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Daily Cholecalciferol Supplementation during Pregnancy Alters Markers of Regulatory Immunity, Inflammation, and Clinical Outcomes in a Randomized Controlled Trial[1–4]

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Abstract

Background: Vitamin D deficiency is widespread in pregnancy and has been associated with adverse health conditions in mothers and infants. Vitamin D supplementation in pregnancy may support the maintenance of pregnancy by its effects on innate and adaptive immunity.

Objective: We assessed the effects of vitamin D supplementation during pregnancy on vitamin D status and markers of immune function associated with adverse pregnancy outcomes.

Methods: We conducted a randomized, controlled, double-blind intervention of 2 doses of cholecalciferol (400 and 2000 IU/d) from <20 wk to delivery in 57 pregnant women. Vitamin D status, regulatory and inflammatory T cells, markers of innate immunity and systemic inflammation, and clinical outcomes including maternal blood pressure and birth weight were assessed at 26 and 36 wk of pregnancy.

Results: Supplementation with 2000 IU/d vitamin D had a greater effect on the change in vitamin D status over pregnancy ($P < 0.0001$) and the final value at 36 wk ($P < 0.0001$) than 400 IU/d, increasing serum 25-hydroxyvitamin D from 81.1 nmol/L at baseline to 116 nmol/L at 36 wk and from 69.6 nmol/L at baseline to 85.6 nmol/L at 36 wk, respectively. The 2000-IU/d group had 36% more interleukin-10$^+$ regulatory CD4$^+$ T cells at 36 wk than did the 400-IU/d group ($P < 0.007$). The daily intake of 2000 compared with 400 IU/d tended to dampen the pregnancy-related increase in diastolic blood pressure by 1.3-fold ($P = 0.06$) and increase birth weight by 8.6% ($P = 0.06$), but these differences were not statistically significant.

Conclusions: Supplementation with 2000 IU/d is more effective at increasing vitamin D status in pregnant women than 400 IU/d and is associated with increased regulatory T cell immunity that may prevent adverse outcomes caused by excess inflammation. This trial was registered at clinicaltrials.gov as NCT01417351. *J Nutr* 2016;146:2388–97.

Keywords: clinical trial, vitamin D, pregnancy, cytokines, T cell

Introduction

Insufficient vitamin D status during pregnancy is widespread in the United States. Sixty-nine percent of pregnant women surveyed in NHANES 2001–2006 were found to have serum 25-hydroxyvitamin D [25(OH)D][9] <75 nmol/L and 33% <50 nmol/L (1). Vitamin D deficiency in infants and young children has been well described and is known to cause rickets (2). Low maternal vitamin D status during pregnancy is associated with an increased risk of adverse pregnancy outcomes, including preeclampsia (3–9), bacterial vaginosis (10–13), gestational diabetes (14–16), and periodontal disease (17), and is associated with adverse health outcomes in infants, including a small size for gestational age (18) and increased risks of asthma and wheezing (19–21), respiratory infections by the age of 3 mo (22),

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[4] Supplemental Figures 1–4, Supplemental Tables 1–7, and Supplemental Methods are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at http://jn.nutrition.org.

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[9] Abbreviations used: GLM, general linear model; ICAM, intercellular adhesion molecule 1; PBMC, peripheral blood mononuclear cell; Th, T helper; TLR, toll-like receptor; Treg, regulatory T; UC, University of California; UCDCM, UC Davis Medical Center; VCAM-1, vascular cell adhesion molecule 1; WHNRC, Western Human Nutrition Research Center; 1,25(OH)$_2$D, 1,25-dihydroxyvitamin D; 24,25(OH)$_2$D, 24,25-dihydroxyvitamin D; 25(OH)D, 25-hydroxyvitamin D.

