could detect TAG in the range of 0-200 mg/dL. One

sample had a TAG concentration higher than 200 mg/dL, and its concentration was calculated from the absorbance value using the standard curve equation. The intra-assay and the inter-assay coefficients of variations for the triglyceride assay kit were 1.34% and 3.17% respectively (62). The TAG assay procedure is described in Appendix B.

Statistical Analysis

Continuous variables are presented as mean ± standard deviation, and categorical variables as the number of observations (percentage). The characteristics of the subjects were reported by race. The characteristics of the subjects with adverse pregnancy outcomes were evaluated by comparing them to subjects without experience any adverse pregnancy outcomes during the index pregnancy. The risks for preeclampsia, GDM, and cesarean section among pregnant women with plasma 25(OH)D concentrations and the 25(OH)D/TAG ratio below the medians were compared to those above the medians, and were presented as odds ratios and 95% confidence intervals (95% CI). Mean ± standard deviation of plasma 25(OH)D, TAG, and 25(OH)D/TAG ratio within each category of various maternal factors was reported to assess their effects on maternal vitamin D status.

The differences in maternal characteristics between groups were assessed by independent sample t-tests for continuous variables and by chi-square tests for categorical variables. Fisher's exact test was used in place of chi-square test when more than 20% of the cells have an expected frequency below five. Plasma 25(OH)D concentrations, TAG concentrations, 25(OH)D/TAG ratios, and maternal years of education were log-transformed before t-tests were performed, because the distributions of these variables were skewed.

Results were considered statistical significant if two-tailed *P* values <0.05. Statistical analyses were performed using PASW Statistics 18.

Chapter 4

RESULTS

This study was conducted to determine the vitamin D status of the cohort of pregnant women, to assess its relationship with adverse pregnancy outcomes, and to examine if plasma 25(OH)D/TAG ratio would be a better indicator of vitamin D status.

Subject Characteristics

In this study, African American women and Hispanic women were younger, had a higher BMI, were less educated, were more likely to be multiparous, and tended to start taking prenatal vitamins later in gestation than Caucasian women (Table 1). Hispanic women tended to have a lower systolic blood pressure at enrollment compared with African American women (108 ± 10.6 mm Hg vs. 114 ± 10.2 mm Hg; $P = 0.02$) and Caucasian women (108 ± 10.6 mm Hg vs. 115 ± 9.4 mm Hg; $P = 0.001$). No significant differences between race groups in the gestational age when blood was drawn (data not shown) and the season of blood collection were observed.

Maternal 25(OH)D concentrations of the Caucasian women were the highest among the three race groups, and were almost two times the 25(OH)D concentrations of African American women (64.2 ± 32.2 nmol/L vs. 33.9 ± 20.8 nmol/L; $P < 0.001$). Hispanic women had maternal 25(OH)D concentrations (54.3 ± 34.5 nmol/L) lower than Caucasian women ($P = 0.04$), but higher than African American women ($P = 0.001$). Vitamin D deficiency (25(OH)D < 50 nmol/L) was found in 86% of African American women, 35% of Caucasian women, and 56% of Hispanic women, whereas vitamin D insufficiency (25(OH)D 50-75 nmol/L) was found in 10% of African American women, 40% of Caucasian women, and 16% of Hispanic women in this cohort. African American women also had lower TAG concentrations than Caucasian ($P = 0.002$) and Hispanic women ($P = 0.003$). Similar to plasmas 25(OH)D concentrations, Caucasian women

had higher 25(OH)D/TAG ratios than African American women (1.85 ± 1.34 vs. 1.14 ± 0.94; *P* < 0.001) and Hispanic women 1.85 ± 1.34 vs. 1.40 ± 1.26; *P* = 0.03) (Table 1).

