CHANGES IN MATERNAL VITAMIN D STATUS THROUGHOUT PREGNANCY AND THE EFFECTS OF SUPPLEMENTATION

By

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ABSTRACT

BACKGROUND: Pregnant women make up a special population at increased risk for vitamin D insufficiency and deficiency. Maternal 25 hydroxyvitamin D (25(OH)D) status has implications for the health of the mother and fetus. Because blood lipids change during pregnancy, it may be important to measure not only 25(OH)D but also to ratio 24(OH)D to plasma triacylglycerol (TAG).

METHODS: Blood samples were collected at enrollment (8-22 weeks gestation) and delivery from a mixed race population of pregnant women (n=299) living in the Kansas City metropolitan area at latitude 39°06' north. Plasma 25(OH)D was measured by enzyme immunoassay (EIA). TAG was measured by enzymatic hydrolysis assay.

RESULTS: Rates of deficiency, insufficiency, sufficiency, and toxicity were 25.8%, 30.4%, 40.8% and 3.0% respectively at enrollment and 54.9%, 29.6%, 15.6% and 0% respectively at delivery. Mean 25(OH)D concentration of African American women at enrollment was 12.39 ± 2.94 ng/ml and 6.80 ± 3.20 ng/ml at delivery. Caucasian women had mean levels of 23.84 ± 3.80 ng/ml at enrollment and levels of 13.37 ± 3.36 ng/ml at delivery. Vitamin D levels of Caucasian women were significantly higher than African American women at both enrollment ($p < 0.00001$) and delivery ($p < 0.0001$). Maternal BMI was associated with lower 25(OH)D status at enrollment ($p < 0.001$) and delivery ($p = 0.018$). Season of blood collection significantly affected 25(OH)D status at delivery ($p < 0.001$). Vitamin D supplementation was significantly associated with higher 25(OH)D status at delivery ($p = 0.033$). Maternal concentration of TAG was not significantly related to 25(OH)D status at enrollment or delivery.

CONCLUSION: Despite vitamin D supplementation, maternal 25(OH)D status decreased by 40% from 8-20 weeks gestation to infant delivery. The 40% increase in maternal plasma volume between 8 and 32 weeks gestation may influence 25(OH)D status at infant delivery.

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TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

TABLES

FIGURES

Chapter 1

INTRODUCTION

Vitamin D deficiency was common at the end of the 19th century in areas of increased industrialization in North America and Europe, causing rickets (the index disease of vitamin D deficiency) to emerge in great numbers. Although the deficiency disease was largely eradiated by public health policies such as vitamin D fortification of milk, we are now aware that less severe forms of vitamin D insufficiency and deficiency still exist in the 21st century (1). The biomarker of vitamin D status used routinely is 25(OH)D. Various studies have begun to link 25(OH)D to functional outcomes and several groups have set guidelines for vitamin D status based on plasma/serum 25(OH)D. However, there is still not a consensus on the concentration that should be considered adequate, insufficient or deficient. In addition, research uses different standards so comparison among studies is difficult.

There is not one cause for the increase in vitamin D deficiency. Some causes are believed to be aging, latitude, skin pigmentation, increased sunscreen use, decreased exposure to direct sunlight, increased obesity, and increased incidence of fat malabsorption disorders (2). In pregnancy, vitamin D is important for maternal health and fetal growth. Pregnancy creates increased demands for calcium absorption and bone remodeling in the fetus and therefore creates an increased demand for vitamin D and risk for vitamin D insufficiency and deficiency (3-6).

There is controversy regarding the vitamin D supplement dosage women should consume during pregnancy (7-12). This is in part due to the fact that functional studies in pregnant women are lacking. However, there are only a few studies that have measured 25(OH)D in pregnancy. One of the goals of the present study is to measure 25(OH)D at enrollment (between 8 and 20 weeks gestation) and immediately following delivery and to determine if these

concentrations are influenced by plasma TAG concentration. This study also aims to explore the predictors of vitamin D status in women as there is even less information on the amount of vitamin D supplementation taken by US women and whether or not supplementation improves vitamin D status.

The Institute of Medicine (IOM) guidelines from 2011 states the Dietary Reference Intake (DRI) for vitamin D for adults as 600 International Units (IU) and the tolerable upper limit of intake is 4,000 IU, with no distinction for pregnancy (13). The US Endocrine Society released a different statement recommending pregnant women should take 1,400 IU of supplemental vitamin D per day (10). The key differences between the recommendations is the IOM guidelines are designed for the general healthy population and the Endocrine Society guidelines are designed to prevent and treat deficiencies (10). It is unknown which recommendations are most appropriate to support a healthy pregnancy. Several studies show pregnant women have inadequate vitamin D status (<20 ng/ml) throughout gestation, suggesting the US Endocrine Society's recommendations may be more applicable to pregnant women in the US (14-19). It is necessary to determine if there is significant evidence to change the vitamin D recommendations during pregnancy.

Several factors can affect vitamin D status. Some of the most notable variables are body mass index (BMI), race, latitude and season of blood draw (10-12, 20). Maternal fatty acid mobilization to the fetus is increased dramatically during the first two trimesters of pregnancy (21). In addition, plasma triacylglycerol (TAG) is greatly increased during pregnancy creating a state of maternal hypertriglyceridemia important for fatty acid delivery across the placenta to the fetus. Vitamin D is a fat-soluble vitamin and few studies show a relationship between vitamin D

status and TAG status (22-25). More research is necessary to examine the changes in 25(OH)D status from enrollment to delivery in relation to TAG levels.

Statement of Purpose

The purpose of the study is:

1) To determine how vitamin D levels change from enrollment (8-20 weeks gestation) to delivery in a cohort of pregnant women living in the Kansas City metropolitan area.
2) To examine the changes in 25(OH)D concentration as it relates to supplemental vitamin D intake throughout pregnancy.

Research Questions:

Primary Research Question: How does vitamin D status of US women change from enrollment (8-20 weeks gestation) to delivery in relation to intake of vitamin D from prenatal supplements.

Secondary Research Questions:

1) Does supplemental vitamin D intake decrease the incidence of vitamin D deficiency and insufficiency at delivery compared to enrollment (8-20 weeks gestation)?
2) Does circulating triacylglycerol concentration in relation to circulating 25(OH)D concentration change from mid pregnancy to delivery?
3) What variables predict vitamin D status at enrollment and delivery?

