Clinical Trials

Vitamin D Supplementation during Pregnancy: Double Blind, Randomized Clinical Trial of Safety and Effectiveness

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Disclosures

Bruce W. Hollis, Ph.D. serves as a consultant for Diasorin Inc., Stillwater, MN.

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Abstract

Background: The need, safety and effectiveness of vitamin D supplementation during pregnancy remain controversial.

Design: In this randomized controlled trial, women with a singleton pregnancy at 12-16 weeks' gestation received 400, 2000 or 4000 IU vitamin D_3/day until delivery. The primary outcome was maternal/neonatal circulating 25(OH)D at delivery, with secondary outcomes 25(OH)D ≥80 nmol/L achieved and 25(OH)D concentration required to achieve maximal $1,25(OH)_2D$ production.

Results: Of the 494 women enrolled, 350 women continued until delivery: Mean 25(OH)D by group at delivery and 1-month before delivery were significantly different ($p<0.0001$), and percent who achieved sufficiency was significantly different by group, greatest in 4000 IU group ($p<0.0001$). The relative risk (RR) for achieving ≥80 nmol/L within one month of delivery was significantly different between 2000 vs. 400 IU (RR 1.52 [CI 1.24-1.86]); 4000 vs. 400 (RR 1.60 [CI 1.32-1.95]), but not between 4000 vs. 2000 (RR 1.06 [CI 0.93-1.19]). Circulating 25(OH)D had a direct influence on circulating $1,25(OH)_2D$ concentrations throughout pregnancy ($p<0.0001$) with maximal production of $1,25(OH)_2D$ in all strata in the 4000 IU group. There were no differences between groups on any safety measure. Not a single adverse event was attributed to vitamin D supplementation or circulating 25(OH)D levels.

Conclusions: Vitamin D supplementation of 4,000 IU/day for pregnant women was safe and most effective in achieving sufficiency in all women and their neonates regardless of race while the current estimated average requirement was comparatively ineffective at achieving adequate circulating 25(OH)D, especially in African Americans.

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Introduction

The function of vitamin D during pregnancy for both mother and fetus remains largely undefined. Vitamin D is known to be involved in skeletal homeostasis during pregnancy as evidenced by a recent publication dealing with craniotabes in the newborn, and severe vitamin D deficiency may lead to neonatal seizures in those neonates with profound hypocalcemia (1-5). The function of vitamin D during this sensitive period, however, may also have potential effects on other systems, including immune (6-10), pancreatic (11-13), musculoskeletal (14-17), and cardiovascular function (18-20) as well as neural development (21-24). Recent publications suggest relationships between maternal vitamin D status and adverse pregnancy outcomes such as preeclampsia and cesarean section (25-28).

A Cochrane Review published in 2000 highlighted the relative dearth of data dealing with vitamin D supplementation during human pregnancy (29). This review listed seven studies on the topic (30-36), of which four reported clinical outcomes (30-32,36). From these limited data, the Cochrane Review concluded that there was insufficient evidence to evaluate the effects of vitamin D supplementation during pregnancy (29). Since that time, there have been few studies that have addressed this issue (37-39).

In 2004, we initiated an NICHD-sponsored 6-year randomized double-blind placebo-control trial of vitamin D supplementation during pregnancy to assess safety and pregnancy outcomes with an approved Investigational Drug Application from the FDA (#66,346). We hypothesized that 4000 IU/day vitamin D_3 would be more efficacious and effective than the standard dosing regimen of 400 IU/day and 2000 IU (the former upper limit for vitamin D)(40)/day dosing regimen in achieving a total circulating 25(OH)D level of at least 80 nmoL/L (32/ng/mL) in pregnant women regardless of race throughout pregnancy and at the time of delivery without causing any safety concerns. This minimal value of 80 nmol/L was based on years of research with regard to circulating 25(OH)D levels suppressing secondary hyperthyroidism, optimal intestinal calcium absorption and bone mineral density (41). These results are presented here.

Methods

Study Design: This study was a single center, randomized, controlled, double-blinded study of vitamin D supplementation stratified by race (FDA IND #66,346; ClinicalTrials.gov # NCT00292591). Women less than 16 weeks' gestation with a singleton pregnancy were eligible for participation in the study.

Study Participants and Setting: This study was approved by MUSC's Institutional Review Board for Human Research (HR# 10725), and was conducted from January 4, 2004 through July 31, 2009 at the Medical University of South Carolina (MUSC), Charleston, South Carolina. The inclusion criteria for the subjects included the following: (1) maternal age of 16 years or greater at the time of consent; (2) confirmed singleton pregnancy of less than 16 completed weeks of gestation at the time of consent; (3) planned to receive ongoing prenatal care in the Charleston, SC area; and (4) the ability to provide written informed consent at the first visit. If a woman received her obstetrical care at a facility separate from MUSC, then she came to MUSC's Clinical and Translational Research Center (CTRC) outpatient research facility for each of the study visits. Women were consented at their first prenatal visit, at which time baseline 25(0H)D levels were measured. Irrespective of gestational age at enrollment, subjects began vitamin D supplementation between the start of the 12^{th} and the start of the 16^{th} weeks of gestation (12 $0/7^{th}$ and 16 $0/7^{th}$ weeks) as defined by their last menstrual period.

Exclusion Criteria: Those women with a pregnancy greater than 16 weeks of gestation as calculated by last menstrual period were not eligible to participate. Pregnant women with pre-existing calcium or parathyroid conditions, or who required chronic diuretic or cardiac medication therapy including calcium channel blockers, or chronic hypertension were not eligible for enrollment into the study. Pregnant women with active thyroid disease (e.g., Graves, Hashimoto's or thyroiditis) also were excluded; however, mothers on thyroid supplement with normal serological parameters could participate in the study if they were without any other endocrine dysfunction

Study Protocol

Gestational Age at Enrollment: Subjects could be consented and enrolled into the study before the

initiation of vitamin D supplementation at 12-16 weeks of gestation. Gestational age was based on last menstrual period. If a woman was unsure of her gestational age, the obstetrical estimate at the time of the visit was used. If, at the 20-week fetal ultrasound it was determined by the obstetrician that the gestational age was incorrect, the revised gestational age was used and the discrepancy noted.

Initial Study Visit: Baseline blood and urine samples were obtained following subject consent at the initial visit; however, the earliest time of randomization following measurement of baseline total circulating 25(OH)D was 12 weeks' gestation with the target upper limit of gestation of 16 weeks' gestation. Irrespective of enrollment gestational age, vitamin D supplementation did not begin before the twelve week of gestation (12 and $0/7^{th}$ weeks).

Subsequent Study Visits: Subjects were followed with monthly study visits, which continued until delivery. The visits coincided with routine obstetrical visits or were performed in conjunction with those visits if the obstetrical care was provided outside of MUSC. The subjects also were seen at the GCRC/CTRC for a study visit at 16 weeks of gestation, and with their infant at 2 weeks' postpartum.

Completion of Questionnaires: Following their written, informed consent, mothers completed questionnaires regarding sociodemographic information, baseline health status and medical history at the first visit. At the second visit, the Block Food Frequency Questionnaire was completed to ascertain generalized eating pattern, with specific calculation of calcium and vitamin D intake (Block, Berkeley, California).(42-47). Each completed FFQ form was sent to the processing center (Berkeley, California) and this data were later reviewed for accuracy by a registered dietician who was blinded to subject treatment group assignment. Total caloric intake, vitamin D and calcium intake were recorded for each subject.

An interim maternal health history questionnaire also was completed at each visit with the assistance of the study coordinator to ascertain adverse events, discussing type and frequency of acute illnesses such as respiratory, gastrointestinal, and other viral and/or bacterial illnesses. A review of medications and doctor's visits was obtained at that time.

After delivery, the newborn record of each infant was reviewed for mode of delivery and level of

neonatal care required (normal newborn nursery, Level 2 or Level 3 intensive care). Birth weight (grams) and gestational age also were recorded.

Blood and Urine Samples: Maternal blood and urine samples were collected at each visit. Cord blood was obtained at delivery. If the cord blood sample could not be obtained, a neonatal blood sample was drawn within two weeks of delivery.

Intervention

Multi-Vitamin and Vitamin D Supplementation: Pregnant women who presented for prenatal care at ≤16 weeks' gestation were randomized into one of three treatment regimens of vitamin D_3 after establishing their baseline serum 25(OH)D level. All patients received a total of two pills daily: a standard prenatal multivitamin vitamin containing 400 IU of vitamin D and an additional vitamin D_3 supplement of 0 IU (placebo), 1600 IU, or 3600 IU of vitamin D_3 for a total of 400 IU, 2000 IU and 4000 IU of vitamin D supplementation, respectively.

In order to obtain Institutional Review Board approval for the study, the following safety measure was put into place: baseline total circulating 25(OH)D levels were measured and women with levels ≤100 nmol/L (40 ng/mL) were eligible for randomization into one of the three arms (400, 2000 or 4000 IU vitamin D_3/day) with further substratification by race within each treatment group. Women with baseline 25(OH)D levels >100 nmol/L to 150 nmol/L (>40-60 ng/mL, levels considered to be in the normal range at the time of study implementation) were randomized into one of two treatment groups (400 or 2000 IU vitamin D_3/day), while women with a baseline 25(OH)D level >150 nmol/L (>60 ng/mL) were given 400 IU vitamin D_3/day. The doses of vitamin D utilized in our study were selected based on current recommendations (400 IU/day), the upper safe intake level established in 1997 (2000 IU/day)(40) and the amount we calculated to be required to achieve nutritional vitamin D sufficiency (4000 IU/day) (48) .

Adherence to Medication Regimen: Adherence to the prescribed vitamin D supplementation regimen of 1 prenatal vitamin and the vitamin D supplement was measured by maternal self-report and

pill counts at each follow-up visit (49). The number of vitamin D pills returned was divided by the expected number of pills that would have been taken between study visits to generate a percentage that served as a measure of adherence of medication regimen between study visits. The adherence measures were used to generate an average adherence for each subject (49). If a woman missed one prenatal visit, her next month supply of vitamins was either mailed to her or dropped off at her residence. In such cases, medication adherence was based on the pill count from the date of the last visit to the current prenatal visit over the expected number of pills taken. If a woman had more than two missed visits or if she failed to take at least 50% of the prescribed vitamin D pills, she was exited from the study.