Table 1. Maternal Characteristics by Race

Maternal Characteristics	African American n = 115	Caucasian n = 159	Hispanic and Other n = 25	*P*-value[a]	*P*-value[b]	*P*-value[c]
Maternal age (yr)	23.6 ± 4.5	26.9 ± 4.4	24.6 ± 5.2	<0.001	0.34	0.02
Maternal BMI (kg/m^2)	28.4 ± 5.4	26.0 ± 4.8	28.5 ± 6.0[d]	<0.001	0.92	0.02
Maternal years of education	12.3 ± 1.7	15.1 ± 3.0	13.0 ± 2.4	<0.001	0.27	0.001
Gravidity, n (%)				0.04	0.42	0.05
1	35 (30.4)	69 (43.4)	5 (20.0)			
≥2	80 (69.6)	90 (56.6)	20 (80.0)			
Smoking in pregnancy, n (%)	45 (39.1)	46 (28.9)	8 (32.0)	0.10	0.66	0.94
Blood pressure at enrollment						
Systolic (mmHg)	114 ± 10.2	115 ± 9.4	108 ± 10.6	0.13	0.02	0.001
Diastolic (mmHg)	68 ± 7.8	69 ± 8.0	66 ± 7.8	0.42	0.11	0.04
Gestational age when prenatal vitamin use started (wk)[e]	9.1 ± 4.0	4.3 ± 4.4	7.5 ± 4.7	<0.001	0.08	0.001
Plasma 25(OH)D (nmol/L)	33.9 ± 20.8	64.2 ± 32.2	54.3 ± 34.5	<0.001	0.001	0.04
Vitamin D status, n (%)				<0.001	<0.001	0.05
Deficient	99 (86.1)	55 (34.6)	14 (56.0)			
Insufficient	12 (10.4)	63 (39.6)	4 (16.0)			
Sufficient	4 (3.5)	41 (25.8)	7 (28.0)			
Plasma TAG (mg/dL)	36.4 ± 17.4	45.3 ± 30.7	53.3 ± 37.2	0.002	0.003	0.24
25(OH)D/TAG ratio[f]	1.14 ± 0.94	1.85 ± 1.34	1.40 ± 1.26	<0.001	0.33	0.03

Table 1. Maternal Characteristics by Race (Continued)

Maternal Characteristics	African American n = 115	Caucasian n = 159	Hispanic and Other n = 25	*P*-value[a]	*P*-value[b]	*P*-value[c]
Season of blood draw, n (%)						
Spring (Mar-May)	25 (21.7)	45 (28.3)	9 (36.0)	0.66	0.16	0.35
Summer (Jun-Aug)	38 (33.0)	47 (29.6)	6 (24.0)			
Autumn (Sep-Nov)	26 (22.6)	35 (22.0)	8 (32.0)			
Winter (Dec-Feb)	26 (22.6)	32 (20.1)	2 (8.0)			

[a]*p* value between African American and Caucasian.
[b]*p* value between African American and Hispanic and Other.
[c]*p* value between Caucasian and Hispanic and Other.
[d]1 subject was excluded from the BMI analysis because measured weight was missing.
[e]7 subjects (5 African Americans and 2 Caucasians) were excluded from these analyses due to lack of information on prenatal vitamin use.
[f]25(OH)D/TAG ratio was calculated by dividing plasma 25(OH)D concentration (nmol/L) by plasma TAG concentration (mg/dL)

Adverse Pregnancy Outcomes

There were five cases of preeclampsia and 15 cases of GDM in this cohort. Although both preeclampsia and GDM were approximately two times more prevalent in the African American women compared with Caucasian women (data not shown), these differences did not reach statistical significance ($P = 0.65$ and $P = 0.50$ respectively). Eighty-nine (30%) women in this cohort had a cesarean delivery. No statistical significant differences in the rate of cesarean delivery were found between the race groups.

Women with adverse pregnancy outcomes tended to have higher BMI, have higher blood pressure at enrollment, deliver their infants earlier, and have a larger chance of delivering a preterm infant, when compared with women with no adverse pregnancy outcomes in the index pregnancy (Table 2). No significant differences in gravidity, prenatal vitamin use, and smoking

status between women with adverse pregnancy outcomes and women without were observed. However, women with adverse pregnancy outcomes tended to have higher circulating TAG concentrations (46.9 ± 35.7 mg/dL vs. 40.2 ± 22.3 mg/dL; $P = 0.04$).

Table 2. Characteristics between Women with and without Adverse Pregnancy Outcomes

	Women without adverse pregnancy outcomes n = 189	**Women with adverse pregnancy outcomes n = 92**	***P-value***
Maternal age	25.0 ± 4.6	26.6 ± 5.1	0.01
Maternal BMI (kg/m^2)	26.6 ± 5.4[a]	28.3 ± 4.8	0.01
Gravidity, n (%)			0.36
1	68 (36.0)	39 (42.4)	
≥2	121 (64.0)	53 (57.6)	
Blood pressure at enrollment (mm Hg)			
Systolic	113 ± 9.1	117 ± 11.0	0.002
Diastolic	68 ± 7.7	70 ± 8.3	0.04
Prenatal vitamin use, n (%)[b]	184 (98.4)	91 (98.9)	0.99
Smoking during pregnancy, n (%)	60 (31.7)	35 (38.0)	0.36
Plasma 25(OH)D concentrations (nmol/L)	50.4 ±31.7	55.2 ± 31.9	0.22
Plasma TAG concentrations (mg/dL)	40.2 ± 22.3	46.9 ± 35.7	0.04
25(OH)D/TAG ratio[c]	1.56 ± 1.22	1.56 ± 1.35	0.79
Gestational age at delivery (wk)	39.5 ± 1.3	38.9 ± 2.2	0.008
Preterm <37 wk, n (%)	6 (3.2)	12 (13.0)	0.004