Chapter 2

REVIEW OF LITERATURE

Vitamin D Metabolism and Physiological Functions

Vitamin D is a unique fat-soluble vitamin with hormonal qualities (1, 26, 27). Synthesis of vitamin D occurs through direct contact with sunlight or through the ingestion of vitamin D containing foods or supplements. There are two major forms of vitamin D. Vitamin D_2, also known as ergocalciferol, is a 28-carbon compound produced by yeast and mushrooms when exposed to ultraviolet B (UVB) radiation. Vitamin D_3, also known as cholecaliferol, is produced in the skin when exposed to sunlight (1, 2, 28). Both vitamin D_2 and vitamin D_3 can be used to effectively raise vitamin D levels in children and adults (29). Supplemental vitamin D_2 and D_3 are both included in the present analysis.

After exposure to sunlight, 7-dehydrocholesterol in the skin is converted to previtamin D_3. Previtamin D_3 is readily converted to vitamin D_3 through a heat dependent process and is re-routed to form inactive precursors when vitamin D levels are sufficient (1, 13, 30, 31). Vitamin D ingested through the diet or supplements is first absorbed by the small intestine, where it is incorporated into chylomicrons and enters the lymphatic system (10). In the circulation, vitamin D (represents D_2 or D_3) is bound tightly to vitamin D binding protein (DBP). Vitamin D bound to DBP travels to the liver where 25-hydroxylase converts vitamin D to 25(OH)D. Although biologically inactive, 25(OH)D is the form used to measure vitamin D status because of its longer half life and greater concentration (1, 32, 33). After hydroxylation in the liver, 25(OH)D travels to the kidney where another hydroxylation reaction occurs through the action of the enzyme 25-hydroxyvitaminD-1hydroxylase to form $1,25(OH)_2D$, the active form of vitamin D (1, 10).

The physiological functions of vitamin D are dependent on the interaction between $1,25(OH)_2D$ and vitamin D receptors (VDR) (27). Recent research shows VDR are present in many tissues in the body, including the intestine, kidneys, bone, breast, brain, prostrate, pancreas, immune cells, and placenta (11, 27, 28). The active form of vitamin D can be formed in some of these tissues where it functions as an autocrine factor (2) .Vitamin D, through interaction with VDR, aids in the maintenance of calcium and phosphorus homeostasis and the promotion of healthy bone remodeling. Newer research addresses other important roles of vitamin D in enhancement of immune function, promotion of insulin production, inhibition of cellular proliferation, and inhibition of renin production (10). The widespread presence of VDR in the body and the multiple physiological responsibilities of vitamin D are evidence of the physiological importance of vitamin D.

Assessment of Vitamin D Status

In 2011, the Institute of Medicine (IOM) released the DRIs for calcium and vitamin D and defined 25(OH)D blood level cut points associated with four levels of vitamin D status including: vitamin D deficiency, levels generally considered inadequate for bone and overall health, levels generally considered adequate for bone and overall health, and levels associated with possible adverse effects (13). It is important to note the IOM's recommendations were designed specifically for healthy individuals living in North America. The reviews used to complete the reference values were conducted by the US Agency for Healthcare Research and Quality (AHRQ) (34, 35).

Table 1: Institute of Medicine: 25(OH)D Concentrations and Health (13)

Status	**nmol/L**	**ng/ml**
Associated with vitamin D deficiency leading to rickets in children and osteomalacia in adults	<30	<12
Generally considered inadequate for bone and overall health in healthy individuals	30-50	12-19.99
Generally considered adequate for bone and overall health in healthy individuals	≥ 50	≥20
Possible adverse effects	> 125	>50

In 2011, the US Endocrine Society released a set of guidelines composed by the Endocrine Practice Guidelines Committee for the evaluation, treatment and prevention of vitamin D deficiency (Table 2) (10). The IOM and the US Endocrine Society aimed to address different goals, as the IOM evaluated the healthy subset of the North American population at large, and the Endocrine Society evaluated the needs of those at risk for deficiency (12).

Table 2: Endocrine Society: 25(OH)D Concentrations and Status (10)

Status	**nmol/L**	**ng/ml**
Deficiency	<50	<20
Insufficiency	52.5-72.5	20-29
Sufficiency	72.6-250	30-100
Possible adverse effects	> 250	>100

Vitamin D status is typically assessed by measuring plasma 25(OH)D levels. There are advantages and disadvantages for the types of assays used to measure plasma 25(OH)D (2, 36-38). Direct detection methods for measuring plasma 25(OH)D are high-performance liquid chromatography (HPLC) and liquid chromatography-mass spectrophotometry (LC-MS). HPLC and LC-MS methods separate the $25(OH)D_2$ from the $25(OH)D_3$ (2). Both direct detection methods require highly trained laboratory technologists, and when done correctly are highly

accurate techniques. However, LC-MS has a much higher sample output than HPLC (39). Antibody-based assays assess vitamin D status through measurement of total 25(OH)D. Antibody-based assays cannot detect the difference between vitamin D_2 and vitamin D_3. Research shows that antibody-based assays such as the enzyme immunoassay (EIA) used in the present analysis show similar levels of plasma 25(OH)D as is found with radioimmunoassay (RIA) (40). However, EIA methods are subject to calibration errors and measurement bias. In December of 2011, the National Institute of Standards and Technology (NIST) developed a Standard Reference Material (SRM) in order to assist with reliable and accurate measurements of 25(OH)D. The SRM 972 uses three different isotope dilution mass spectrometry approaches, making this reference material the first to use the method of ID liquid chromatography-mass spectrophotometry (LC-MS) to assess 25(OH)D status (32, 33). The SRM 972 was not used in the current analysis because plasma 25(OH)D was assessed prior to its release.

Prevalence and Causes of Vitamin D Deficiency

According to the US Endocrine Society cut points listed in Table 2, vitamin D deficiency and insufficiency affect more than 1 billion people worldwide (2). Rickets was first described almost 18 centuries before its etiology was determined to be due to vitamin D deficiency in the early 20th century. Rickets was prevalent in the18th and early 19th century in industrial cities of Europe and North America (1) and by 1820 it was known that cod liver oil could cure rickets. In the present day, there are several well-known factors contributing to vitamin D insufficiency and deficiency. Some notable factors related to vitamin D deficiency are sunscreen use, skin pigment, aging, season, latitude, time of day during sun exposure, fat malabsorption disorders, obesity, breast feeding, liver failure, nephrotic syndrome, chronic kidney disease, and certain heritable and acquired disorders (2).