Randomization

Our study utilized stratified blocked randomization to balance by ethnicity and also to balance by enrollment (as a cautionary measure against a potential temporal or seasonal bias). A randomization scheme was separately developed for each of the three ethnic groups (i.e., the stratum). Within each stratum, the treatments were assigned within blocks. Since there were three treatment groups, the block size had to be divisible by three; the data team selected a block size of six, which was unknown to the investigators or the pharmacists. In this way, at the end of each block (i.e., enrollment of six subjects), each ethnic group was balanced in the number randomly assigned to 400, 2000, and 4000 IU treatment groups.

Materials

Source of Vitamin D: Vitamin D tablets were manufactured by Tishcon Corporation, Westbury, NY, a Good-Manufacturing-Practice (GMP) facility. The cholecalciferol contained in the vitamin D tablet was supplied to Tishcon Corporation by Hoffman-La Roche Ltd., Basel, Switzerland. The tablet vitamin D concentration was verified by the company every 6 months and by an independent laboratory chosen by the investigators (Heartland Assays, Ames, IO) using HPLC-UV to ensure the tablets met label claim

throughout the study; these results were reported to the Investigational Drugs Department at MUSC. Tablets were maintained in MUSC's Research Pharmacy until the time that they were dispensed to each enrolled subject.

Source of Prenatal Vitamins: Prenatal vitamins prescribed at the time of each subject's enrollment were Myadec Multivitamin-Multimineral Supplement (distributed by Pfizer Consumer Healthcare, Morris Plains, NJ) with 400 IU vitamin D_3/tablet. Those mothers who were unable to swallow a prenatal vitamin were given Flintstones™ Complete chewable vitamin (Bayer Healthcare, Morristown, NJ), which provided 400 IU vitamin D_3 per vitamin.

Measures

Maternal Sociodemographic Measures included maternal age at time of enrollment, her self-defined race, insurance status, educational status, occupation and employment outside of the home.

Pregnancy Health Status, and Labor and Delivery Characteristics and Complications: Characteristics of each mother's health status and complications during pregnancy, labor, and delivery were recorded and reviewed by an obstetrician (DDJ blinded to treatment). If the mother required hospitalization, a copy of the hospital record was obtained after she had signed a release of medical information form. Any acute illnesses, hospitalizations or development of pregnancy-related conditions that were not preexisting also were recorded. The Data Monitoring and Safety Committee (DSMC) was notified of all such events.

Anthropomorphic Measurements: Pre-pregnancy height and weight of each mother were recorded at the first outpatient visit to determine Body Mass Index or BMI (weight [kg]/height^2 [m^2]). During subsequent visits, only the subject's weight was recorded. Birth weight (grams) was recorded for each infant.

Laboratory Measurements

Maternal and Cord Blood/Neonatal Vitamin D and Metabolite Assays: *Circulating vitamin D_2 and D_3* were measured in serum using direct ultraviolet detection preceded by organic extraction and high performance liquid chromatography as previously described (50). This assay has a coefficient of variation of ≤10% and a 5 nmol/L vitamin D detection limit. There is no normal established circulating range of vitamins D_2 or D_3 in human subjects.

A rapid, direct RIA developed in the Hollis laboratory and manufactured by Diasorin Corporation (Stillwater, MN) was used to measure *total circulating 25(OH)D* concentration in serum samples (51). This RIA is an FDA-cleared device and, in fact, is the FDA predicate device for the measurement of circulating 25(OH)D in humans.

Based on clinical laboratory classifications (52,53), *a priori*, deficiency was defined as total circulating 25(OH)D <50 nmol/L (20 ng/mL), insufficiency as ≥50 to <80 nmol/L (≥20 to <32 ng/mL), and sufficiency as ≥80 nmol/L (≥32 ng/mL) (41,53-55). The inter- and intra-assay coefficient of variation is ≤10%.

An RIA manufactured by Diasorin Corporation and developed in the Hollis laboratory was used to measure total circulating $1,25(OH)_2D$ concentration (56). This assay uses an I^{125}-labeled tracer and samples are processed using acetonitrile followed by solid-phase extraction and quantitation. This RIA is an FDA-cleared device. The normal circulating level of $1,25(OH)_2D$ is 48-120 pmol/L (20-50 pg/mL). The inter- and intra-assay coefficient of variation is ≤15%.

Maternal and Cord Blood/Neonatal Circulating intact PTH Concentrations: Intact PTH (iPTH) was measured by immunoradiometric (IRMA) that utilizes 2 different polyclonal antibodies (Diasorin, Stillwater, MN). The first antibody, specific for PTH 39-84, is bound to a solid phase bead. The second antibody is specific for PTH 1-34 and is labeled with ^{125}I. The adult normal range for iPTH in our laboratory is 1.3-5.4 pmol/L. Higher vitamin D levels are associated with lower iPTH; as vitamin D status improves iPTH declines (57).

Maternal Baseline and Follow-up Serum Calcium, Creatinine and Phosphorus Studies: Maternal serum total calcium, creatinine and inorganic phosphorus were measured by MUSC's Clinical Chemistry

Laboratory using standard methodology and laboratory normative data. Results were reported to the PI and downloaded to the research database from the clinical chemistry registry. All results were reviewed by the Clinical PI of the study on a weekly basis for any abnormal values and reported to the DSMC.

Circulating Vitamin D Binding Protein (VDBP): VDBP was measured using a commercial ELISA purchased from R&D Systems (Minneapolis, MN). Circulating VDBP levels in normal individuals using this ELISA is stated by the manufacturer to be 3.93 ± 1.62 μmol/L.

Maternal Urinary Calcium/Creatinine Ratio: A non-fasting urine sample was obtained from the mother at each obstetrical visit and was sent to the Clinical Chemistry Laboratory at MUSC for urinary calcium (Ca) and creatinine (Cr) measurements[1] and derivation of the urinary Ca (mg/dL): Cr (mg/dL) ratio (converted to mmol/L/mmol/L).

Safety Measures throughout the Study: All study subjects were monitored for hypervitaminosis D. The circulating 25(OH)D level of 225 nmol/L (90 ng/mL) was used to define hypervitaminosis D as required by the FDA and our IRB. This conservative maternal level was arbitrarily chosen to ensure the safety of all study patients, particularly those assigned to the 4,000 IU vitamin D_3/day regimen (58). Subsequent vitamin D supplementation trials have demonstrated that circulating levels of 25(OH)D exceeding 300 nmol/L (120 ng/mL) do not cause hypercalciuria, the first indicator of hypervitaminosis D (48). Even in women who are vitamin D deficient, urinary calcium excretion increases during pregnancy secondary to increased glomerular filtration rate (59). Given this, urinary calcium: creatinine ratio was used and is the most sensitive, early indicator of hypervitaminosis D. Operationally, we defined *a priori* caution limits for hypervitaminosis D as a non-fasting urinary calcium/creatinine ratio ≥0.8 mg/ mg or 2.27 mmol/mmol.

Whenever any patient exceeded the caution limit or had an abnormal clinical chemistry value, a specific case study by the Data Safety and Monitoring Committee (DSMC) was to be initiated to examine the contribution of confounding factors (e.g., diet, sunlight exposure, etc.). Operationally, vitamin D_3

[1] To convert mg/dL of calcium to mmol/L, multiply the value by 0.25. To convert mg/dL of creatinine to mmol/L, multiply by 0.088.

supplementation stopped if the urinary calcium: creatinine ratio exceeded 1.0 (mg/dL/mg/dL) or if the circulating 25(OH)D level exceeded 225 nmol/L (90 ng/mL).

Statistical Methods

Sample Size and Power Considerations:

To detect a statistically significant increase in 25(OH)D by 10 ng/mL between any two groups was calculated to require a minimum of 32 patients per group at 90% power, alpha = 0.05, two tailed test for the primary analysis. This calculation assumed that the standard deviation of 25(OH)D measurements at a single time point was approximately 10, that there would be a low correlation (r = 0.25) between the baseline and final measurements, and that a substantial proportion (up to 50%) of participants may be lost to follow-up due to moving, termination of care, or discontinuation of participation. Because the primary outcome—maternal and neonatal vitamin D status at or around the time of delivery, a prerequisite for inclusion in the final analysis was that the mother had to have had a livebirth and had to have been a study participate until the day of delivery. Lastly, as one of the secondary goals of this study was to explore vitamin D differences by ethnicity, the three supplemented groups (400, 2,000 and 4,000 IU/day) were balanced by ethnicity (equal numbers of Caucasian, African American, and Hispanic).

Statistical Analysis

The main variables of interest were: (1) differences in mean maternal and infant total circulating 25(OH)D at the time of delivery between supplement groups (ANOVA); and (2) differences, between supplement groups, in the proportion of women achieving 25(OH)D of ≥80 nmol/L within one month and at the time of delivery (Chi-square). Secondary analyses employed Chi-square for categorical variables, ANOVA or Student's t-test, as appropriate, for normally distributed variables (with the Bonferonni option for pair-wise analysis in ANOVA), and paired Student's t-test for within group changes from baseline to delivery. Multiple regression was used to assess the association between final vitamin D concentrations with baseline values, ethnicity, dose group and the dose *race interaction. Stratified

analysis was used to more fully explore any evidence of interaction. Variables that were not normally distributed were analyzed with Wilcoxon-Mann-Whitney test. The association between vitamin D, 25(OH)D and $1,25(OH)_2D$; D_3; and urinary calcium: creatinine ratio were explored with a combination of exponential and linear models. Data were analyzed with SAS 9.22(60) (Cary, NC) and SigmaPlot software (Systat Software, Inc., San Jose, CA).