[a]1 subject was excluded from the BMI analysis because measured weight was missing.
[b]7 subjects were excluded from this analysis due to lack of information on prenatal vitamin use.
[c]25(OH)D/TAG ratio was calculated by dividing plasma 25(OH)D concentration (nmol/L) by plasma TAG concentration (mg/dL)

Maternal 25(OH)D Concentrations and Adverse Pregnancy Outcomes

No statistically significant differences in maternal plasma 25(OH)D concentrations were found between non-preeclamptic women and preeclamptic women, between women without GDM and women with GDM, and between women delivered vaginally and women delivered by cesarean section (Table 3). Lower maternal 25(OH)D concentrations were associated with higher odds for preeclampsia (OR, 4.05; 95% CI, 0.45-36.71) and GDM (OR, 2.12; 95% CI, 0.71-6.38), but were not statistically significant (Table 4). Lower maternal 25(OH)D concentrations were also associated with lower odds for cesarean delivery (OR, 0.54; 95% CI, 0.32-0.89; $P < 0.05$). Exclusion of repeat cesarean deliveries from the analysis did not significantly affect the result (data not shown). Overall, lower maternal 25(OH)D concentrations were associated with lower odds for adverse pregnancy outcomes (OR, 0.65; 95% CI, 0.40-1.08), but results were not statistically significant.

Maternal 25(OH)D/TAG Ratios and Adverse Pregnancy Outcomes

Significantly lower 25(OH)D/TAG ratios were found in women with preeclampsia (0.67 ± 0.56 vs. 1.55 ± 1.24; $P = 0.04$), and women with GDM (0.98 ± 0.83 vs. 1.59 ± 1.27; $P = 0.02$) (Table 3). Lower maternal 25(OH)D/TAG ratios were associated with higher odds for preeclampsia (OR, 4.05; 95% CI, 0.45-36.71), but not statistically significant (Table 4). However, the association between lower 25(OH)D/TAG ratios and increased odds for GDM (OR, 2.96, 95% CI, 0.92-9.55) was almost significant ($P = 0.07$).

There were no statistically significant differences in 25(OH)D/TAG ratios between women delivered vaginally and women delivered by cesarean section. Lower maternal 25(OH)D/TAG ratios were associated with lower odds for cesarean delivery (OR, 0.74; 95% CI, 0.45-1.22) and adverse pregnancy outcomes (OR, 0.80, 95% CI, 0.48-1.31), but results were not statistically

significant. Exclusion of repeat cesarean deliveries from the analysis did not significantly affect the result (data not shown).

Table 3. Plasma 25(OH)D Concentrations and 25(OH)D/TAG Ratios Between Cases and Controls

	Non-Preeclamptic n = 294	Preeclamptic n = 5	Non-GDM n = 266	GDM n = 15	Vaginal n = 210	Cesarean n = 89
25(OH)D (nmol/L)	52.1 ± 32.0	30.4 ± 14.1	52.6 ± 32.1	41.1 ± 24.6	49.9 ± 31.8	56.0 ± 31.8
25(OH)D/ TAG[a]	1.55 ± 1.24	0.67 ± 0.56*	1.59 ± 1.27	0.98 ± 0.83*	1.52 ± 1.19	1.58 ± 1.35

[a]25(OH)D/TAG ratio was calculated by dividing plasma 25(OH)D concentration (nmol/L) by plasma TAG concentration (mg/dL)
**P*-value <0.05

Table 4. Odds Ratios and 95% Confidence Intervals for Pregnancy Outcomes According to 2 Measures of Vitamin D Status

	25(OH)D		25(OH)D/TAG[a]	
	< median	>median	<median	>median
Preeclampsia	4.05 [0.45-36.71]	Ref	4.05 [0.45-36.71]	Ref
GDM[b]	2.12 [0.71-6.38]	Ref	2.96 [0.92-9.55]	Ref
Cesarean delivery	0.54 [0.32-0.89]*	Ref	0.74 [0.45-1.22]	Ref
Adverse pregnancy outcomes[b]	0.65 [0.40-1.08]	Ref	0.80 [0.48-1.31]	Ref