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and type 1 diabetes (23). Changes to the maternal immune system during normal pregnancy allow maternal tolerance to fetal alloantigens derived from the father while maintaining a robust immune response to microbial infections (24). Tolerance to alloantigens by the adaptive immune system involves FoxP3$^+$ regulatory T (Treg) cells and other T cells that produce regulatory cytokines, including IL-10$^+$ CD4$^+$ T cells. T helper 1 (Th1) cells that mediate inflammatory responses that target paternal alloantigens are dampened by Treg cells and, to some degree, by Th2 cells (24, 25). Vitamin D acts on the adaptive immune system to enhance regulatory immunity and on the innate immune system to maintain antimicrobial defenses (8, 26). Vitamin D promotes the development of FoxP3$^+$ Treg and IL-10$^+$ CD4 T cells (27–30) and Th2 cells (31) and supports innate immune defenses, including the production of antimicrobial peptides (32). The effects of vitamin D on immune function may explain the association between vitamin D deficiency in pregnancy with the increased risk of bacterial vaginosis (11) and periodontal disease (17), which could result from impaired innate immunity, and of preterm birth (33) and preeclampsia (4), which could result from impaired tolerance of the adaptive immune system to fetal alloantigens. The Institute of Medicine's RDA for vitamin D was increased from 400 to 600 IU/d (15 μg/d) in 2011 (34) for adults (including pregnant women), a concentration considered adequate for maintaining a serum 25(OH)D concentration of 50 nmol/L. Some investigators have suggested that 75 nmol/L is needed for a variety of health outcomes, including pregnancy-related outcomes (35, 36). Supplementation with >2000 IU/d to achieve this target concentration is supported by trials in pregnant women (37, 38) and nonpregnant adults (39).

The primary objective of the study was to compare the relative effects of 2 doses of vitamin D_3 supplementation (2000 and 400 IU/d) on markers of adaptive immune function (focusing on Treg cells and inflammatory T cell subsets) that may be linked to adverse outcomes in pregnancy. Secondary goals were to assess the relative effects of these interventions on markers of innate immune function and systemic inflammation, maternal vitamin D status, and the risk of adverse maternal and fetal outcomes.

Methods

Study design, setting, and subject recruitment

This study (NCT01417351) was a randomized, double-blind, placebo-controlled trial of supplemental vitamin D administration. The study was approved by the University of California (UC), Davis, institutional review board. Participants were recruited from the obstetrics and gynecology clinics at the UC Davis Medical Center (UCDMC) and from the Davis, California, community with study visits at the USDA Western Human Nutrition Research Center (WHNRC) between August 2010 and June 2013. Potential subjects were screened with the use of questionnaires for study exclusion criteria, including high vitamin D exposure.

Inclusion and exclusion criteria

Participants aged >18 y and with a singleton pregnancy of <20 wk at enrollment were included in this study. Vitamin D exposure was assessed with a screening questionnaire designed to indirectly assess vitamin D status based on sun exposure and vitamin D intake. Categories of self-assessment questions included *1*) time spent outdoors on weekdays and weekends; *2*) typical outdoor activities; *3*) use of sunscreen; *4*) sun-avoidance behaviors (seeking shade, wearing clothing that covers exposed skin); *5*) use of tanning beds; and *6*) frequency of intake of vitamin D-containing foods (e.g., dairy, fatty fish) and supplements. The questionnaire was based on observations made previously by our group regarding the association of sun exposure and vitamin D intake on vitamin D status in our geographical area (40). Exclusion criteria included regular and recent use of vitamin D supplements (>600 IU/d); a recent history of tanning bed use; regular and midday sun exposure >90 min/d; a history of hypertensive, digestive, or endocrinologic diseases, autoimmune disease, or type 1 diabetes; use of anticonvulsant therapy or other medications known to affect vitamin D or calcium metabolism; and previously diagnosed digestive or absorptive problems.

Randomization and intervention

This study was a double-blind randomized study. Eligible women were randomly assigned to a daily vitamin D intake of 2000 IU vitamin D_3 (treatment) or 400 IU vitamin D_3 (control). All women took a daily prenatal multivitamin and multimineral that contained 400 IU vitamin D_3 plus a daily study supplement (1600 IU or placebo). Study supplement capsules (1600 IU cholecalciferol and matching placebo containing rice flour) were manufactured by NOW Foods. All study supplements were stored in a secure temperature-controlled facility at the UCDMC Investigational Drug Service until they were dispensed to enrolled subjects. A UCDMC Investigational Drug Service pharmacist generated a single block-randomized list (block size of 4) and distributed the study supplements to participants in sequential order as they were enrolled. Study staff and participants were blinded to the treatment group for the duration of the study.

Study visits and sample collection

All subjects gave written informed consent before starting the study. Study visits took place at either UCDMC or the USDA WHNRC human studies unit. Three study visits occurred at specific gestational age ranges: <20 (baseline), 26–28, and 35–36 wk. All efforts were made to help participants comply with these date ranges. We collected health history, educational, and sociodemographic information with the use of a questionnaire during the first visit.