The analysis was conducted as an intention-to-treat (ITT) (61). The ITT approach (effectiveness) compares the outcomes between supplement groups, as assigned, and makes no assumption regarding whether or not subjects were adherent to the dosing regimen.[2] The intention-to-treat design was used as a measure of the effectiveness of increasing vitamin D levels via oral dosing. This approach presents a conservative finding of potential benefits that could be shown from a population- or public health-based intervention.[3]

Results

Study Population

Figure 1 shows the enrollment, allocation, and follow-up of the women who participated in the trial. A total of 516 women were interviewed and 502 consented to participate in this study and were randomly assigned to a treatment group: 166 were assigned to Group 1 (400 IU group); 167 were assigned to Group 2 (2000 IU group); and 169 were assigned to Group 3 (4000 IU group). Of the 502 women consented, there were 23 women with a valid initial 25(OH)D greater than 100 nmol/L (40 ng/mL) who were not eligible for enrollment in the 4000 IU group: 2 African American, 6 Hispanic and 15 Caucasian women. Of those, 12 were enrolled into the 400 IU group, 10 were enrolled into the 2000 IU group, and 1 was enrolled in the 4000 IU group (the latter being a protocol deviation early in the study where one woman with a baseline 25(OH)D of 41 was randomized to the 4000 IU group): 17

[2] Data of adherent subjects will be made available to any investigator upon request following publication.
[3] Adherence efficacy and outcome data with detailed pharmacokinetics will be presented in a separate paper.

continued until delivery: 1 African American, 6 Hispanic and 10 Caucasian women; 10 were in the 400 IU group, and 6 were in the 2000 IU group, and 1 was in the 4000 IU group. Finally, there was one Caucasian woman whose baseline 25(OH)D level was 172.5 nmoL/L (69 ng/mL) who was placed into the 400 IU group. After allocation into treatment groups, there were no statistically significant differences among the groups with regard to lost to follow-up, drop-outs or pregnancy losses.

The sociodemographic characteristics of the active cohort are found in **Table 1**. Baseline characteristics were similar between the groups on the basis of race/ethnicity, maternal age, gestational age at enrollment, educational and employment status, health rating, planned pregnancy, body mass index and season at study entry. There was a trend toward differences between the groups on the basis of maternal gravidity and parity and insurance status. A total of 62 women (12.4%) were taking a prenatal vitamin at the time of randomization. Of the 502 women who were randomized to treatment, 350 women continued in the study until delivery and had outcome data available for analysis: 98 African American, 137 Hispanic and 115 Caucasian women evenly distributed into the 3 treatment groups, with 111 controls, 122 in 2000 IU and 117 in 4000 IU groups. There were no differences in baseline vitamin D status among treatment groups.

A comparison of those women who completed the study and those who electively discontinued the study is found in **Table 2**. Women who had a pregnancy loss or who moved away were excluded in this analysis. Individuals who exited the study did not differ by treatment group. Those women who electively exited the study were more likely to be African American than Hispanic or Caucasian. Compared to women who continued in the study until delivery, women who exited the study were more likely to be younger ($p=0.01$), African American ($p=0.003$), of higher gravidity ($p<0.0001$), less educated ($p=0.01$), employed at entrance into the study ($p=0.04$), with an unplanned pregnancy ($p=0.01$), and with a BMI <30 ($p=0.02$). Baseline vitamin D status by ethnicity of those who completed vs. those who exited also did not differ (see **Table 2**).

With regard to pregnancy losses, there were 8 women in the 400 IU group (baseline mean ± SD: 16.5 ± 7.6 weeks; median 15.5; range 10.0-34.0); 5 in the 2000 IU group (baseline mean ± SD: 17.2 ±4.6;

median 15.0; range 12.0-23.0); and 10 in the 4000 IU group (baseline mean ± SD: 16.4 ± 6.3; median 16.0; range 9-32) who experienced a loss after enrollment into the study. The 25(OH)D level around or at the time of the loss did not differ by treatment groups (p=0.8) There were no statistically significant differences in mean gestational age at loss among the treatment groups (p=0.9) or in the % losses per treatment group (p=0.4). When looking at baseline 25(OH)D of those women who delivered a liveborn infant vs. those who experienced a pregnancy loss, the mean levels were 57.8 ± 24.4 vs. 50.5 ± 23.3 nmol/L; however, this did not reach statistical significance.

Among the 350 women who continued in the study until delivery, the median ratio of the number of study capsules taken to the number that should have been taken between the time of randomization and delivery was similar between the groups. Adherence to protocol was not statistically different between treatment groups: 69% (400 IU group), 68% (2000 IU group), and 69% (4000 IU group) (p=0.9).

Study Outcomes

As shown in **Table 3**, the primary outcome—mean circulating 25(OH)D one month prior to delivery and at delivery was statistically different between treatment groups, with the highest mean level achieved in the 4000 IU group.. Overall, the mean 25(OH)D by dose group one month before delivery and at delivery, and as chronic levels measured the average from 20-36 weeks of gestation were significantly different between control and 2000, control and 4000, and 2000 vs. 4000 ($p<0.0001$).

The secondary outcome measure of attaining a total circulating 25(OH)D of at least 80 nmol/L at the time of delivery was met by 43/86 (50%) women in the 400 IU group, 63/80 (70.8%) in the 2000 IU Group, and 68/83 (82%) in the 4000 IU Group (see **Table 3**); however, there were 92 women with missing levels at delivery ($p<0.0001$). Because of the high correlation ($r=0.72$, $p<0.0001$) between one month prior to delivery and delivery 25(OH)D values, one month prior to delivery values were used as a surrogate for delivery values for the women with missing delivery room values. When combined, 57/109 (52.3%) women in the 400 IU Group, 93/117 (79.5%) in the 2000 IU Group, and 94/112 (83.9%) women in the 4000 IU Group achieved a minimal circulating 25(OH)D level of at least 80 nmol/L around the time

of delivery ($p<0.0001$). Expressed as relative risk ratios, as shown in **Table 4**, there were significant differences between the 2000 vs. 400 IU groups (RR 1.52; 95% CI 1.24-1.86) and between the 4000 vs. 400 IU groups (RR 1.60; 95% CI 1.32-1.95); however, there was not a significant difference between the 2000 and 4000 IU groups in this regard.

Vitamin D supplementation at various treatment doses given to our pregnant population had a variable effect on circulating levels of vitamin D_3 and its metabolites (**Figure 2a**). Supplementation of vitamin D_3 at double the prior 1997 IOM recommendation of 200 IU/d(40) and the current IOM EAR (62) provided essentially no increase in circulating vitamin D_3 levels and only a minimal 5 ng/mL rise in circulating 25(OH)D levels over the duration of the study (**Figures 2a, 2b**). Conversely, supplementing 2000 or 4000 IU/d vitamin D_3 had a profound effect on increasing both circulating levels of vitamin D_3 and 25(OH)D (**Figures 2a, 2b and Table 5a and b**). **Figure 3a** describes the substrate-product relationship in all patients between vitamin D_3 and 25(OH)D. The relationship is biphasic with respect to 25(OH)D production requiring at least 10 ng/mLl circulating vitamin D_3 to saturate the vitamin D-25-hydroxylase.

Table 5a and b provide additional information with respect to circulating 25(OH)D concentrations analyzed by race, vitamin D dose, and stage of gestation. Clearly, race and duration of supplementation have profound effects on the circulating level of 25(OH)D attained. African Americans lag at every time point and dose in relation to circulating 25(OH)D. This is especially noticeable in the 400 IU group. In contrast, a greater proportion of African American women achieved 25(OH)D ≥80 nmol/L by the second trimester in the 4000 IU group when compared to both the 400 and 2000 IU groups, (see **Table 5b**).

One of the most interesting biochemical findings in our study was the association between circulating $1,25(OH)_2D$ levels and of circulating 25(OH)D (**Figures 2c and 3b**). In exploring the association between 25(OH)D and $1,25(OH)_2D$, 25(OH)D was found to have a direct influence on $1,25(OH)_2D$ levels throughout pregnancy ($p<0.0001$). While the baseline $1,25(OH)_2D$ level in all groups at 12 weeks' gestation were not significantly different (**Figure 2c**), within a few weeks, however, the circulating $1,25(OH)_2D$ levels became significantly elevated in the 2,000 and 4,000 IU groups as opposed to the 400 IU group.

The relationship between these vitamin D metabolites is examined more closely in **Figure 3b**. This figure clearly demonstrates a biphasic relationship between circulating 25(OH)D and $1,25(OH)_2D$ with circulating levels of 25(OH)D of at least 100 nmol/L (40 ng/ml) required to support maximum $1,25(OH)_2D$ output in the pregnant women. It is also worthy to note that circulating $1,25(OH)_2D$ levels at 12 weeks' gestation are approximately triple that of normal, nonpregnant female and normal male subjects as previously reported (Fig. 2c) (56).

Figure 2d and **Table 5c and d** also display circulating intact PTH levels. The trend of PTH in all subjects was higher as the subjects progressed through pregnancy, but was not significantly different by treatment group. Decreases in circulating PTH was observed if the levels attained were analyzed by race. The African American group clearly had decreased circulating PTH as circulating 25(OH)D levels increased (**Table 5c and d**).

Circulating levels of VDBP were measured in 80 selected subjects based on their circulating $1,25(OH)_2D$ levels, which ranged from 224.9 to 768.0 pmol/L at various stages of gestation. The average level of VDBP with this ELISA detected in these subjects was 5.45 ± 1.26 μmol/L, which represented a 39% increase as compared to normal subjects. Further, using linear regression, no relationship was observed between circulating VDBP and $1,25(OH)_2D$.

With respect to the effect of circulating 25(OH)D on either blood calcium or urinary calcium, no significant effects were observed with one exception: that being the relationship between low circulating 25(OH)D and urinary calcium levels (**Figures 4a, 4b, 5**). From **Figure 5**, it would appear that approximately 75 nmol/L (30 ng/mL) circulating 25(OH)D was required in the pregnant women to normalize urinary calcium excretion. Above that threshold, 25(OH)D appeared not to influence urinary calcium and subsequent excretion.