Skin pigmentation and subsequent melanin production is one of the greatest predictors of vitamin D deficiency. African Americans and Hispanics are 50-90% less efficient in forming vitamin D_3 in their skin than their white counterparts (1). The third National Health and Nutrition Examination Survey (NAHNES) analyzed the prevalence of insufficient vitamin D defined by levels ≤ 37.5 ng/ml among premenopausal white and African American women randomly selected from 81 counties across the US. According to their findings, 40% of African American women were vitamin D insufficient and 0.7% of white women were vitamin D insufficient after adjusting for season and BMI (41). In a study completed in Pittsburgh, black and white pregnant women and their neonates were assessed for vitamin D deficiency and insufficiency. At delivery, 88.3% of the black women and 47% of the white women were insufficient or deficient in 25(OH)D levels as defined by 15 nmol/L and <37.5 nmol/L respectively (19). In another study, the vitamin D status of pregnant women in South Carolina was assessed, revealing 97% of African Americans, 81% of Hispanics, and 67% of white women were either insufficient or deficient, as defined by 25(OH)D levels at 20-32 ng/ml and <20 ng/ml respectively (17). Seasonality was not a significant covariate in this study as would be expected given the latitude of South Carolina. Factors such as avoidance of sunlight and increased use of sunscreen are possible contributors to the deficiency rates in pregnant women in South Carolina.

Latitude and seasonal exposure are important factors affecting vitamin D status. According to a study published in the *Harvard Women's Health Watch*, those people living above 37 degrees north or below 37 degrees south cannot absorb vitamin D through their skin during fall, winter, and spring months (42). Latitude cannot be examined alone as it is not an exclusive variable in exposure to vitamin D. Variables such as sunscreen use, clothing coverage, the amount of time spent outdoors, ethnicity, nutritional practices, and cultural customs are all

factors must also be considered, as vitamin D deficiency has been observed in several equatorial populations where sunshine is prevalent (27). Chan et al (43) determined season of blood draw had no significant effect on mid pregnancy 25(OH)D status of pregnant women enrolled in the Kansas University DHA Outcomes Study (KUDOS) cohort.

Obesity also affects vitamin D status. Because vitamin D is a fat-soluble vitamin, studies suggest it can be sequestered in adipose tissue (1, 2, 20). Several studies show an inverse relationship between 25(OH)D status and BMI (2, 13, 20, 44, 45). One study challenged the sequestration mechanism in obesity, demonstrating a hyperbolic volumetric dilution model is a better indicator of 25(OH)D status. In this study, there was an inverse relationship between body weight and vitamin D status in all body weight ranges from normal to obese. The major finding in this study revealed a person with greater tissue mass requires more vitamin D supplementation to achieve plasma 25(OH)D in the sufficient range than does a person with a smaller tissue mass (46). According to the US Endocrine Society, obese individuals (BMI>30 kg/m_2) given 50,000 IU of vitamin D_2 either through an oral supplement or UVB exposure increased their 25(OH)D levels to 50% of the level of non-obese individual administered the same regimen (10). The US Endocrine Society recommends that vitamin D deficient, obese individuals age 19 and older receive 10,000 IU per day to correct deficiency until the person reaches sufficient ranges (10).

Vitamin D in Pregnancy

Pregnant women make up a special population at increased risk for vitamin D deficiency. A multitude of studies verify the high prevalence of insufficient and deficient vitamin D status of pregnant women (3, 8, 14, 16, 17, 19, 26, 31, 34, 35, 41, 47-56). The placenta is one of several extra-renal sites where VDR helps to convert 25(OH)D to 1,25$(OH)_2$D (57, 58). The presence of 25-hydroxyvitamin D-1alpha-hydroxylase (CYP24A1) and VDR are significantly increased in

the placenta during the first and second trimesters of gestation compared to placenta at term and endometrial tissue in women not pregnant (59, 60). In the third trimester, the fetus begins to calcify its skeleton creating an increased demand for maternal calcium transfer to the fetus. Circulating levels of $1,25(OH)_2D$ are elevated during the third trimester of pregnancy in order to meet the fetal calcium needs through increased maternal calcium absorption (10, 61).

Research shows a correlation between vitamin D deficiency and risk of several adverse events in pregnancy. Gestational diabetes (31), preeclampsia (35), small for gestational age births (14), asthma (62), and schizophrenia (50) are associated with low vitamin D status in pregnancy. Poor vitamin D status in pregnancy reveals detrimental effects on calcium homeostasis in both the mothers and infants (52).

Vitamin D Supplementation in Pregnancy

According to the IOM, the current estimated average requirement (EAR) for vitamin D for healthy pregnant women is 400 IU per day, and the DRI is 600 IU per day (13). The US Endocrine Society recommends pregnant women take at least 600 IU of vitamin D per day and claim it may take up to 1500-2000 IU per day to achieve and maintain 25(OH)D blood levels at 30 ng/ml (10). Some sources recommend supplementation in pregnancy with levels higher than the tolerable upper limit defined as 4000 IU by the IOM in order to maintain adequate levels of blood 25(OH)D (63). Since pregnant women are at an increased risk for vitamin D insufficiency and deficiency, some researchers believe prenatal supplements, containing 400 IU of vitamin D on average are inadequate to achieve adequate levels of 25(OH)D (10, 64, 65).

In a double blind randomized control trial, pregnant women between 12 and 14 weeks gestation were randomized to consume no vitamin D supplements or 400 IU, 2000 IU, and 4000 IU per day until delivery (66). There was no evidence of vitamin D toxicity and no adverse

events reported in any supplementation group. Women in the 4000 IU group had the lowest percentage of insufficiency and deficiency. (66). Research in Turkey revealed supplementation with 400 IU of vitamin D and 1000 mg of calcium per day throughout pregnancy was insufficient to maintain normal vitamin D levels (23). Vitamin D status throughout pregnancy was assessed in a group of Canadian women between 20 and 35 weeks of gestation with 80% of the women consuming a prenatal supplement containing at least 400 IU of vitamin D(52). Despite supplement use, vitamin D insufficiency was observed in 89% of women, as defined by between 50 and 75 nmol/l (52).

Vitamin D and Triacylglycerol

Maternal fat accumulation occurs at an increased rate in pregnancy when compared to non-pregnant counterparts (21). There is an increase in production of hepatic TAG in pregnant women during the first and second trimesters (21). Vitamin D is a fat-soluble vitamin, and may be affected by blood lipid concentration in pregnancy. Interestingly, the evidence favors an inverse relationship. Two prior studies found an inverse relationship between TAG levels and serum 25(OH)D levels in pregnancy (24, 25). Speeckaert et al. (67) examined the relationship between vitamin D binding protein (VDP) and lipoprotein fractions in male subjects, and reported a significant negative correlation between serum TAG levels and the ratio of 25(OH)D and vitamin D binding protein. Chan et al. (43) proposed a ratio of plasma 25(OH)D concentration to TAG concentration is a better indicator of vitamin D status in pregnancy and a better predictor of adverse outcomes in pregnancy. She examined women from the KUDOS cohort at mid pregnancy, and observed lower 25(OH)D/TAG ratios in women with preeclampsia (n=5) and gestational diabetes mellitus (n=15). The change in 25(OH)D ratios from enrollment

(8-20 weeks gestation) to delivery may provide more insight into the relationship between vitamin D status during pregnancy.