[a]25(OH)D/TAG was calculated by dividing plasma 25(OH)D concentration (nmol/L) by plasma TAG concentration (mg/dL)
[b]18 subjects were excluded from these analyses due to lack of complete OGTT results.
**P*-value <0.05

Effects of Potential Confounders on Vitamin D Status

Non-obese women (BMI < 30) tended to have higher plasma 25(OH)D concentrations ($P < 0.001$), lower plasma TAG concentrations ($P = 0.01$), and higher 25(OH)D/TAG ratios ($P < 0.001$) than obese women (BMI ≥ 30). Nulliparous women had higher 25(OH)D concentrations ($P = 0.006$) and 25(OH)D/TAG ratios ($P = 0.02$). There were no significant differences in 25(OH)D and TAG concentrations or 25(OH)D/TAG ratios between women in different age groups, smokers and non-smokers, prenatal vitamin users and non-users at enrollment, and plasma samples collected in different seasons (Table 5).

Table 5. Plasma 25(OH)D Concentrations, TAG Concentrations and 25(OH)D/TAG Ratios between Maternal Groups

	Plasma 25(OH)D concentrations (nmol/L)	*P*-value	Plasma TAG concentrations (mg/dL)	*P*-value	25(OH)D/TAG ratios[a]	*P*-value
Maternal age						
<30 yrs	50.9 ± 32.1	0.26	42.1 ± 28.8	0.17	1.54 ± 1.26	0.93
≥30 yrs	55.3 ± 31.1		44.7 ± 20.9		1.52 ± 1.13	
Maternal BMI[b]						
<30	57.0 ± 32.7	<0.001	39.9 ± 21.0	0.01	1.74 ± 1.28	<0.001
≥30	38.6 ± 25.7		49.2 ± 39.0		1.05 ± 0.96	
Gravidity						
1	57.3 ± 35.1	0.006	41.4 ± 22.1	0.70	1.74 ± 1.41	0.02
≥2	48.5 ± 29.5		43.3 ± 30.2		1.42 ± 1.12	
Smoking during pregnancy						
Yes	46.8 ± 26.8	0.20	44.6 ± 24.9	0.31	1.32 ± 0.96	0.10
No	54.1 ± 33.9		41.5 ± 28.7		1.65 ± 1.34	
Prenatal vitamin use[c]						
Yes	52.2 ± 32.0	0.15	42.6 ± 27.7	0.56	1.56 ± 1.24	0.12
No	30.1 ± 18.6		45.9 ± 18.2		0.68 ± 0.28	
Season of blood collection						
Spring	56.1 ± 33.2	>0.05	45.1 ± 24.8	>0.05	1.47 ± 1.08	>0.05
Summer	52.2 ± 33.2		43.1 ± 34.9		1.55 ± 1.09	
Autumn	50.2 ± 25.5		42.2 ± 25.8		1.64 ± 1.58	
Winter	46.9 ± 34.6		38.8 ± 18.8		1.49 ± 1.22	

[a]25(OH)D/TAG ratios were calculated by dividing plasma 25(OH)D concentrations (nmol/L) by plasma TAG concentrations (mg/dL)
[b]1 subject was excluded from the BMI analysis because measured weight was missing.
[c]7 subjects were excluded from this analysis due to lack of information on prenatal vitamin use.

Chapter 5

DISCUSSION

Maternal 25(OH)D Concentrations and Adverse Pregnancy Outcomes

Our findings suggested an association between lower maternal plasma 25(OH)D concentrations during early pregnancy with an increased subsequent risk for preeclampsia (OR, 4.05; 95% CI, 0.45-36.71). This inverse association supports the findings of other studies on maternal vitamin D status and preeclampsia risk (8-11). However, the association reported in this study did not reach statistically significant level. The paucity of cases likely plays a role in this lack of statistical significance. This study only had 5 cases of preeclampsia. In contrast, the nested case-control study by Bodnar et al. (9) contained 55 cases and 219 controls, and found every 50-nmol/L decrease in serum 25(OH)D concentration <22 weeks of gestation doubled the risk of preeclampsia (adjusted OR, 2.4; 95% CI, 1.1-5.4).