Throughout the study, there were no statistically significant differences between groups on any safety measure: serum calcium, creatinine, and phosphorus, and urinary calcium: creatinine ratios (p-value not significant [pNS] between groups). Review of adverse events by the DSMC showed that not a single adverse event in this trial was attributed to vitamin D supplementation or circulating 25(OH)D

levels. There was one safety measure stop implementation: in the 4000 IU group, one woman with a baseline circulating 25(OH)D level of 29.3 nmol/L (13.3 ng/mL) increased to 233.3 nmol/L (93.3 ng/mL) at visit 2. Her follow-up circulating 25(OH)D level at the return visit prior to stopping supplementation had decreased to 66.6 ng/mL. Although her urinary calcium/creatinine ratio and all serum biochemical indices were within normal limits, the woman ceased supplementation per protocol.

Mode of delivery and neonatal characteristics by maternal treatment group are found in **Table 6**. There were no differences between the groups in terms of gestational age at delivery or birth weight. In addition, there were no significant differences in level of care (newborn nursery vs. higher level of care [level 2 or neonatal intensive care admission]) or increased adverse outcomes of pregnancy related to maternal vitamin D intake. There were several differences, however, in terms of neonatal vitamin D status by treatment group: Neonatal 25(OH)D was significantly correlated with maternal 25(OH)D overall, 1-month prior, and at delivery (r^2=0.6; OR 0.50); and was significantly different by treatment group: 45.5±25.3 nmol/L (18.2±10.1 ng/mL, control), 57.0±24.5 nmol/L (22.8±9.8 ng/mL, 2000 IU) and 66.3±25.8 nmol/L (26.5 ±10.3 ng/mL, 4000 IU), ($p<0.0001$). By treatment group, using IOM guidelines for sufficiency (total circulating 25(OH)D ≥50 nmol/L or 20 ng/mL) (62), 31/78 (39.7%) neonates in the 400 IU group, 53/91 (58.2%) in the 2000 IU group, and 66/84 (78.6%) in the 4000 IU group had a cord blood/neonatal 25(OH)D level in the deficient range ($p<0.0001$).

Discussion

In this randomized controlled trial of a diverse group of pregnant women living at latitude 32^{o}N supplemented from 12-16 weeks of gestation until delivery, compared to the 400 (control) and 2000 IU groups, a daily vitamin D dose of 4000 IU was associated with improved vitamin D status at throughout pregnancy, one month prior, and at delivery in both mother and neonate. Irrespective of race and ethnicity, this improvement in vitamin D status was achieved without any evidence of hypervitaminosis D or an increase in adverse events during pregnancy and with optimization of 25(OH)D and 1,25$(OH)_2$D.

From a standpoint of enzyme kinetics, this simply means that in the case of vitamin D being converted to 25(OH)D and subsequently to $1,25(OH)_2D$, enzyme saturation is occurring, i.e., reaction rates are moving from first order to zero order enzyme kinetics. In simple terms, it means that an appropriate amount of substrate is being supplied to produce maximum product, i.e., 25(OH)D and $1,25(OH)_2D$: as such, no substrate "starvation" is occurring.

At no point in human nutrition is it more critical to ensure adequate nutrient intake than during the state of pregnancy. Folate intake during pregnancy and its role in the development of neural tube defect serves as a stark example (63,64). The limited clinical investigation into meaningful dietary vitamin D supplementation during pregnancy can be traced back to post-World War II Britain. Because of the British experience with idiopathic infantile hypercalcemia attributed to hypervitaminosis D, an inaccurate association occurred that had a profound effect on the potential of vitamin D supplementation, not only during infancy but also during pregnancy. In 1963, Black and Bonham-Carter(65) recognized that elfin facies observed in patients with severe idiopathic infantile hypercalcemia resembled the peculiar facies observed in patients with supravalvular aortic stenosis syndrome. By 1966 vitamin D was viewed by the medical community as the cause of SAS syndrome.(66,67) With the advent of molecular genetics, the children with SAS Syndrome were discovered to have Williams Syndrome, an example of unipaternal disomy, with abnormal vitamin D metabolism.(68-75)

The perception that vitamin D can inflict harm during pregnancy still lives on today as many obstetrical specialists are afraid to undertake vitamin D repletion during this period. Research efforts in this area were further hampered when in 1997, the Institute of Medicine issued guidelines that defined the adequate intake (AI) for vitamin D during pregnancy to be 200 IU/d with intakes greater than 2000 IU/d causing potential harm.(40) Recently, the IOM issued new guidelines with respect to pregnant women that define the estimated average requirement (EAR) and recommended dietary allowance (RDA) to be 400 and 600 IU/day, respectively. They also increased the tolerable upper intake limit (UL) to 4000 IU/day.(62) These new guidelines, with the exception of the UL, are based on old data since

limited new data exist. The result of prior and current guidelines is that most prenatal vitamins only contain 400 IU of vitamin D. In our experience, many of today's practicing obstetricians are unaware of the vitamin D content in prenatal vitamins or have a fear of administering additional vitamin D supplements to the pregnant women.

Our study was based on two previous vitamin D supplementation studies in non-pregnant adults that appeared to be safe.(48,58) Prior to undertaking the NIH-funded study described here, however, we had to obtain an Investigational Drug Number from the FDA which entailed writing a complete investigational drug application. This was required by the FDA since we proposed using a vitamin D_3 dose of 4,000 IU/d, 20 times the adequate intake (AI), and twice the safe limit put forth by the IOM in 1997,(40) but currently put forth as the UL.(62) Thus, our study is the first one to test this current UL in pregnant women.

The only known avenue of vitamin D toxicity is manifested through hypercalcemia and hypercalciuria,(76) neither of which was observed in our RCT. In fact, our Data and Safety Monitoring Committee concluded that not a single adverse event in this RCT could be attributed to vitamin D intake. Hypervitaminosis D is largely arbitrarily defined as circulating levels of 25(OH)D that exceed 375 nmol/L (150 ng/mL), a level we never attained with our dosing regimen. As has been observed in other human supplementation studies, the conversion of vitamin D to 25(OH)D appears to be controlled.(77) Further, it has been known for decades that during pregnancy $1,25(OH)_2D$ levels become extremely elevated.(78,79) This increase in circulating $1,25(OH)_2D$ levels has particularly been attributed to an increase in the serum vitamin D-binding protein that would regulate the amount of "free" $1,25(OH)_2D$ available in the circulation.(79) While this rise in VDBP during pregnancy has been shown to be 46-103%, depending on the assay employed,(80) it cannot account, however, for a nearly 3-4-fold increase in circulating $1,25(OH)_2D$ observed in our study. Bikle, et al., (81) clearly demonstrated that free $1,25(OH)_2D$ levels are increased during pregnancy despite the significant increase in VDBP levels. We were unable to measure "free" circulating levels of $1,25(OH)_2D$ in our subjects, however, our data agree with that of Bikle, et al., in that no relationship was observed during pregnancy between circulating

VDBP and "total" circulating $1,25(OH)_2D$ (81). New data from our study suggests that a circulating 25(OH)D level of approximately 100 nmol/L (40 ng/mL) is required to optimize production of $1,25(OH)_2D$ during human pregnancy through renal and/or placental production of the hormone (Figure 2c, 3b). It is also of great interest that production of circulating $1,25(OH)_2D$ in the fetus is directly linked to circulating 25(OH)D (10).

Clearly, vitamin D metabolism is greatly altered during pregnancy, and that pregnancy itself is the primary driver for these extraordinary circulating $1,25(OH)_2D$ levels. From our data, it is evident that production of $1,25(OH)_2D$ is really not under the control of the classic regulators of calcium, phosphorus and PTH. The dramatic rise in maternal circulating $1,25(OH)_2D$ following conception is remarkable for many reasons: by 12 weeks of gestation, maternal circulating $1,25(OH)_2D$ levels are already triple that of the nonpregnant female (Figure 2c). From that point in gestation, the $1,25(OH)_2D$ levels rise much higher and are driven by substrate—25(OH)D availability (Figure 3b). This substrate dependence of $1,25(OH)_2D$ production is never observed in normal human physiology driven by classical calcium homeostasis (10,82,83).

Another remarkable factor in pregnant women is how they can attain supra-physiologic levels of $1,25(OH)_2D$, sometimes exceeding 700 pmol/L in our study, and never exhibit hypercalciuria or hypercalcemia. These tremendous circulating levels of $1,25(OH)_2D$ during pregnancy are possibly of placental origin or from the renal 1-α-hydroxylase that would have to be uncoupled from feedback control and for reasons other than maintaining calcium homeostasis. The second scenario is most likely because women with nonfunctional renal 1-α-hydroxylase and normal placental function fail to increase circulating $1,25(OH)_2D$ during pregnancy (84). The increased levels of $1,25(OH)_2D$ may be due to the methylation of the catabolic CYP24A1 placental gene (85). It is possible that calcitonin may be a contributor to this process in that calcitonin rises during pregnancy (86), is known to stimulate the renal 1-α-hydroxylase gene independent of calcium levels (87,88), and also protects by opposing hypercalcemia (89). Another possible stimulator of the 1-α-hydroxylase during pregnancy is prolactin (90). If prolactin was a major contributor, however, the effect should continue into lactation, which we

do not see; and would be accompanied by elevated circulating $1,25(OH)_2D$, which also is not seen (91). Further, the physiologic function of this altered vitamin D metabolism may be related to increased reliance on innate immune function during pregnancy as well as decreased adaptive immune responses (7,8,10,92), protecting the newborn from respiratory infection and subsequent wheezing (93,94), and possibly epigenetic alterations in invariant NKT cells, which can lead to increased autoimmune disease prevalence (95,96). As supported by this and prior studies, it is important to remember that for cord blood to attain 25(OH)D of 50 nmol/L, the maternal 25(OH)D level would need to be at least 80 nmol/L (97).