Chapter 3

METHODS

Overview

The subjects' measurements in this study originated from the Kansas University DHA Outcomes Study (KUDOS). The purpose of this study was to determine the change in vitamin D status from enrollment (8-20 weeks gestation) to delivery, to assess the effect of supplementation, and to examine if the plasma TAG changes from enrollment to delivery in relation to 25(OH)D levels.

Sample

The sample for the proposed study includes women from the KUDOS cohort. The primary goal of the KUDOS study was to determine if supplementation with docosahexanoic acid (DHA) would improve pregnancy outcomes of the mother and child, specifically duration of gestation, and infant visual and cognitive function. The KUDOS study is a randomized, double-blind, placebo controlled Phase III clinical trial. The study randomly assigned 350 women who met the following inclusion criteria to 600 mg of DHA or a placebo: Age 16-35.9, within 8-20 weeks gestation, able to communicate in English, BMI< 40. Women were excluded from the study if they had any serious health condition at risk of damaging the development of their fetus in utero, postnatally, or could affect the heath of the mother. Specific exclusion criteria included: diagnosis of cancer, lupus, hepatitis, diabetes mellitus (Type I, Type II, gestational), and HIV/AIDS at baseline. Women were also excluded if they were expecting multiple infants, or had hypertension (systolic ≥140 mm Hg). The women in the cohort were randomized to receive either a DHA oil capsule or an oil capsule (with no DHA) three times each day (total of 600mg) from the point of enrollment until delivery. For the Vitamin D analysis, all 299 women with

delivery information were included. The exclusion criteria remained the same as the original KUDOS trial. The sample for the vitamin D analysis study is n = 299 at enrollment (8-20 weeks) and n = 237 at delivery.

Setting

The KUDOS study was conducted in the Kansas City metropolitan area. Recruitment for the study was conducted at three hospitals: Truman Medical Center, Kansas University Medical Center, and St. Luke's. Women were recruited by word of mouth, through Kansas University Medical Center email listserv or through information provided in their hospital. All of the women delivered in a hospital in the Kansas City metropolitan area between October 2005 and May 2009.

Ethics

The Human Subjects Committee at the University of Kansas Medical Center evaluated the protocol, procedures, amendments, and informed consent used in the KUDOS study. The nutritional status assessment was included in the consent for the KUDOS study (HSC10186).

Procedure:

Informed consent was received from each subject participating in the KUDOS study. Non-fasting blood samples were drawn from women at baseline enrollment and at delivery. The blood samples were separated into plasma and red blood cells and stored at -80 degrees Celsius. At enrollment, weight and blood pressure were measured. BMI was calculated from measured weight and self reported or measured height. Supplement use, including prenatal vitamin use was self-reported at enrollment. Race was self-reported as African American, Caucasian or Other. Subjects within the "Other" category included Hispanic, American Indian, Hawaiian, and Korean.

The plasma samples collected at enrollment and delivery were used for the 25(OH)D and the TAG assays. In accordance with the most recent IOM guidelines, plasma 25(OH)D concentrations used to define vitamin D deficiency, insufficiency, and sufficiency as less than 12 ng/ml; between 12 and 19.9 ng/ml; and greater than 20 ng/ml respectively (13).

The concentration of plasma 25(OH)D was analyzed with a 25(OH)-Vitamin D direct Enzyme-linked Immunoabsorbant Assay (EIA) kit (Imundiagnostik AG, Bensheim, Germany; Article number K2110). The detection limit of 25(OH)D for the assay was 5.6 nmol/L at a sensitivity of n=20. The intra-assay coefficients for variation were 10.7% and the inter-assay coefficients for variation was 13% and 2% for assay 1 and 11% and 8% for assay 2. The assay has 100% specificity for both 25(OH)D_2 and 25(OH)D_3. The assay measured total 25(OH)D, including all 25(OH)D_2 and 25(OH)D_3 present in the samples. (See Appendix A)

The concentration for TAG was analyzed with a TAG assay kit (Cayman Chemical Company, Ann Arbor, Michigan; Item number 10010303). The assay range under standard conditions is 0-200 mg/dl TAG. The intra assay coefficient of variation was 1.34% and the inter-assay coefficient of variation was 3.17%. (See Appendix B)

Analysis of Data

Descriptive statistics were used to determine the mean ± standard deviation of maternal plasma 25(OH)D and circulating TAG at enrollment and delivery. Linear regression was used to determine if vitamin D supplementation or circulating triacylglycerol were related to vitamin D status at enrollment and delivery and, thus, rates of deficiency and insufficiency. Model assumptions were verified using the Kolmogorov-Smimov, Shapiro-Wilk, and Breusch-Pagan tests. Vitamin D status at enrollment and delivery were positively skewed, so a square root transformation was employed to normalize the distribution. The effect of multicollinearity was

examined, and the presence of outliers and influential observations were assessed using studentized deleted residuals, leverage values, Cook's distance, DFFITS, and DFBETAS. All data was analyzed with SPSS Statistics 17.0 software (SPSS, Chicago, IL), and P-values ≤ 0.05 will be considered significant.

Chapter 4

RESULTS

This study was designed to determine how vitamin D levels change from enrollment (8-20 weeks gestation) to delivery in a cohort of pregnant women living in the Kansas City metropolitan area, and to examine the changes in 25(OH)D concentration as it relates to supplemental vitamin D intake throughout pregnancy.

Subject Characteristics

Maternal 25(OH)D status was 40% higher at enrollment than at delivery in a mixed race population of women in the Kansas City metropolitan area (Table 3). Blood samples from enrollment were collected between 8 and 20 weeks gestation (mean ± SD: 14.7 ± 3.6 weeks) and blood samples from delivery were collected the morning after delivery. Women studied had a mean BMI of 27.1 ± 5.2 kg/m^2, and approximately 60% were either overweight or obese when they became pregnant. Weight gain was 28.3 ± 13.9 lbs on average and a large portion of women had a gestational weight gain greater than recommended by the IOM.

Predictors of Vitamin D Status

At enrollment and delivery, African American women and women with a higher BMI were likely to have insufficient vitamin D status ($p < 0.0001$ at enrollment and $p < 0.0001$ at delivery). Vitamin D status at enrollment and delivery was significantly influenced by race ($p < 0.0001$). Caucasian women had significantly higher levels of vitamin D at enrollment (23.84 ± 3.80 ng/ml) and delivery (13.37 ± 3.36 ng/ml) than African-American women at enrollment (12.39 ± 2.94 ng/ml) and delivery (6.80 ± 3.20 ng/ml) ($p < 0.0001$) (Figure 1).