During each visit, 20 mL whole blood was collected aseptically by venipuncture into sodium-heparin Vacutainer tubes (BD Medical). Plasma was separated by centrifugation at 1500 × *g* for 10 min at 25°C and stored at −80°C until analysis. Subjects' height and weight were measured by trained research staff during WHNRC visits. Subjects' blood pressure was measured with the use of a Dinamap ProCare automated blood pressure machine (GE Medical Systems). Subjects' temperature was measured by a digital oral thermometer to confirm that they did not have a fever. For subjects receiving prenatal care at the UCDMC obstetrics and gynecology clinics, height, weight, and blood pressure at the time of phlebotomy were collected in the clinical setting, and the data were retrieved from the electronic medical record (Epic Systems).

For study participants at UCDMC, the electronic medical record was used to collect data on the results of the oral glucose tolerance test and birth outcomes. For study participants seen at WHNRC, a questionnaire to determine the results of the oral glucose tolerance test and birth outcomes was mailed to the study participants after the birth of the infant. The questionnaire was completed by the participant and returned to the study team.

Laboratory analyses

Vitamin D analysis. The plasma concentration of 4 metabolites—25(OH)D_3, 25(OH)D_2, 24,25-dihydroxyvitamin D_3 [24,25(OH)$_2D_3$], and 1α,25-dihydroxyvitamin D_3—was measured with the use of an ultrahigh-performance LC-electrospray ionization–triple quadrupole mass spectrometer as described in **Supplemental Methods** and **Supplemental Table 1**.

Immune function analysis. Maternal immune function was assessed by measuring absolute counts of cell subsets in whole blood, T cell subsets by flow cytometry, and cytokine expression of stimulated peripheral blood mononuclear cells (PBMCs) (Supplemental Methods).

Sample size

This study was conducted to assess the feasibility of conducting a vitamin D intervention during pregnancy in this clinical setting, to assess the adequacy of these doses of vitamin D for increasing vitamin D status, and

to determine the magnitude and variability of changes in maternal outcomes of interest for a healthy pregnancy (e.g., changes in inflammation markers, maintenance of blood pressure). The study sample size was based on an evaluation of previous interventions in nonpregnant adults that examined the effects on inflammation [42–50/group based on Schleithoff et al. (41)] and immune markers [30/group based on Bock et al. (42)] and blood pressure [~60/group based on Pfeifer et al. (43)]. The sample size of 25/group allows differences between group means of 0.8 SDs to be detected with ≥80% power; the actual power for any variable may be greater depending on the predictive power of the covariates.

Statistical analysis

Statistical analyses were performed with the use of SAS version 9.3 (SAS Institute). The normality of continuous variables was assessed with the use of the UNIVARIATE procedure. Variables that were not normally distributed (Shapiro-Wilk statistic >0.97) were transformed to $\log_{10}$ or square root; otherwise, the analysis was done on normalized ranks. The level of significance was set at $P \leq 0.05$. The serum concentration of 25-hydroxycholecalciferol was used to determine the effect of the supplementation intervention. The concentration of total 25(OH)D (25-hydroxycholecalciferol + 25-hydroxyergocalciferol) was used for all other analyses because this concentration represents the total biologically available substrate pool.

The effect of vitamin D supplementation was analyzed as intention to treat. All subjects for whom data were available were included in the group comparisons, and adherence to the supplementation protocol was not considered in the tests for differences by treatment group. The effect of vitamin D treatment on clinical outcomes was tested with the use of ANCOVA [general linear model (GLM) procedure] and controlled for baseline 25(OH)D. The concentration of vitamin D metabolites, percentages of cell types in peripheral blood, concentrations of inflammatory markers in peripheral blood, and cell-culture supernatants were compared by mixed-model ANOVA for differences by vitamin D treatment, study visit, and group-by-time interaction. If significant group differences at visits 2 or 3 were detected with the use of the mixed model, P values for the group comparison at a single study visit were determined with ANCOVA (GLM procedure) and controlled for baseline 25(OH)D. Group differences in the change in outcome variables over time (defined as the difference in outcome variable between visits 3 and 1) were determined by ANCOVA [GLM procedure adjusted for baseline 25(OH)D] with the use of rank-transformed change variables. The effect of pregnancy on outcome variables for the entire study population, regardless of vitamin D treatment group, was determined by mixed-model analysis. Pearson correlation analysis was used to determine the association between vitamin D metabolites (25(OH)D or 1,25-dihydroxyvitamin D [1,25$(OH)_2$D]) and clinical outcomes. An additional post hoc regression analysis was conducted to determine whether immune function outcome variables at 35–36 wk gestation were associated with the serum 25(OH)D concentration at 35–36 wk gestation. In this analysis, a regression model was generated (GLM procedure) that included the 35–36-wk serum 25(OH)D concentration and predicted the 35–36-wk value of the immune outcome variable, adjusting for the baseline value of the outcome variable.