Our data also suggest that a circulating level of approximately 75 nmol/L (30 ng/mL) 25(OH)D is required to normalize calcium excretion into the urine. Interestingly, this value is virtually identical to the value obtained by Heaney *et al.* with respect to the equilibration of intestinal calcium absorption (98). This increased level of circulating 25(OH)D in the pregnant woman also appears to reduce circulating parathyroid hormone, especially in African American subjects. It is also important to compare our study results with respect to two recent reports dealing with vitamin D supplementation during pregnancy (62,99). The IOM report recommends a vitamin D intake of 400-600 IU/day and states that this level can be obtained solely from the diet. Further, this intake level would be sufficient to meet their circulating 25(OH)D target of 20 ng/mL (50 nmol/L) (62). Even using this conservative 25(OH)D level, their recommendation would have left >50% of our total cohort and >80% of African American women in the cohort deficient at study entry. The Endocrine Society's recommendation of a daily vitamin D intake of 1,500-2,000 IU and target 25(OH)D level of >30 ng/mL (75 nmol/L) (99) is more sound advice yet is still conservative when compared to our study results. It must be pointed out that the purpose of the IOM report was to guide food manufacturers and fortifiers and is not intended to guide clinical practice (62). On the other hand, clinical practice guidance is precisely the purpose of The Endocrine Society's recommendations (99).

This study has certain limitations: this study was conducted at a southernly latitude, and therefore, the vitamin D requirements of women living at more northern latitudes could be greater. While women

with preexisting hypertension and diabetes were excluded from the study, these women may be at greater risk of vitamin D deficiency, and therefore, may receive the greatest benefit from vitamin D supplementation of 4000 IU/day. Because of safety concerns, women were not allowed to remain in the study if their total circulating 25(OH)D level rose above 225 nmol/L. There were three women who attained this threshold, none of whom had any associated hypercalciuria or hypercalcemia. Lastly, due to safety concerns that surrounded the use of 4000 IU vitamin D supplementation during pregnancy, the study was designed to begin supplementation starting at the 12th week of gestation, beyond the period of early organogenesis. Hence, we cannot ensure the safety before the 12th week of gestation. With regard to vitamin D intake during pregnancy, it is interesting that our study largely confirms the observations of Obermer in England more than 60 years ago (100). Obermer's suggestions largely were ignored because of greatly flawed associations between vitamin D and Supravalvular Aortic Stenosis Syndrome (65,66,101). The data in our paper put us back on the path suggested by Obermer with respect to vitamin D intake during pregnancy. Additional studies will be necessary to ascertain safety of 4000 IU/day vitamin D supplementation before the 12th week of gestation.

Conclusions

In summary, starting at 12-16 weeks of gestation, vitamin D supplementation with 4,000 IU/day was most effective in achieving vitamin D sufficiency throughout pregnancy, one month prior to delivery and at delivery in a diverse group of women and their neonates without increased risk of toxicity. These findings suggest that the current vitamin D EAR and RDA for pregnancy women issued in 2010 by the Institute of Medicine (62) should be raised to 4,000 IU vitamin D per day so that all women regardless of race attain optimal nutritional and hormonal vitamin D status throughout pregnancy.

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Authors' roles

Study design: all authors contributed to study design (BWH, DDJ, TCH, ME, CLW).

Study conduct: all authors contributed to study conduct (BWH, DDJ, TCH, ME, CLW).

Data collection: all authors contributed to data collection (BWH, DDJ, TCH, ME, CLW).

Data analysis: BHW, TCH, ME, CLW

Data interpretation: all authors contributed to data interpretation (BWH, DDJ, TCH, ME, CLW).

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TCH and ME take responsibility for the integrity of the data analysis.

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Legends for Tables

Legend for Table 1: Maternal Sociodemographic and Clinical Characteristics at Study Enrollment by Vitamin D Supplementation Group

[1]Race/Ethnicity as defined by mother.

[2]Self-reported maternal health status rating from 1 (poor) - 10 (excellent)

[3]BMI, Prepregnancy body mass index (BMI)

[4]International Units, IU: dietary intake calculated from Block 1998.2 Food Frequency Questionnaire; amount did not include prenatal vitamin intake

Legend for Table 2: Subjects Who Completed the Study Compared to Subjects Who Exited[1] before Delivery

[1]Exited included those patients who chose not to continue. It does not include those with pregnancy losses, who became medically ineligible or who moved from geographic area.

[2]Race/Ethnicity as defined by mother

[3]Gestational age at enrollment based on last menstrual period; change of gestational age occurred in 11 cases at time of 20 week fetal ultrasound.

[4]Self-reported maternal health status rating from 1 (poor) to 10 (excellent)

[5]BMI, Prepregnancy body mass index (BMI)

Legend for Table 3. Total Circulating 25(OH)D Concentration (nmoL) during Pregnancy by Treatment Group

Table 4. Secondary Outcome: Achieving Total Circulating 25(OH)D ≥ 80 nmoL around Time of Delivery by Treatment Group

Vitamin D sufficiency defined *a priori* as a total circulating 25(OH)D concentration of ≥80 nmoL (32 ng/mL). The following comparisons were made: 2000 IU group vs. the 400 IU group; 4000 IU group vs. 400 IU group; and lastly, the 4000 IU group vs. the 2000 IU group. Risk ratios and risk differences were reported for each comparison with 95% confidence intervals (CI).

Legend for Table 5. Pill Adherence, Mode of Delivery, and Neonatal Characteristics by Vitamin D Supplementation Group

[1]Mode of delivery was categorized *a priori* as either a vaginal delivery (defined as spontaneous vaginal delivery or assisted vaginal delivery [which included use of forceps or vacuum extraction]) or cesarean section (further subdivided as cesarean following labor, cesarean without labor, and repeat elective cesarean) Primary cesarean section included women who had undergone a cesarean section with or without labor for either a maternal or fetal indication and did not include women who underwent a repeat, elective cesarean section.

[2]Pill Adherence Measure was calculated as follows: the number of pills taken divided by the number of pills predicted to have been taken based on the number of days between visits. Of note, women who missed a study visit had additional vitamin D study pills delivered to them to ensure a continued supply of the study drug. If a woman missed two consecutive visits, she exited the study.

Legend for Table 6. Circulating 25(OH)D and PTH Changes during Pregnancy by Treatment Group and Race/Ethnicity

[1]2nd trimester mean value was the average 25(OH)D value obtained at visits between 16 and 24 weeks of gestation.

[2]Δ baseline to 1 month prior connotes the change from the baseline 25(OH)D level to the level achieved at one month prior to delivery.

[3]2nd trimester mean value was the average PTH value obtained at visits between 16 and 24 weeks of gestation.

Legends for Figures

Legend for Figure 1. Flow Diagram of Pregnancy Study

[1]IU denotes International Units

Legend for Figure 2. Circulating vitamin D_3, its metabolites and intact parathyroid hormone as a function of vitamin D_3 dose and time during pregnancy.

Panels A, B, C and **D** show the mean (± S.E.M.) circulating concentrations of vitamin D_3, 25(OH)D, $1,25(OH)_2D$ and intact parathyroid hormone at defined time points during pregnancy.

Legend for Figure 3. Substrate-Product Relationships of Vitamin D Metabolites during Pregnancy

Panel A demonstrates the relationship between circulating vitamin D_3 to control the production of 25(OH)D during pregnancy. **Panel B** demonstrates the relationship of circulating 25(OH)D to control the production of $1,25(OH)_2D$ during pregnancy. All data points for all subjects in all groups were included in this analysis.

Legend for Figure 4. Serum Calcium and Urinary Calcium/Creatinine Ratio as a Function of Vitamin D_3 Dose and Time during Pregnancy

Panels A and **B** show the mean (± S.D.) serum calcium and urinary calcium/creatinine ratio at defined time points during pregnancy.

Legend for Figure 5. Relationship of Circulating 25(OH)D on the Urinary Calcium: Creatinine Ratio during Pregnancy.

All data points are included for all study patients. Urinary calcium and urinary creatinine were measured in mmol/L. Ratio was calculated from measurement of urinary calcium (mmol/L) divided by measurement of urinary creatinine (mmol/L).

Table 1. Maternal Sociodemographic and Clinical Characteristics at Study Enrollment by Vitamin D Supplementation Group

Maternal Characteristic	400 IU Group N=111	2000 IU Group N=122	4000 IU Group N=117	p-value
Race/Ethnicity,[1] N (%)				0.9
African American	28 (25.2)	37 (30.3)	33 (28.2)	
Hispanic	45 (40.5)	48 (39.3)	44 (37.6)	
Caucasian	38 (34.2)	37 (30.3)	40 (34.2)	
Maternal Age (years), Mean ± SD	27.0 ± 5.6	27.4 ± 5.7	26.6 ± 5.4	0.6
Range	18 - 41	17 - 41	17 - 44	
Gestation at Enrollment, weeks				
Mean ± SD	12.5 ± 1.9	12.6 ± 1.6	12.4 ± 2.0	0.8
Range	7.1 – 18.4	8.4 – 17.6	6.4 – 21.4	
Maternal Gravidity, Median	2	2	2	0.08
Range	1 - 8	1 - 7	1 -9	
Maternal Parity, Median	2	2	1	0.052
Range	0-5	0-7	0 - 9	
Education, N (%)				0.4
< HS Education	18 (17.3)	23 (19.7)	13 (11.6)	
HS graduate	17 (16.4)	24 (20.5)	22 (19.6)	
College or more	69 (66.4)	70 (59.8)	77 (68.8)	
Employed at Study Entrance, N (%)	61 (55.0)	67 (54.9)	65 (55.6)	0.9
Insurance, N (%)				0.07