Table 3. Maternal Characteristics at enrollment (n=299) and delivery (n=237)

Maternal Characteristic	Mean	SD	Range
At Enrollment (8-20 weeks gestation)			
Age (years)	25.40	4.80	16.1 – 36.0
Weight (lbs)	159.2	32.1	100.0 – 282.0
BMI (kg/m^2)	27.10	5.20	16.6 – 42.6
Education Level (year)	13.80	2.80	8 – 20
Race (n %)			
Caucasian	60.2%		
African American	38.5%		
Other	1.3%		
Gestational age (weeks)	14.70	3.60	7.3 – 22.3
25(OH)D (ng/ml)	20.71	12.78	2.03 – 90.64
Plasma TAG (mg/dl)	42.56	27.50	13.0 – 318.0
From Enrollment to Delivery			
Supplemental Vitamin D (IU/d)	331.80	122.70	0 – 916.7
Gestational weight gain (lbs)	28.30	13.9	(-9.0) – 63.0
25(OH)D at delivery (ng/ml)	12.5	8.5	0.1 – 41.9
Plasma TAG (mg/dl)	62.7	58.0	1.5 – 359.2

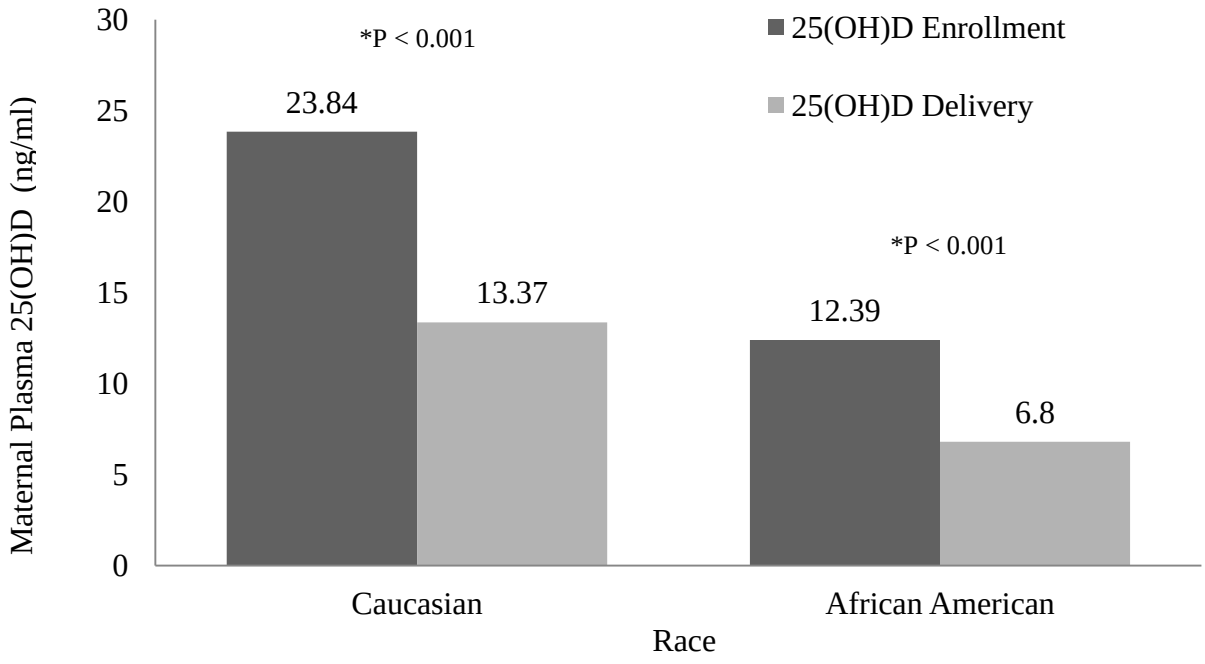

Figure 1. Effect of race on 25(OH)D status at enrollment and delivery. The difference between Caucasian subjects at enrollment and delivery was significant ($p<0.001$). The difference between African American subjects at enrollment and delivery was also significant ($p < 0.001$). Caucasian and African-American women had different 25(OH)D at enrollment ($p<0.001$) and delivery ($p<0.0001$).

Figure 2. Maternal 25(OH)D status as percent deficient, insufficient, sufficient and toxic at enrollment (n=299) and delivery (n=237).

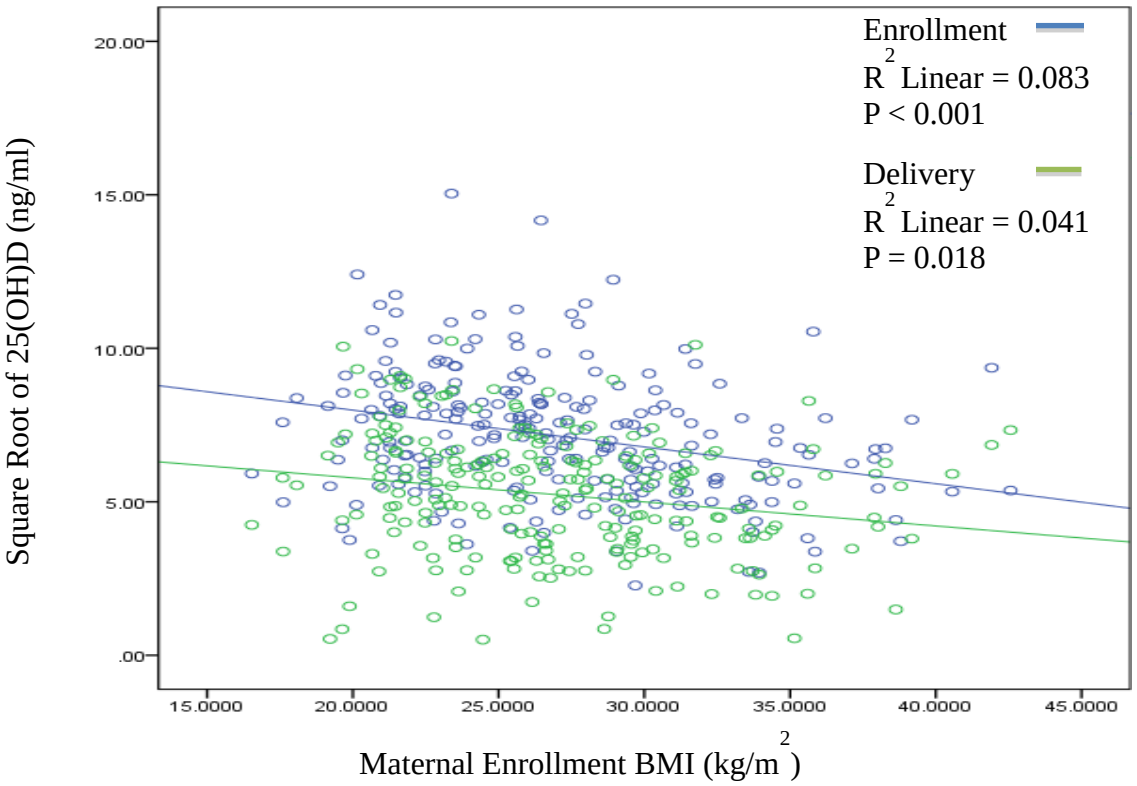

Figure 3. Maternal BMI and square root adjusted 25(OH)D status at enrollment ($p < 0.001$) and delivery ($p = 0.018$).