Results

Study population and adherence to supplementation protocol

Study participant recruitment and retention are summarized in **Figure 1**. The median gestational age at enrollment (visit 1) was 14 wk (IQR: 9, 17). The median gestational ages at visits 2 and 3 were 27 and 36 wk, respectively. For those subjects who returned remaining study supplements after the completion of the trial ($n = 43$), the median adherence to the study supplement (calculated as the percentage number of pills taken to the expected number of pills taken by number of days on protocol) was 94% (IQR: 81, 97). The 2000-IU/d treatment group had a higher median percentage adherence than the 400-IU/d control group (96% and 87%, respectively; $P = 0.05$). The median duration of supplementation was 175 d and did not differ by treatment group (2000-IU/d treatment: 175 d, range: 126–252 d; 400-IU/d control: 168 d, range: 112–252 d; $P = 0.60$).

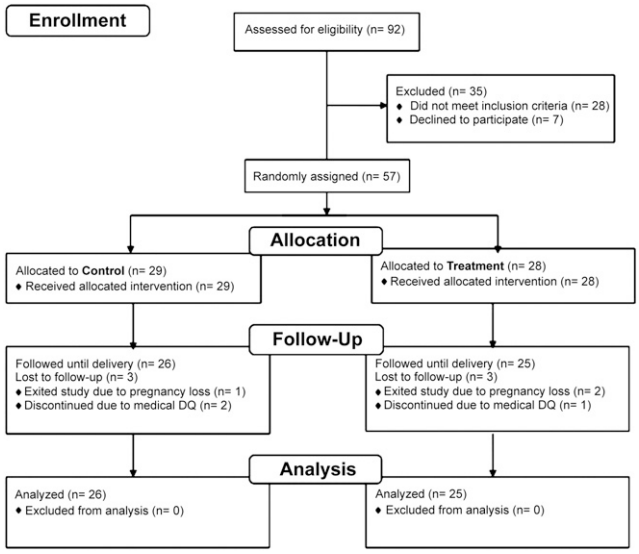

FIGURE 1 Flow diagram of pregnant women in the Vitamin D in Pregnancy study. DQ, disqualification.

Baseline characteristics and vitamin D status

A comparison of the study groups at baseline is shown in **Table 1**. Thirty-seven women were recruited at UCDMC (46% 2000-IU/d treatment; 54% 400-IU/d control), and 20 women were recruited at WHNRC (55% 2000-IU/d treatment; 45% 400-IU/d control). Participants at WHNRC tended to be older ($P = 0.02$), have a lower prepregnancy BMI (in kg/m^2) ($P = 0.005$), have a lower baseline systolic blood pressure ($P = 0.006$), and start the intervention later in pregnancy ($P = 0.0006$).

The mean baseline 25(OH)D concentration was 75.3 nmol/L. Despite randomization, the treatment groups differed in serum 25(OH)D status at baseline, with mean serum 25(OH)D concentrations for the 2000-IU/d treatment and 400-IU/d control groups of 81.1 and 69.6 nmol/L, respectively ($P = 0.016$) (**Figure 2, Supplemental Table 2**). At baseline, 60.5% of the study population had a 25(OH)D status <75 nmol/L, and 14% had a 25(OH)D status <50 nmol/L. 25(OH)D status at baseline was positively associated with gestational age at baseline ($r = 0.42$; $P = 0.001$) and negatively associated with prepregnancy BMI ($r = -0.44$; $P = 0.0006$). Similarly, 1,25$(OH)_2$D was positively associated with baseline gestational age ($r = 0.39$; $P = 0.003$) and negatively associated with prepregnancy BMI ($r = -0.37$; $P = 0.004$). By the end of the study, 81.4% of the women had a 25(OH)D concentration >75 nmol/L.

There was an association of season with total 25(OH)D concentration at baseline. Women who enrolled in the summer and fall had a greater baseline 25(OH)D than those who enrolled in winter and spring (79.8 and 69.1 nmol/L, respectively; $P = 0.028$). This difference did not persist when the groups were compared at the third study visit (98.9 and 103 nmol/L, respectively; $P = 0.60$). There was no difference in baseline 25(OH)D by ethnicity ($P = 0.51$).