Our findings also reported an association between lower maternal plasma 25(OH)D concentrations during early pregnancy with an increased subsequent risk for GDM (OR, 2.12; 95% CI, 0.71-6.38). Similar associations were found in several studies on maternal vitamin D status and GDM risk (12-14). Likewise, the association reported in this study was not statistically significant, probably due to the small number of GDM cases (n = 15). A nested case-control study including 57 GDM cases and 114 controls found that the risk for GDM in women with 25(OH)D concentrations <50 nmol/L in early pregnancy was 2.66 times higher than those with 25(OH)D concentrations $\geq$75 nmol/L (adjusted OR, 2.66; 95% CI, 1.01-7.02) (12).

The results of this study suggested lower maternal 25(OH)D concentrations were significantly associated with a lower risk of cesarean delivery (OR, 0.54; 95% CI, 0.32-0.89). This finding is in contrast with the findings of another study, which reported a four-fold

increased risk for primary cesarean delivery associated with women with 25(OH)D concentrations <37.5 nmol/L (OR, 3.84; 95% CI, 1.71-8.62) (15). Our findings may not be comparable with that study because they measured their subjects' serum 25(OH)D concentrations after delivery. Their results may reflect the effects of cesarean delivery on maternal vitamin D status instead. Bowyer et al. (25) also included both primary and repeat cesarean deliveries in their study, and they found no significant association between maternal vitamin D status in the third trimester and risk for cesarean delivery.

Maternal 25(OH)D/TAG Ratios and Adverse Pregnancy Outcomes

We hypothesized the 25(OH)D/TAG ratio may be a better indicator of vitamin D status, and thus a better predictor of adverse pregnancy outcomes. Women with preeclampsia and GDM had significantly lower 25(OH)D/TAG ratios than women without preeclampsia and GDM respectively, but their plasma 25(OH)D concentrations were not significantly different. Similar to plasma 25(OH)D concentrations, our findings suggested an association between lower maternal 25(OH)D/TAG ratios and an increased subsequent risk for preeclampsia (OR, 4.05; 95% CI, 0.45-36.71), but this association did not reach statistical significance. Our findings also suggested a 3-fold increased risk for GDM associated with lower maternal 25(OH)D/TAG ratios (OR, 2.96; 95% CI 0.92-9.55). This association was near to statistically significant ($P = 0.07$), and was much more significant than the association between maternal 25(OH)D concentrations and GDM risk ($P = 0.19$). Likewise, the small number of diagnoses of preeclampsia and GDM in this cohort could contribute to the lack of statistical significance in the findings on preeclampsia and GDM. Our findings also suggested a positive association between maternal 25(OH)D/TAG ratios and risk for cesarean delivery (OR, 0.74; 95% CI, 0.45-1.22), but this association was not statistically significant.

Although neither 25(OH)D concentrations nor 25(OH)D/TAG ratios were proved to be significantly associated with the risk of preeclampsia and GDM in our study, our hypothesis that 25(OH)D/TAG is a better indicator of maternal vitamin D status is still possible. In our study, 25(OH)D/TAG ratios were associated with the risk of the adverse pregnancy outcomes in a manner similar to 25(OH)D concentrations, and the associations were more significant compared to 25(OH)D concentration alone.

Effects of Potential Confounders on Vitamin D Status

In this study, obesity was found to be associated with lower plasma 25(OH)D concentrations ($P < 0.001$), and 25(OH)D/TAG ratios ($P < 0.001$). This is consistent with previous findings of lower bioavailability of vitamin D in obese people because of possible sequestration of vitamin D in subcutaneous fat (63). Also, nulliparity was associated with higher 25(OH)D concentrations ($P = 0.006$) and 25(OH)D/TAG ratios ($P = 0.02$). These maternal factors may have confounding effects on the results and contribute to the lack of statistical significance of our findings.

Seasonal effects on circulating 25(OH)D concentrations were not found in this study. This suggests that vitamin D synthesis in the skin was not a significant contributor to vitamin D status of pregnant women in this cohort.

Limitations

The present study has several limitations. Firstly, only a few subjects were diagnosed with preeclampsia or GDM. Therefore, the findings regarding these pregnancy outcomes did not reach statistical significance, and thus conclusion of whether or not maternal vitamin D status has an effect on these two outcomes could not be made in this study. Secondly, the reasons for cesarean delivery were not collected, so the adverse effect of higher maternal vitamin D status on the risk of cesarean delivery suggested by our findings could not be explained in this study.