Supplemental vitamin D intake predicted higher 25(OH)D status at delivery ($p = 0.033$). Mean supplemental vitamin D intake was less than the RDA for pregnant women (333.1 ± 122.7). Despite supplement use, rates of insufficiency and deficiency increased significantly from enrollment to delivery ($p < 0.0001$) (Figure 2).

Women with higher pre-pregnancy BMI had significantly lower concentration of plasma 25(OH)D at enrollment and delivery ($p < 0.0001$; $p = 0.018$ respectively) (Figure 3). When the model was adjusted to determine if maternal body weight predicted 25(OH)D status, in other words, when BMI was normalized, a non significant relationship between maternal body weight was observed at enrollment or delivery ($p=0.731$; $p=0.734$; data not shown). Gestational weight gain was not related to 25(OH)D status at delivery ($p=0.813$).

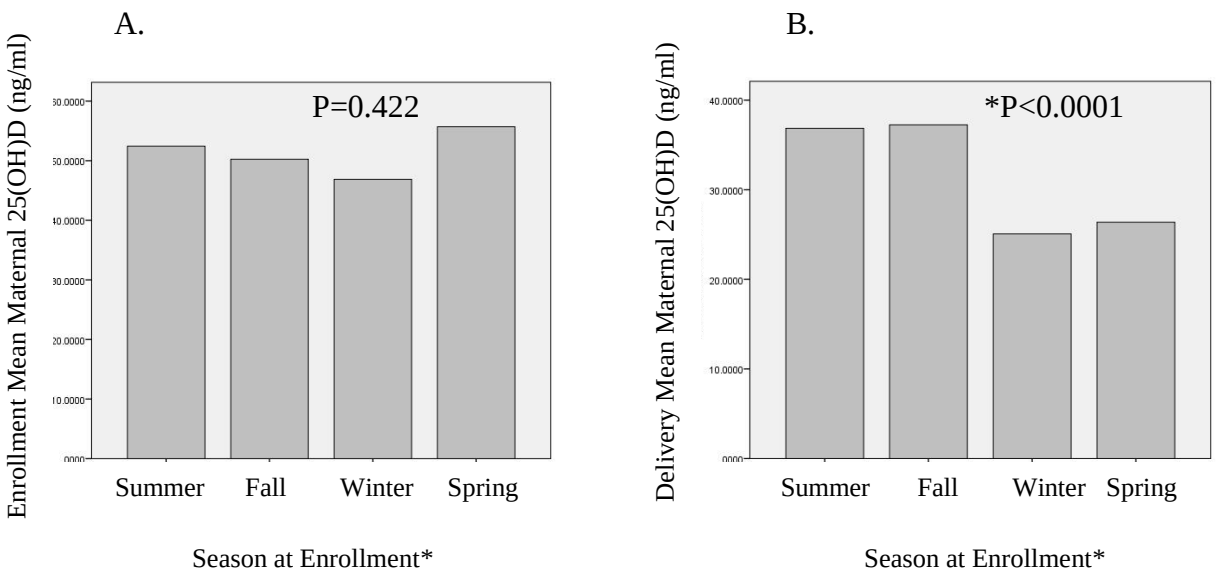

Figure 4. *Summer months: June 1-Augusut 31; Fall months: September 1-November 30; Winter months: December 1-Februray 29; Spring months: March 1-May 31.

A. Association between season of enrollment blood draw and 25OH)D status at enrollment among all races (p = 0.422). B. Association between season of delivery blood draw and 25(OH)D status at delivery among all races (p < 0.0001).

Season of blood collection for the study subjects all living at 39°06' north latitude was related to 25(OH)D status at delivery but not at enrollment (p <0.001 and p = 0.442 respectively) (Figure 4). Enrollment blood draws revealed non significant differences between mean levels of 25(OH)D between seasons. However, the differences between season of blood draw were statistically significant at delivery with the greatest mean levels of 25(OH)D during fall and summer months and least during winter and spring.

Concentrations of maternal TAG at enrollment and delivery were not related to 25(OH)D at delivery (data not shown). Maternal TAG concentration was 45% higher at delivery than TAG concentration at enrollment; however, maternal TAG was not a significant predictor of 25(OH)D status at enrollment or delivery.

Chapter 5

DISCUSSION

Maternal 25(OH)D Concentration from Enrollment to Delivery

Our research team investigated the changes in 25(OH)D status from enrollment through delivery among women living in the Kansas City metropolitan area. We hypothesized there would be a decrease in 25(OH)D status from enrollment to delivery, and an increased number of women insufficient and deficient at delivery when compared to enrollment. In support of our hypothesis, we report vitamin D status decreased by 40% from enrollment to delivery. Skin pigmentation, season of blood collection, and BMI were all significantly related to 25(OH)D status at delivery.

To the best of our knowledge, the dramatic change in vitamin D status from 8-20 weeks gestation to delivery was observed in only one other study by Milman et al (53). In this study, 25(OH)D concentration was measured in Danish women at 18, 32, 39 weeks gestation and 8 weeks postpartum. The Danish study revealed a significant increase in 25(OH)D levels from 18-32 weeks and a significant decrease from 32-39 weeks with the greatest decrease 39 weeks to 8 weeks postpartum. Although much of the postnatal decrease was attributed to lactation, the overall decrease throughout gestation is in agreement with our results. The striking change in 25(OH)D status observed in our study and the Danish study have several possible explanations. Blood volume increases approximately 40% from gestational week 8 to week 32. A dilutional effect previously mentioned may be the cause of decreased 25(OH)D status throughout pregnancy, however, the exact cause of such marked decreases is unknown.

The high rate of maternal vitamin D insufficiency and deficiency in this study raises important questions about the definition of vitamin D deficiency in pregnancy. Physiological

changes in pregnancy show an average 40% increase in maternal blood volume from 8 to 32 weeks gestation. Consequently, an oral dose of vitamin D would produce a smaller rise in plasma 25(OH)D in a pregnant women between 8-32 weeks gestation than the same dose would produce in that woman at her pre-pregnant blood volume. With this in mind, it is difficult to determine if the women in our study were actually vitamin D deficient, or if the increase in blood volume had a dilution effect on plasma 25(OH)D levels, causing them to appear deficient.