Medicaid/None	62 (55.9)	85 (69.7)	69 (59.0)	
Commercial	49 (44.1)	37 (30.3)	48 (41.0)	
Subjective Health Rating Scale				0.4
Median[2]	9	10	10	
Range	5-10	5-10	1-10	
Planned Pregnancy, N (%)	59 (54.6)	61 (50.4)	59 (50.4)	0.8
BMI, N (%)[3]				0.6
≤30	78 (70.3)	87 (71.3)	89 (76.1)	
> 30	33 (29.7)	35 (28.7)	28 (23.9)	
Season at study entry, N (%)				0.9
April - September	54 (48.7)	60 (49.2)	56 (47.9)	
October – March	57 (51.4)	62 (50.8)	61 (52.1)	
Vitamin D Intake in IU,[4] **Mean ± SD**	181.6 ± 108.4	195.8 ± 135.0	204 .2 ± 148.2	0.6
Range	21.4 – 470.6	8.2 - 693.8	5.3 – 737.3	
Calcium Intake, mg/day, Mean ± SD	1063.6 ± 539.6	993.9 ± 514.0	1073.6 ± 491.9	0.6
Range	252.9 – 2888.1	285.4 – 2754.1	275.6 –2925.9	
Kcal Intake, Mean ± SD	2148.3 ± 778.6	2059.4 ± 803	2212.9 ± 920.8	0.5
Range	977.3- 4668.2	993.4 – 4793.4	929.3 – 5516	

Table 2. Subjects Who Completed the Study Compared to Subjects Exited Before Delivery[1]

Maternal Characteristic	Delivered N=350	Exited N=129	p-value
Treatment Group, N (%):			0.5
400 IU	111 (74.0)	39 (26.0)	
2000IU	122 (79.7)	31 (20.3)	
4000IU	117 (75.5)	38 (24.5)	
Ethnicity,[2] N (%):			**0.003**
African American	98 (66.7)	49 (33.3)	
Hispanic	137 (81.1)	32 (18.9)	
Caucasian	115 (81.0)	27 (19.0)	
Maternal Age (years), Mean ± SD	27.0 ± 5.6	25.5 ± 5.1	**0.01**
Range	17-44	18-42	
Gestational Age at Enrollment (weeks),			0.053
Mean ± SD	12.5 ± 1.8	12.1 ± 2.1	
Range[3]	6.3-21.4	6.1-17.7	
Maternal Gravidity, Median (range)	2.0 (0-9)	3.0 (1-10)	**<.0001**
Education, N (%):			**0.01**
< High School Education	54 (73.0)	20 (27.0)	
High School graduate	63 (71.6)	25 (28.4)	
College or more	216 (84.4)	40 (15.6)	

Employed at Entrance into Study, N (%)			**0.04**
Yes	193 (81.1)	45 (18.9)	
No	157 (44.9)	48 (51.6)	
Insurance, N (%):			0.4
Medicaid/None	216 (75.3)	71 (24.7)	
Commercial	134 (78.4)	37 (34.3)	
Subjective Health Rating Scale,[4] Median (range)	9.0 (1-10)	9.0 (5-10)	0.6
Planned Pregnancy, N (%)			**0.01**
Yes	179 (51.7)	34 (37.4)	
No	167 (74.6)	57 (25.5)	
BMI,[5] N (%):			**0.02**
≤30	254 (73.8)	90 (26.2)	
> 30	96 (84.2)	18 (15.8)	
Season at study entry, N (%):			0.6
April - September	170 (75.9)	54 (24.1)	
October - March	180 (77.9)	51 (22.1)	
Baseline 25(OH)D, nmol/L, Mean ± S.D. (range)			
Total	59.5 ± 23.8 (6.0-172.5)	50.5 ± 25.1 (6.5-120.5)	0.001
African American	39.4 ± 18.6 (6.0-108.8)	37.4 ± 17.6 (6.5-87.8)	0.6
Hispanic	59.3 ± 20.0 (17.3-103.8)	54.7 ± 20.6 (23.0-95.3)	0.3
Caucasian	74.6 ± 20.2 (29.5-172.5)	68.8 ± 28.8 (23.3-120.5)	0.2

Table 3.Total Circulating 25(OH)D Concentrations (nmol/L) during Pregnancy

Measure	400 IU Group	2000 IU Group	4000 IU Group	p-value
25(OH)D at baseline, Mean ± SD	61.6± 27.1	58.3± 22.3	58.2± 21.8	0.5
(Range)	(6.0 – 172.5)	(14.0 – 115.3)	(11.8 – 109.3)	
25(OH)D 1 month before delivery				
Mean ± SD	79.4± 34.3	105.4± 35.7	118.5± 34.9	<0.0001
(Range)	(16.0 – 193.0)	(17.3 – 176)	(26.3 – 243.5)	
25(OH)D at delivery, Mean ± SD	78.9 ± 36.5	98.3 ± 34.2	111.0 ± 40.4	<0.0001
(Range)	(12.5 – 159.5)	18.0 – 177.0	25.0 – 251.0	
25(OH)D, 20-36 weeks,[1]Mean ± SD	79.1 ± 29.5	94.4 ± 26.1	110.8 ± 28.3	<0.0001
(Range)	(17.1 – 162.3)	(16.7 – 149.1)	(26.5 – 212.3)	
Achieved 25(OH)D Level ≥80 nmoL at 1 month prior to delivery, N (%)	51 (50.0)	82 (73.9)	91 (82.0)	<0.0001
Achieved 25(OH)D Level ≥80 nmoL at delivery, N (%)	43 (50.0)	63 (70.8)	68 (82.0)	<0.0001
Achieved 25(OH)D Level ≥80 nmoL at 1 month prior to delivery or at delivery, N (%)	57 (52.3)	93 (79.5)	94 (83.9)	<0.0001
Infant birth 25(OH)D, Mean ± SD	18.2 ± 10.1	22.8 ± 9.8	26.5 ± 10.3	<0.0001
(Range)	(2.4 – 48.4)	(3.6 – 47.9)	(2.4 – 52.0)	

Table 4. Secondary Outcome: Achieving Total Circulating 25(OH)D ≥80 nmol/L Around Time of Delivery by Treatment Group

25(OH)D nmol/L	2000 IU N (%)	400 IU N (%)	Risk ratio (95% CI)	Risk difference (95% CI)
≥80 nmol/L	93 (79.5)	57 (52.3)	1.5200 (1.2426-1.8594)	0.2719 (0.1530-0.3909)
	4000 IU N (%)	400 IU N (%)	Risk ratio (95% CI)	Risk difference (95% CI)
≥80 nmol/L	94 (83.9)	57 (52.3)	1.6049 (1.3183-1.9540)	0.3163 (0.2005-0.4322)
≥80 nmol/L	4000 IU N (%)	2000 IU N (%)	Risk ratio (95% CI)	Risk difference (95% CI)
	94 (83.9)	93 (79.5)	1.0559 (0.9340-1.1936)	0.0444 (-0.0555-0.1443)

Table 5. Circulating 25(OH)D and PTH Changes during Pregnancy by Treatment Group and Race/Ethnicity

a. Circulating 25(OH)D (nmol/L) by Trimester Stratified by Treatment Group

Treatment Group	Baseline 25(OH)D Mean ± SD	2nd Trimester[1] Mean ± SD	1 month prior delivery Mean ± SD
400 IU	61.2 ± 27.1	76.1 ± 27.5	81.2 ± 35.9
2000IU	57.5 ± 22.4	84.2 ± 23.0	102.6 ± 36.4
4000IU	59.8 ± 25.4	98.6 ± 27.3	114.2 ± 35.5
p-value	0.5	**<0.0001**	**<0.0001**

b. Circulating 25(OH)D (nmol/L) By Trimester Stratified by Treatment Group and Race/Ethnicity

	400 IU				2000 IU				4000 IU			
Characteristic	Baseline 25(OH)D Mean ± SD	2nd Trimester Mean ± SD	1 month prior to delivery Mean ± SD	Δ baseline to 1 month prior[2] (p-value)	Baseline 25(OH)D Mean ± SD	2nd Trimester Mean ± SD	1 month prior to delivery Mean ± SD	Δ baseline to 1 month prior (p-value)	Baseline 25(OH)D Mean ± SD	2nd Trimester Mean ± SD	1 month prior to delivery Mean ± SD	Δ baseline to 1 month prior (p-value)
African American	37.3 ± 17.1	48.8 ± 21.1	49.4 ± 28.4	12.7(**0.009**)	41.0 ± 19.1	72.2± 28.4	91.2 ± 45.1	49.4 (**<0.0001**)	40.7 ± 20.1	81.0 ± 26.4	97.8 ± 42.4	57.4 (**<0.0001**)
Hispanic	59.1 ± 21.6	76.9 ± 21.7	79.5 ±30.3	20.3 (**<0.0001**)	59.2 ± 18.9	85.2 ± 16.8	102.1 ± 28.7	42.1 (**<0.0001**)	63.3 ±27.6	101.4 ± 28.2	121.1 ± 30.9	60.1 (**<0.0001**)
Caucasian	81.3 ± 23.8	95.2 ± 20.6	106.9 ± 26.4	25.0 (**<0.0001**)	71.9 ± 19.0	94.9 ± 18.3	115.7 ± 31.8	44.4 (**<0.0001**)	71.3 ± 17.3	109.8 ± 19.2	120.4 ± 29.7	50.4 (**<0.0001**)
p-value	**<0.0001**	**<0.0001**	**<0.0001**		**<0.0001**	**<0.0001**	**0.02**		**<0.0001**	**<0.0001**	**0.008**	

c. Intact PTH (pmol/L) by Trimester Stratified by Treatment Group

Treatment Group	Baseline PTH Mean ± SD	2nd Trimester[3] Mean ± SD	1 month prior delivery Mean ± SD
Control	1.9 ± 1.0	1.9 ± 1.0	2.2 ± 1.3
2000IU	1.8 ± 0.9	1.7 ± 0.9	2.1 ± 1.1
4000IU	1.8 ± 1.1	1.6 ± 0.8	1.9 ± 1.1
p-value	0.5	0.1	0.1

d. PTH (pmol/L) By Trimester Stratified by Treatment Group and Race/Ethnicity

	400 IU			2000IU			4000IU		
Characteristic	Baseline PTH Mean ± SD p-value	2nd Trimester Mean ± SD p-value	1 month prior Mean ± SD p-value	Baseline PTH Mean ± SD p-value	2nd Trimester Mean ± SD p-value	1 month prior Mean ± SD p-value	Baseline PTH Mean ± SD p-value	2nd Trimester Mean ± SD p-value	1 month prior Mean ± SD p-value
African American	2.5 ± 1.2	2.6 ± 1.2	3.1 ± 1.8	2.1 ± 1.1	2.0 ± 0.9	2.3 ± 1.3	2.0 ± 0.9	1.9 ± 0.9	2.3 ± 1.1
Hispanic	1.8 ± 0.9	1.8 ± 0.7	2.0 ± 1.0	1.7 ± 0.9	1.8 ± 1.0	2.2 ± 1.1	1.8 ± 1.1	1.5 ± 0.7	1.7 ± 0.9
Caucasian	1.6 ± 0.9	1.4 ± 0.8	1.7 ± 1.0	1.6 ± 0.7	1.5 ± 0.7	1.9 ± 1.0	1.7 ± 1.2	1.6 ± 0.8	1.7 ± 1.3
p-value	**0.001**	**<0.0001**	**0.0002**	**0.01**	0.055	0.3	0.6	0.06	0.06