The fact that the study subjects might have been taking DHA capsules during their pregnancy may be a limitation as well, because higher n-3 fatty acid intake may also reduce the risk of preeclampsia (10). Our findings may have been confounded by the effect of DHA on pregnancy outcomes. Assessment of the confounding effect of DHA is not possible before October 2011 because we are still blinded to the capsule assignment until then. Another limitation is the plasma samples have been stored for up to five years and have been thawed and refrozen. However, serum 25(OH)D and TAG has been reported to be stable in storage and was unaffected after up to four freeze-thaw cycles (64-67). To assess the effect of storage on 25(OH)D and TAG concentrations in the samples, we divided the plasma samples into two groups—samples collected in 2006-2007 and samples collected in 2008-2009. The mean 25(OH)D concentration of older samples was about 7 nmol/L lower than the mean of newer samples, though not statistically significant ($P = 0.07$), but there were no significant differences in TAG concentrations and 25(OH)D/TAG ratios between older and new samples. Therefore, sample storage may not significantly affect our findings. Also, this study used a sample of convenience, and the results may not sufficiently represent all pregnant women in the US. Finally, the ELISA kit has a 100% cross-reactivity of $25(OH)D_3$ and $24,25(OH)_2D_3$, and a 67.8% cross-reactivity of $25(OH)D_2$ (61). The accuracy of measurements could thus be compromised because the assay measured $24,25(OH)_2D_3$ as well, and plasma 25(OH)D concentrations would be underestimated when a subject's major source of vitamin D was in the form of vitamin D_2.

Implications

We measured maternal vitamin D status before the diagnosis of preeclampsia and GDM. Therefore, the increased risk associated with lower plasma 25(OH)D concentrations and 25(OH)D/TAG ratios suggested by our findings, though not statistically significant, is more

likely to be a consequence, rather than a cause, of suboptimal maternal vitamin D status. This suggests that maternal vitamin D status during early pregnancy may be an important predictor of pregnancy outcomes.

Although more than 98% of our subjects reported prenatal vitamin consumption at enrollment, 56% and 26% of the pregnant women in our study were vitamin D deficient (25(OH)D < 50 nmol/L) and insufficient (25(OH)D 50-75 nmol/L), respectively. This large number of pregnant women with suboptimal vitamin D status in our study suggests that the amount of vitamin D in prenatal vitamin supplements may be inadequate.

The mean plasma 25(OH)D concentration of African American women was almost half of that of Caucasian women in this study (33.9 ± 20.8 nmol/L vs. 64.2 ± 32.2 nmol/L; $P < 0.001$). This racial disparity in vitamin D status during pregnancy may partly explain the higher morbidity and mortality rates among African Americans due to the potential adverse effects caused by poor vitamin D status during pregnancy and infancy. Maintaining a sufficient vitamin D status is possibly a means to reduce the health disparities in the US.

Future Studies

The statistical significance of our findings was limited by the small number of preeclampsia and GDM cases in the cohort. Similar studies with higher number of cases are needed to yield statistically significant findings to confirm the effects of maternal vitamin D status on pregnancy outcomes, and whether 25(OH)D/TAG ratio predicts pregnancy outcomes better than plasma 25(OH)D concentration alone. Maternal vitamin D status during pregnancy was inversely associated with the subsequent risk of cesarean delivery in this study. Further analyses exploring the association between maternal vitamin D status and specific indications for cesarean delivery are needed to clarify the role of vitamin D in delivery. Future randomized control trials to verify

the benefits of vitamin D supplementation on pregnancy outcomes are also required to justify the need to revise the recommendations regarding vitamin D intake and supplementation during pregnancy and the need for future vitamin D-related policies or programs to improve the vitamin D status of pregnant women in the US.

Conclusion

Our findings found no significant relationship between maternal vitamin D status and the risks of preeclampsia and GDM due to the small number of these events in the cohort. When using 25(OH)D/TAG ratio, in place of 25(OH)D concentrations, as the indicator of vitamin D status, an almost statistically significant association between lower maternal vitamin D status and increased GDM risk was observed.

Our study also showed a reduced risk of cesarean delivery in women with lower 25(OH)D concentrations. When using 25(OH)D/TAG ratio was used in the analysis instead, this association was attenuated and no longer significant. Additional delivery information is needed to explain this association between maternal vitamin D status and the risk of cesarean delivery.

Maternal 25(OH)D/TAG ratio may be a better indicator of vitamin D status during pregnancy. Due to the lack of statistically significant findings in our study, further investigations into this hypothesis should be done in studies with a larger number of adverse pregnancy events.