Clearly more work is needed as well to evaluate infants and children born to women with variable vitamin D status. As noted earlier, the placenta is one organ that can make active vitamin D from 25(OH)D, but it is not know if the placenta (and ultimately the fetus) is protected from low concentrations of plasma 25(OH)D. Moukarzel (68) did find that lower maternal vitamin D at mid-pregnancy in the population studied here predicted illness in the first 6 months of life; however, when she analyzed infant illness in relation to 25(OH)D at delivery, she did find any relationship.

Predictors of Maternal 25(OH)D Status

In addition, many studies have observed a similar relationship between skin pigmentation and 25(OH)D status. The nearly equal balance of black and white race in our study population allowed us to observe a significant difference between 25(OH)D status of African American women and Caucasian women at both enrollment and delivery. Our findings suggest there may be a need to supplement women with darker skin pigmentation with more vitamin D; however, this would require further study to determine the optimal time to improve their vitamin D status. The mean level of supplemental vitamin D intake of the women in our study was lower than the RDA for pregnant women. Clearly the combination of diet, sun exposure and supplement us was

insufficient to achieve an adequate vitamin D status in most women even at 8-20 weeks gestation.

We agree with a multitude of studies confirming an inverse relationship between BMI and 25(OH)D status. In contrast, Drinic et al (46) found total body weight was a better indicator of 25(OH)D status than BMI in non-pregnant, obese women. His work used a hyperbolic model incorporating the dilution of ingested vitamin D in fat mass suggesting vitamin D requirements must be adjusted for body size. In our analyses, body weight was not significantly related to 25(OH)D status; however, a dilutional effect in pregnancy may suggest why we saw such a marked decrease in vitamin D status from enrollment to delivery.

The significant difference between 25(OH)D status attributed to season of blood collection was in agreement with several other studies ((19, 42, 44, 49). In our study, women who delivered in fall and summer months had higher 25(OH)D levels than women who delivered in winter and spring months. Research continually shows cutaneous vitamin D production is optimal from 10AM to 4PM during summer months and non existent during winter months at latitudes above 35° north (11). The women in our study reside in the Kansas City metropolitan area at latitude 39°06' north, therefore, our findings are in agreement with what is known about cutaneous production of vitamin D. However, we found no significant difference between season of blood collection and 25(OH)D status at enrollment. Although this finding is not in accordance with the literature, I suspect the levels at enrollment were less sensitive to differences in status compared to the levels at delivery because the women in our study had extremely low 25(OH)D at delivery.

There was no relationship between TAG and 25(OH)D levels at either enrollment or delivery, and thus our research shows no reason to take TAG levels into account when assessing

vitamin D status in pregnant women. However, previous research shows TAG concentration has predictive qualities for adverse events in pregnancy (specifically preeclampsia and gestational diabetes) and should therefore be addressed in pregnancy outcomes research.

Potential Confounders on Vitamin D Status

All statistically significant results shown in this study were adjusted for all other factors and show significance after the adjustment. For example, maternal BMI was significant at $p < 0.0001$ even after adjustments for season of blood collection, supplement intake and skin pigmentation have been accounted for. As a result, the model eliminates confounding factors present in other studies examining similar factors.

Limitations

The primary aim of the KUDOS study was to examine the effects of DHA on duration of gestation and infant cognitive and visual function. There may be confounding effects due to the intake of DHA on vitamin D status because vitamin D is a fat-soluble vitamin influenced by the presence of fatty acids. The KUDOS trial has not been un-blinded, and it is not possible to determine the effects of DHA intake on vitamin D status in this cohort. Additionally, the plasma samples used in this cohort have gone through several freeze and thaw cycles, as they have been analyzed for other projects. Studies have shown vitamin D and TAG in serum samples are unaffected by up to four freeze and thaw cycles (39, 69, 70).

Another limitation in this analysis is n=62 samples available for enrollment analyses were not available for postpartum analyses. In some instances, there was not an adequate amount of sample left in order for postpartum analyses, and in other instances samples could not be located. The lack of samples at delivery results in a less powerful analysis than if all samples were available at both time points.

Implications

The results of this study have implications for vitamin D dosage recommendations in pregnancy. Our work showed 25(OH)D levels decreased from enrollment to delivery, despite supplement use. In lieu of this finding, women may require increased supplemental vitamin D throughout pregnancy, with especially increased levels as the pregnancy progresses. Pre-pregnancy vitamin D supplementation, like folic acid supplementation, should be considered as a preventative measure that would decrease the incidence of insufficient and deficient vitamin D status during pregnancy. Women with a higher BMI, darker skin pigmentation, and delivering in winter or fall months may need to supplement with higher levels than their counterparts with a lower BMI, lighter skin, and delivering in summer or spring months. Vitamin D supplementation in pregnancy may require a transition from national recommendations to a more individualized approach.

Low 25(OH)D status in pregnancy has been associated with preeclampsia, gestational diabetes, offspring rickets and reduced bone density. In order to avoid such adverse effects in pregnancy, we must better determine how much vitamin D women need during pregnancy for normal vitamin D status and gather more evidence of the functional effects of vitamin D deficiency on the mother and offspring.

Future Studies

Future studies must include randomized control trials examining the effects of vitamin D supplementation in pregnancy in order to determine how to achieve optimal blood levels for heath of infant and mother. Functional studies are needed to better determine what blood concentrations of 25(OH)D are indeed optimal. For example, future studies must continue to examine the physiological effects of vitamin D as a pro hormone and to determine the roles of

vitamin D in pregnancy. Longitudinal measurements of vitamin D throughout pregnancy will help to determine how status changes throughout gestation and what this means for the mother and fetus. Timing of supplementation and dosage of supplementation are two important factors must be addressed to determine how to achieve optimal health in pregnancy.

Conclusion

Our findings show a significant relationship between maternal BMI, skin pigmentation, supplement use and season of blood collection with 25(OH)D status of pregnant women at delivery. Despite supplement use throughout pregnancy, 81% of women were either insufficient or deficient in vitamin D at delivery. There was no association between TAG concentration and 25(OH)D status at enrollment and delivery and our results suggest TAG concentration need not be taken into account when assessing 25(OH)D status.

In summary, 25(OH)D status decreased significantly from enrollment to delivery in pregnant women living in the Kansas City metropolitan area despite supplement use. Factors such as BMI, skin pigmentation and season of delivery must be taken into account when recommending levels of supplementation in pregnancy.