Table 6. Characteristics at Delivery by Vitamin D Supplementation Group

Characteristic	400 IU Group N=111	2000 IU Group N=122	4000 IU Group N=117	p-value
Maternal age at delivery (years), Mean ± SD	27.4 ± 5.7	28.0 ± 5.7	27.1 ± 5.5	0.49
Mode of Delivery[1]: N (%)				
Uncomplicated vaginal	69 (62.2%)	81 (66.4%)	81 (69.8%)	
Assisted vaginal	2 (1.8%)	4 (3.3%)	9 (7.8%)	
C/S after Labor	23 (20.7%)	19 (15.6%)	19 (16.4%)	
C/S without Labor	17 (15.3%)	18 (14.8%)	7 (6.0%)	
Vaginal, Any type	71 (74.7%)	85 (79.4%)	90 (85.7%)	0.15
Primary Cesarean Section	24 (25.3%)	22 (20.6%)	15 (14.3%)	
Gestational Age (weeks) at Delivery, Mean ± SD	38.6 ± 2.2	38.8 ± 1.8	39.1 ± 1.8	0.17
Birth Weight (grams) at Delivery, Mean ± SD	3221.8 ± 674.9	3360.1 ± 585.0	3284.6 ± 597.6	0.23
Admission to Level II or III, N (%)	12 (10.8%)	14 (11.5%)	11 (9.4%)	0.9

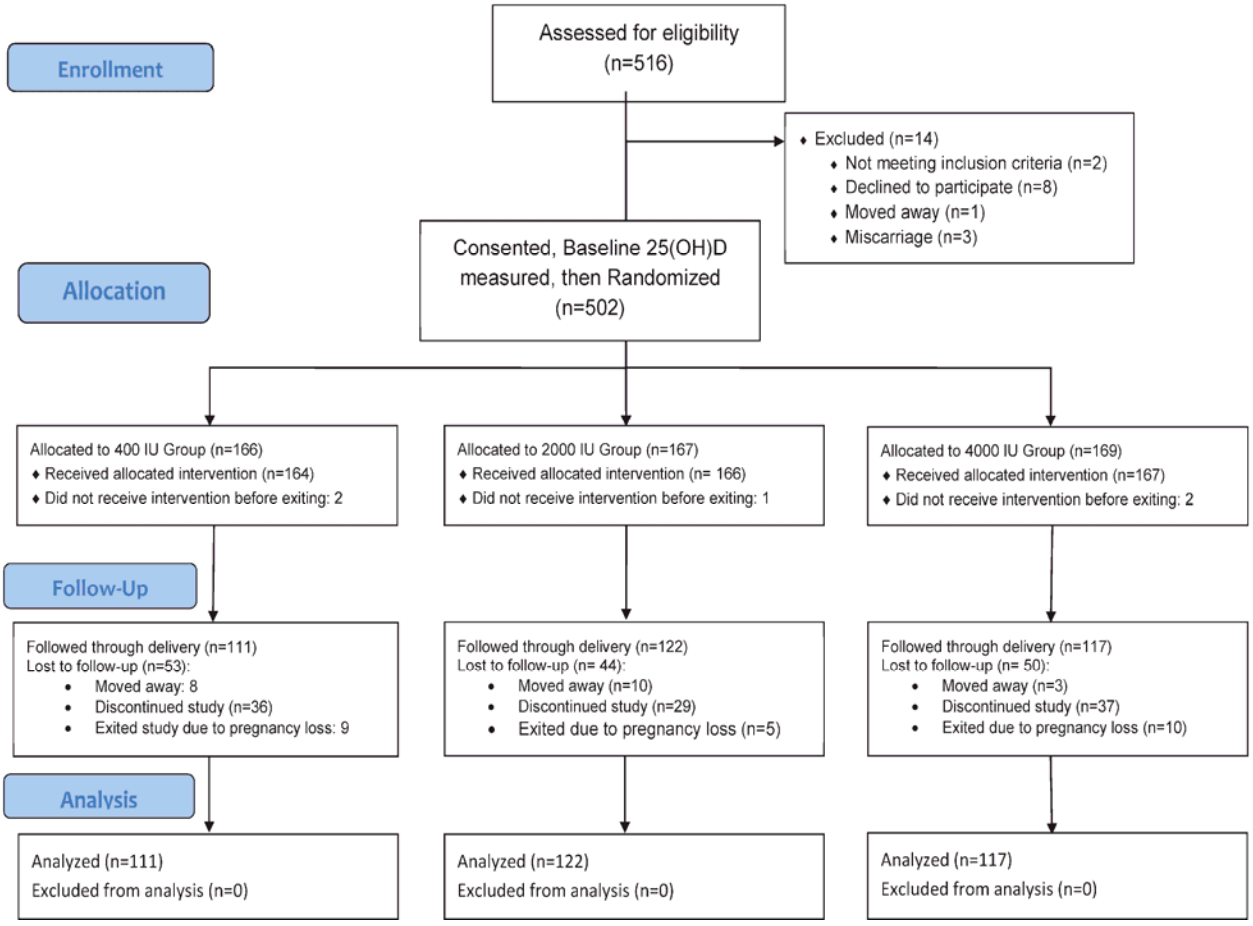
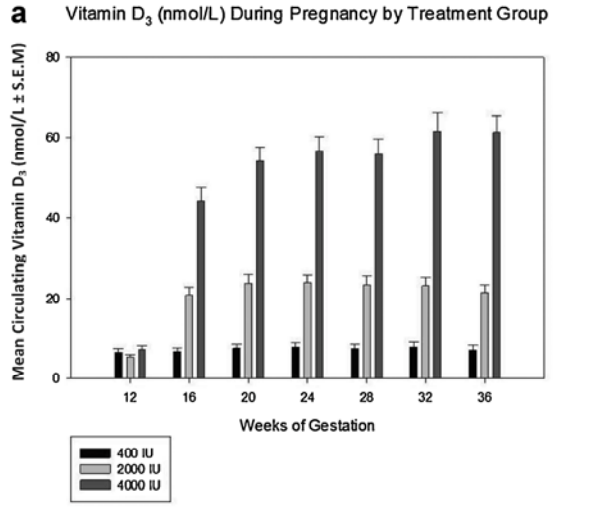
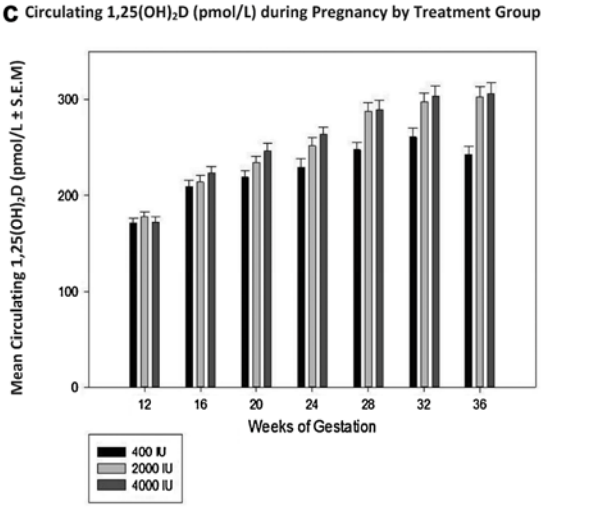


Figure 1



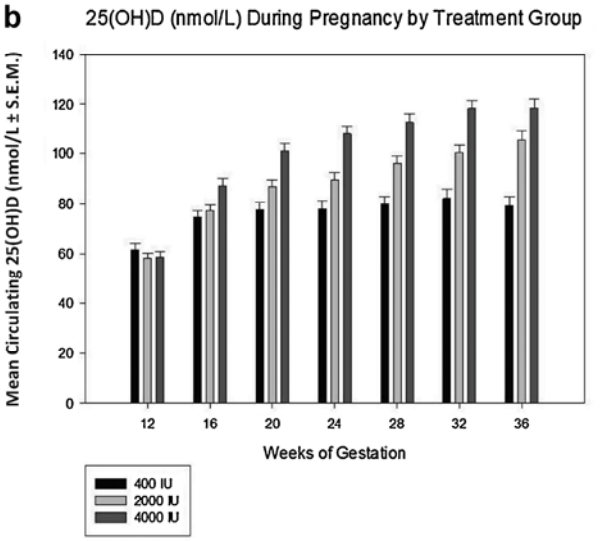
Statistical difference (p-value) in mean Vitamin D_3 between treatment groups at each week of gestation

Treatment Group	12	16	20	24	28	32	36
400 IU vs. 2000 IU	0.3	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
400 IU vs. 4000 IU	0.6	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
2000 IU v 4000 IU	0.1	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001