Chapter 6

SUMMARY

This study aimed to assess the effects of maternal vitamin D status on the subsequent risk for pregnancy complications and to determine if plasma 25(OH)D concentrations should be adjusted for plasma TAG concentrations when assessing vitamin D status in pregnant women.

We obtained the data of 299 pregnant women in the KUDOS trial who had their delivery information available. We measured 25(OH)D and TAG concentrations in the plasma samples collected from these women in their 8^{th} to 20^{th} week of gestation using 25(OH)D ELISA kit and triglyceride assay kit respectively. We then assessed the relationship of plasma 25(OH)D concentrations and 25(OH)D/TAG ratios to the risk of preeclampsia, GDM, and cesarean delivery.

Preeclampsia was diagnosed in five women, GDM was diagnosed in 15 women and 89 cesarean deliveries were observed. 96% of African American women, 74% of Caucasian women, and 72% of Hispanic women in this cohort had plasma 25(OH)D concentrations less than 75 nmol/L. Mean 25(OH)D concentrations of African American, Caucasian, and Hispanic women were 33.9 ± 20.8, 64.2 ± 32.2, and 54.3 ± 34.5 nmol/L respectively. Mean 25(OH)/D ratios of African American, Caucasian, and Hispanic women were 1.14 ± 0.94, 1.85 ± 1.34, and 1.40 ± 1.26 respectively. Vitamin D status of African American women and Hispanic women were significantly lower than Caucasian women ($P < 0.05$). However, African American women and Hispanic women were also higher in BMI and were more likely to be multiparous compared to Caucasian women in this study ($P \leq 0.05$). Both increased BMI and increased parity were associated with lower vitamin D status in these race groups ($P < 0.05$).

Women who subsequently developed preeclampsia (0.67 ± 0.56 vs. 1.55 ± 1.24; $P = 0.04$) or GDM (0.98 ± 0.83 vs. 1.59 ± 1.27; $P = 0.02$) had significantly lower 25(OH)D/TAG ratios, compared to women who did not. The 25(OH)D/TAG ratios were not significantly different between women with different delivery modes.

Women with 25(OH)D concentrations below median had increased odds of preeclampsia (OR, 4.05; 95% CI, 0.45-36.71) and GDM (OR, 2.12; 95% CI, 0.71-6.38). Both associations did not reach statistical significance as a result of the small number of preeclampsia and GDM cases. A reduced risk of cesarean delivery was significantly associated with lower 25(OH)D concentrations (OR, 0.54; 95% CI, 0.32-0.89). This finding was not explained by the inclusion of repeat cesarean delivery in the analysis.

Women with 25(OH)D/TAG ratios below median had increased odds of preeclampsia (OR, 4.05; 95% CI, 0.45-36.71), but this relationship was not statistically significant similarly due to the small number of preeclampsia cases in the study. A close to statistically significant association ($P = 0.07$) between lower 25(OH)D/TAG ratio and increased GDM risk was observed (OR, 2.96, 95% CI, 0.92-9.55). Likewise, an association between lower 25(OH)D/TAG ratio and reduced odds for cesarean delivery, but not statistically significant, was found in our study (OR, 0.74; 95% CI, 0.45-1.22).

Our findings supported our hypothesis that lower maternal vitamin D status is associated with increased risks of preeclampsia and GDM. The small number of preeclampsia and GDM cases in our study may explain the lack of statistical significance of our findings on these adverse outcomes. The positive correlation between maternal vitamin D status and risk of cesarean delivery reported is in conflict with our hypothesis and the finding of a previous study on maternal vitamin D status and risk of primary cesarean delivery. A better evaluation of the

relationship between maternal vitamin D status and the subsequent risk of cesarean delivery may be possible after including the indications for cesarean delivery in the statistical analysis.

Maternal 25(OH)D/TAG may be a better indicator of maternal vitamin D because 25(OH)D/TAG ratios were associated with the risk of the adverse pregnancy outcomes in a manner similar to 25(OH)D concentrations, and the associations were more significant compared to 25(OH)D concentration alone. Future studies with larger number of preeclampsia and GDM diagnoses are needed to confirm the effects of maternal vitamin D status on these pregnancy outcomes and to determine if plasma 25(OH)D concentrations should be adjusted for TAG concentrations to better indicate maternal vitamin D status.

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APPENDIX A

25(OH)D Assay

Plasma 25(OH)D concentration is measured using an ELISA kit (Immundiagnostik AG, Bensheim, Germany). More detailed procedures are available in the kit manual (61).