Chapter 6

SUMMARY

This study aimed to examine the change in 25(OH)D status from enrollment (8-20 weeks) gestation and the effect of supplementation on status in a cohort of pregnant women living in the Kansas City metropolitan area at 39°06' north.

We collected data from 299 pregnant women at enrollment from the KUDOS cohort, of which 237 had delivery information available. We measured 25(OH)D in plasma samples using a 25-OH vitamin D ELISA kit at 8-20 weeks gestation and at delivery using a 25-OH vitamin D EIA Kit. We measured TAG concentrations at enrollment and delivery using a triglyceride assay kit. Supplement use was self reported by the women and used in the analysis to determine associations with 25(OH)D status.

Maternal 25(OH)D status decreased by 40% from 8-20 weeks gestation to delivery. Rates of deficiency, insufficiency, sufficiency, and toxicity were 25.8%, 30.4%, 40.8% and 3.0% respectively at enrollment and 54.9%, 29.6%, 15.6% and 0% respectively at delivery. Mean 25(OH)D concentration of African American women at enrollment was 12.39 ± 2.94 ng/ml and 6.80 ± 3.20 ng/ml at delivery; whereas, Caucasian women had mean levels of 23.84 ± 3.80 ng/ml at enrollment and levels of 13.37 ± 3.36 ng/ml at delivery. Vitamin D levels of Caucasian women were significantly higher than African American women at both enrollment ($p < 0.00001$) and delivery ($p < 0.0001$). Additionally, women with a higher BMI were more likely to have lower 25(OH)D status at enrollment ($p < 0.001$) and delivery ($p = 0.018$). Season of blood collection significantly affected 25(OH)D status at delivery ($p < 0.001$). Vitamin D supplementation was also significantly associated with higher 25(OH)D status at delivery (p

=0.033). Maternal concentration of TAG was not significantly related to 25(OH)D status at enrollment or delivery.

Our hypothesis 25(OH)D status would decrease throughout pregnancy and low levels of supplementation would create sufficient status are in line with the results we observed in our study. Although the women in our cohort were supplementing with mean levels of 331 IU of vitamin D per day, only 15.6% of women had sufficient levels of 25(OH)D at delivery. The findings from our study suggest pregnant women living at a latitude of 39°06' north in Kansas City are not supplementing with enough vitamin D throughout pregnancy in order to reach levels of sufficiency during gestation and at delivery. Because low vitamin D status has serious implications for the mother and fetus, it is pertinent to make revisions to current recommendations. New recommendations should take into account race, BMI, and months of gestation to create individualized approaches to vitamin D supplementation in pregnancy.

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APPENDIX A

25(OH)D Assay

Plasma 25(OH)D was measured using an EIA kit (Immunodiagnostik AG, Bensheim, Germany). More detailed procedures are available in the kit manual.

Principle of Assay

The kit measures 25(OH)D through a competitive binding assay. The assay is based on a competition between 25(OH)D and a 25(OH)D tracer for the binding pocket of vitamin D binding protein. In vivo, all 25(OH)D is bound to VDBP, so the first step of the assay is to precipitate the samples with a precipitating regent to extract the 25(OH)D in the samples. After precipitating the samples, the first incubation step includes adding the sample, calibrator, control, VDBP, VDBP-antibody and an antibody specific for the protein to a solid phase. The 25(OH)D present in the sample and the tracer compete for the VDBP binding site in the coated well. The antibody is bound to the VDBP, causing the VDBP to be immobilized to the well via the tracer. The next incubation step involves the addition of tetramethylbenzidine (TMB) as a host specific peroxidase labeled antibody. Next an acidic stopping solution is added to stop the reaction, causing the contents of the well to turn yellow. The intensity of the yellow color is directly proportional to the 25(OH)D present in the sample. The concentration of 25(OH)D is determined by comparison to a dose response curve determined by the calibrators.

Procedure

1. Label a protocol sheet for every standard, control, and sample to match the placement in the wells.
2. Place the microtiter plate and precipitated samples, standards and controls on a cool block or ice.
3. Allow samples to come to room temperature (18-26°C) and pipette 20 µl of standards, samples, and controls to their respective wells.

4. Add 100 µl VDBP into each well except the NSB well.
5. Add 100 µl assay buffer to the NSB wells.
6. Add 100 µl anti-VDBP antibody into each well.
7. Cover the plate tightly and incubate for 3 hours at 8-10° C in the dark.
8. Aspirate and wash the cells five times with 250 µl of diluted wash buffer, remove remaining wash buffer by hitting the plate against paper towel after the last wash.
9. Add 200 µl conjugate into each well.
10. Cover the plate tightly and incubate for 1 hour at 8-10° C in the dark.
11. Aspirate and wash the cells five times with 250 µl of diluted wash buffer, remove remaining wash buffer by hitting the plate against paper towel after the last wash.
12. Add 200 µl of substrate into each well
13. Incubate for 20-30 minutes at room temperature (18-26°C) in the dark.
14. Add 50 µl of stop solution into each well.
15. Determine the absorption with ELISA reader at 450 nm against 620 nm as a reference.

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.

Principle of the Assay

In this assay, triglycerides are hydrolyzed into glycerol and free fatty acids by lipoprotein lipase. The glycerol is phosphorylated to form glycerol-3-phosphate by glycerol kinase. The glycerol-3-phosphate is oxidized to dihydroxyacetone phosphate and hydrogen peroxide by glycerol phosphate oxidase. The hydrogen peroxide reacts with 4-aminoantipyrine and N-ethyl-N-(3-sulfopropyl)-m-anisidine to form the quinoneimine dye and water. The quinoneimine dye will turn different intensities of purple depending on the triglyceride concentration of the samples. Triglyceride concentrations are determined by comparing absorbance values of the samples with the standard curve of the standard triglyceride samples.

Procedure

1. Label 8 1.5 ml Eppendorf tubes using numbers 1-8.
2. Add 200 µl of the triglyceride standard diluent to tubes 2-8 and 400 µl to tube 1.
3. Add 100 µl of triglyceride standard to tube 1 and mix thoroughly.
4. Serially dilute the triglycerides by removing 200 µl from tube 1 and it to tube 2 and mix thoroughly. Move 200 µl from tube 2 to tube 3 and mix thoroughly. Repeat for tubes 4-7. Tube 8 only contains the triglyceride standard diluent and is used as a blank.
5. Add 10 µl of standard (tubes 1-8) per well in the designated standard wells on the plate
6. Add 10 µl of sample to two or three wells.
7. Add 150 µl of diluted enzyme buffer solution to each well.

8. Carefully shake the microtiter plate for a few seconds to mix and then cover the plate.
9. Incubate the plate for 15 minutes at room temperature
10. Read the absorbance at 530-550 nm using a plate reader.