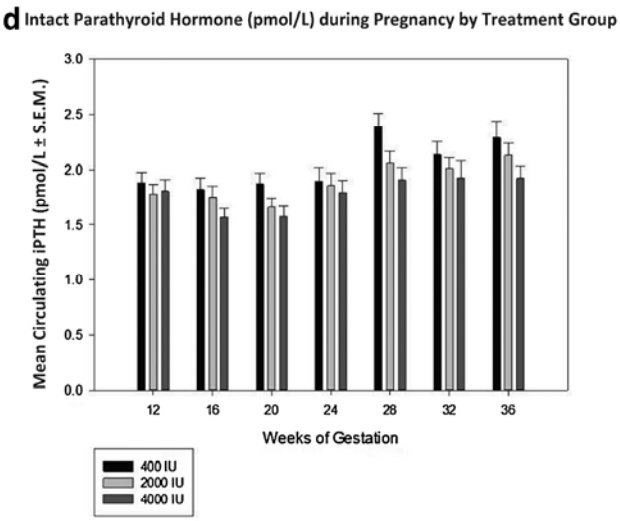
Statistical difference (p-value) in mean circulating $1,25(OH)_2D$ between treatment groups at each week

Treatment Group	12	16	20	24	28	32	36
400 IU vs. 2000 IU	0.3	0.6	0.1	0.06	0.001	0.007	<0.0001
400 IU vs. 4000 IU	0.9	0.1	0.01	0.003	0.001	0.003	<0.0001
2000 IU v 4000 IU	0.4	0.3	0.3	0.3	0.9	0.7	0.8



Statistical difference (p-value) in mean 25(OH)D between treatment groups at each week

Treatment Group	12	16	20	24	28	32	36
400 IU vs. 2000 IU	0.3	0.4	0.02	0.007	0.0001	0.0002	<0.0001
400 IU vs. 4000 IU	0.4	0.002	<.0001	<0.0001	<0.0001	<0.0001	<0.0001
2000 IU v 4000 IU	0.9	0.008	0.009	<0.0001	0.0003	<0.0001	0.009



Statistical difference (p-value) in mean intact PTH between treatment groups at each week

Treatment Group	12	16	20	24	28	32	36
400 IU vs. 2000 IU	0.4	0.6	0.1	0.8	0.049	0.4	0.4
400 IU vs. 4000 IU	0.6	0.06	0.03	0.5	0.006	0.3	0.049
2000 IU v 4000 IU	0.9	0.2	0.5	0.7	0.3	0.6	0.2

Figure 2

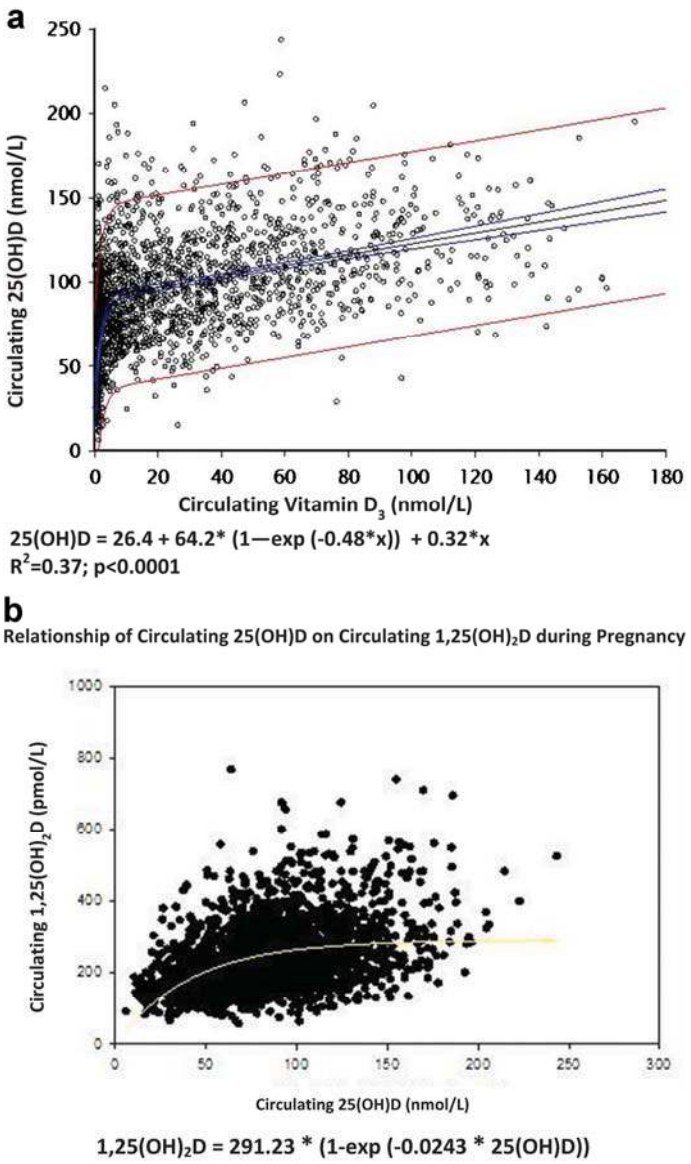
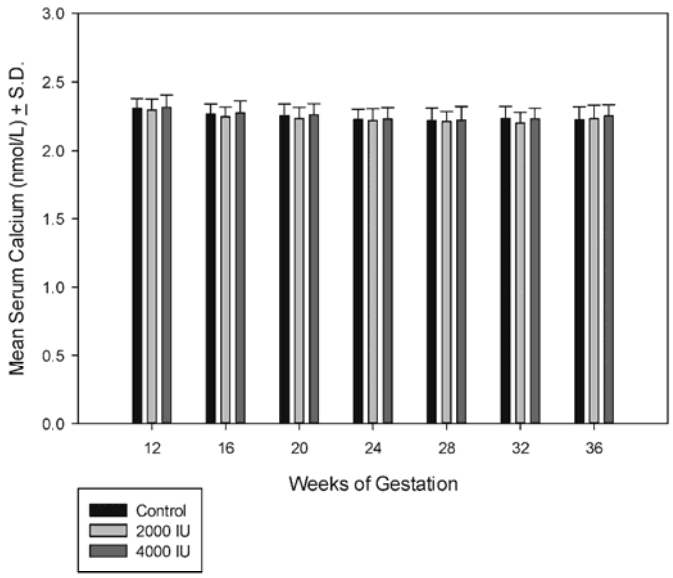


Figure 3

a Serum Calcium (nmol/L) During Pregnancy by Treatment Group



b

Urine Calcium Creatinine Ratio (mmol/L)/(mmol/L) During Pregnancy by Treatment Group

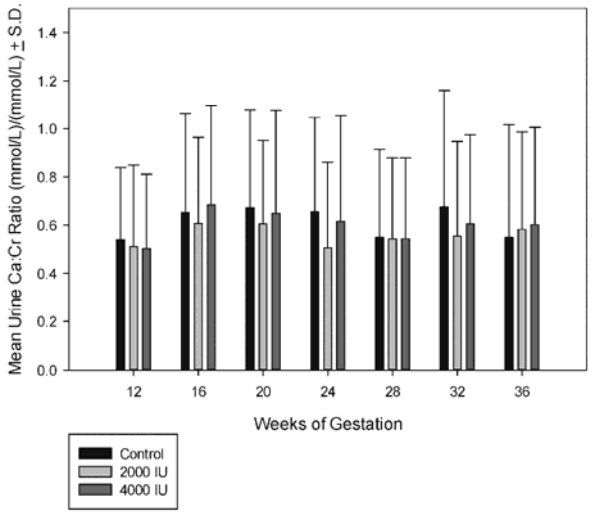
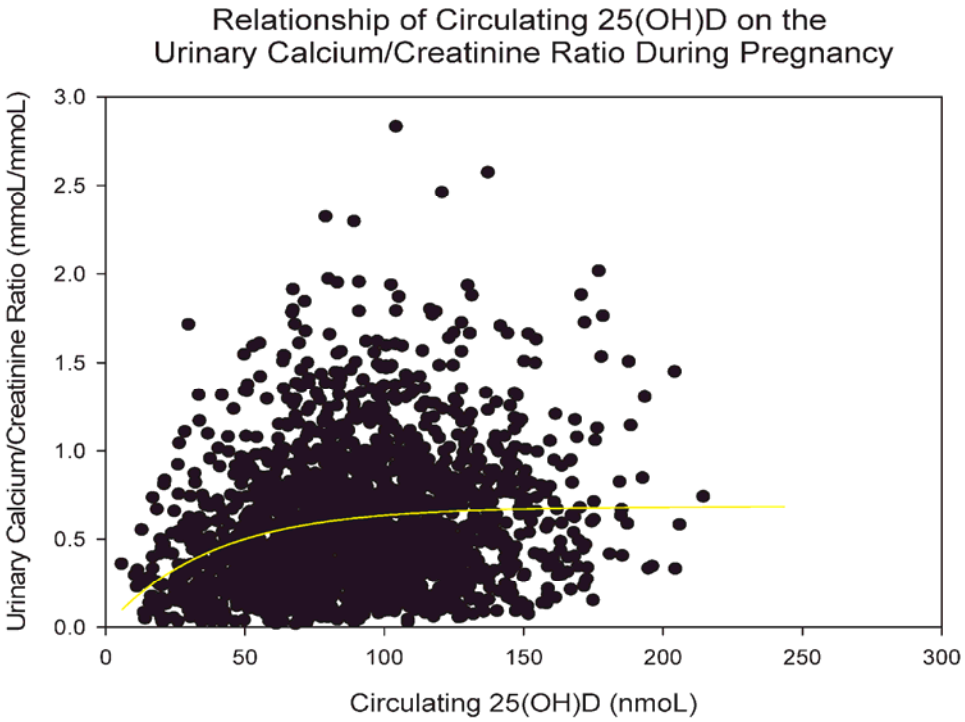


Figure 4



Urinary Calcium/Creatinine Ratio = 0.684 * (1-exp (0.0264 * 25(OH)D))

Figure 5