Principle of the Assay

Plasma samples are incubated with the releasing agent to release DBP-bound 25(OH)D from the DBP. After the addition of the samples and the anti 25(OH)D antibody to the microtiter plate precoated with 25(OH)D, there is a competition between the 25(OH)D in the samples and a fixed quantity of 25(OH)D bound to the wells of the plate for the binding of the antibody. Addition of peroxidase-conjugated antibody to the wells leads to the formation of 25(OH)D-anti 25(OH)D antibody-peroxidase conjugate complexes. Tetramethylbenzidine (TMB) acts as a peroxidase substrate when it is added to the complexes. An acidic solution is then added to stop the reaction. The contents of the wells change from blue to yellow in color. The intensity of the yellow color is inversely proportional to the 25(OH)D concentration of the starting samples. 25(OH)D concentrations of the plasma samples are determined by comparing the absorbance values to the dose response curve obtained from the standards.

Procedure

1. Label a 1.5 mL Eppendorf tube for each standard, control, and plasma sample.
2. Pipette 30 μL of standards or plasma samples or controls into their corresponding tubes.
3. Add 300 μL of releasing reagent into each tube and vortex briefly.
4. Incubate the tubes for 1 hour at 37 °C in a water bath or heating block.
5. Add 600 μL of sample dilution buffer into each tube and vortex carefully.
6. Pipette 100 μL of the mixture of each tube into 2 designated wells, 50 μL each, of the 96-well microtiter plate.
7. Add 150 μL of anti 25(OH)D antibody solution into each well.

8. Cover the plate tightly and incubate for 18-22 hours at 8-10 °C in the dark.
9. Aspirate and wash the wells 5 times with 250 µL of diluted wash buffer using an 8-channel pipette. After the last wash, hit the plate against paper towel to remove the residual wash buffer in the wells.
10. Add 200 µL of peroxidase-conjugated antibody solution into each well
11. Cover the plate tightly and incubate for 1 hour at room temperature while shaking with a horizontal microtiter plate shaker.
12. Aspirate and well the wells 5 times with 250 µL of diluted wash buffer using an 8-channel pipette. After the last wash, hit the plate against paper towel to remove the residual wash buffer in the wells.
13. Add 200 µL of TMB substrate into each well
14. Incubate for 10-15 minutes at room temperature in the dark.
15. Add 50 µL of ELISA stop solution into each well.
16. Read the plate with a microtiter plate reader at 450 nm.

APPENDIX B

Triacylglycerol Assay

Plasma TAG analysis was done by a triglyceride assay kit from Cayman Chemical Company, Michigan, USA. More detailed procedures are found in the kit manual (62).

Principle of the Assay

During the assay, triglycerides are hydrolyzed into glycerol and fatty acids through the action of lipoprotein lipase. The glycerol is phosphorylated by glycerol kinase to form glycerol-3-phosphate, which then reacts with oxygen to produce dihydroxyacetone phosphate and hydrogen peroxide in a reaction catalyzed by glycerol phosphate oxidase. The hydrogen peroxide reacts with 4-aminoantipyrine and N-Ethyl-N-(3-sulfopropyl)-m-anisidine, and the quinoeimine dye forms (with a purple color of various intensities, depending on the TAG concentration to start with). This reaction is catalyzed by peroxidase. TAG concentrations were determined by comparing the absorbance values of the samples with the standard curve obtained from the triglyceride standards.

Procedure

1. Label 8 1.5 mL Eppendorf tubes 1 to 8. Add 200 μL of triglyceride standard diluent to tubes 2-8 and 400 μL to tube 1. Add 100 μL of triglyceride standard to tube 1 and vortex. Transfer 200 μL from tube 1 to tube 2 and vortex tube 2. Transfer 200 μL from tube 2 to tube 3 and vortex tube 3. Repeat this process for tubes 4-7. After this serial dilution, the triglyceride concentrations of tubes 1-8 are 200, 100, 50, 25, 12.5, 6.25, 3.125 and 0 mg/dL respectively.
2. Add 10 μL of the 8 tubes to their designated wells on the 96-well plate in duplicate.
3. Add 10 μL of each plasma sample to their designated wells in duplicate.
4. Add 150 μL of diluted enzyme buffer solution to each well.
5. Mix the solution in the wells by carefully shaking the plate for several seconds with a horizontal microtiter plate shaker.

6. Incubated for 15 minutes at room temperature.
7. Read the absorbance of the wells at 540 nm using a plate reader.