Vitamin D supplementation with 2000 IU/d increased vitamin D status to a greater extent than 400 IU/d

Total 25(OH)D. There was a significant effect of vitamin D treatment group ($P < 0.0001$), visit ($P < 0.0001$), and group-by-visit

TABLE 1 Characteristics of pregnant women at <20 wk gestation by vitamin D_3 treatment group

	All subjects		400 IU/d		2000 IU/d		
	Values	*n*	Values	*n*	Values	*n*	*P*[1]
Age, y	29.6 ± 4.8[2]	57	30.3 ± 5.0	29	28.9 ± 4.6	28	0.30
Prepregnancy BMI, kg/m^2	25.1 (21.3, 29.5)[3]	57	25.7 (22.6, 30.5)	29	23.1 (21.1, 28.1)	28	0.13
Gravidity, %		57		29		28	1.0
0	35		37		32		
≥1	65		63		68		
Parity, %		57		29		28	0.56
0	37		37		36		
≥1	63		63		64		
Gestational age at enrollment, wk	14 (9, 17)	57	15 (10, 17)	29	14 (9, 18.5)	28	0.92
Ethnicity, %		57		29		28	0.15
African American	3.5		0		7.1		
Asian	8.8		10.3		7.1		
Caucasian	50.9		51.7		50		
Hispanic	29.8		24.1		35.7		
Other/mixed	7		13.8		0		
Education, %		47		23		24	0.14
≤High school	0		0		0		
High school graduate	10.6		17.4		4.2		
≥College	89.4		82.6		95.8		
Annual household income, %		47		23		24	0.92
<$40,000	40.4		39.1		41.7		
$40,000–$80,000	27.7		26.1		29.2		
>$80,000	31.9		34.8		29.2		
Marital status, %		52		27		25	0.19
Married or partner	90.4		85.2		96		
Single	9.6		14.8		4		
Employed, %		50		26		24	0.67
Full time	44		42.3		45.8		
Part time	22		26.9		16.7		
Unemployed	34		30.8		37.5		
Insurance, %		52		27		25	0.62
Commercial	63.5		63		64		
MediCal	34.6		33.3		36		
None	1.9		3.7		0		
Season of enrollment, %		57		29		28	0.34
Summer or fall	57.9		51.7		64.3		
Winter or spring	42.1		48.3		35.7		

[1] Difference by vitamin D treatment group.
[2] Mean ± SD (all such values).
[3] Median; IQR in parentheses (all such values).

interaction ($P = 0.006$) on plasma concentration of 25(OH)D. The concentration of 25(OH)D differed by vitamin D treatment group at all study visits (Figure 2A, Supplemental Table 2). A significantly greater proportion of the 2000-IU/d group than the 400-IU/d group achieved a circulating 25(OH)D >75 nmol/L by late pregnancy (96% and 73.1%, respectively; $P = 0.02$). The increase in 25(OH)D between baseline and late pregnancy was significantly greater in the 2000-IU/d group than the 400-IU/d group ($P < 0.0001$) (**Table 2**).

1,25(OH)$_2$D$_3$. The plasma concentration of 1,25(OH)$_2$D increased in both vitamin D treatment groups during pregnancy (Figure 2B), and the plasma concentration of 1,25(OH)$_2$D was positively associated with gestational age ($r = 0.53$; $P < 0.001$).

There was a significant effect of vitamin D treatment group ($P = 0.02$) and visit ($P < 0.0001$) but not group-by-visit interaction ($P = 0.07$) on 1,25(OH)$_2$D$_3$ concentration. The 2000-IU/d treatment caused a greater increase in 1,25(OH)$_2$D$_3$ between baseline and late pregnancy than did the 400-IU/d control ($P = 0.0007$) (Table 2). As expected, plasma 1,25(OH)$_2$D$_3$ correlated positively with plasma 25(OH)D$_3$ ($r = 0.69$; $P < 0.0001$).

24,25(OH)$_2$D$_3$. There was a significant effect of vitamin D treatment ($P = 0.005$), visit ($P < 0.0001$), and group-by-visit interaction ($P = 0.02$) on plasma 24,25(OH)$_2$D$_3$ concentration. Plasma 24,25(OH)$_2$D$_3$ concentration was higher in the 2000-IU/d group at visits 2 and 3, but the difference was only significant at visit 3 [$P = 0.23$ and 0.01, respectively; adjusted for baseline 25(OH)D]. There was a greater increase in 24,25(OH)$_2$D$_3$ from baseline to visit 3 in the 2000-IU/d treatment than 400-IU/d control group ($P = 0.001$) (Table 2).

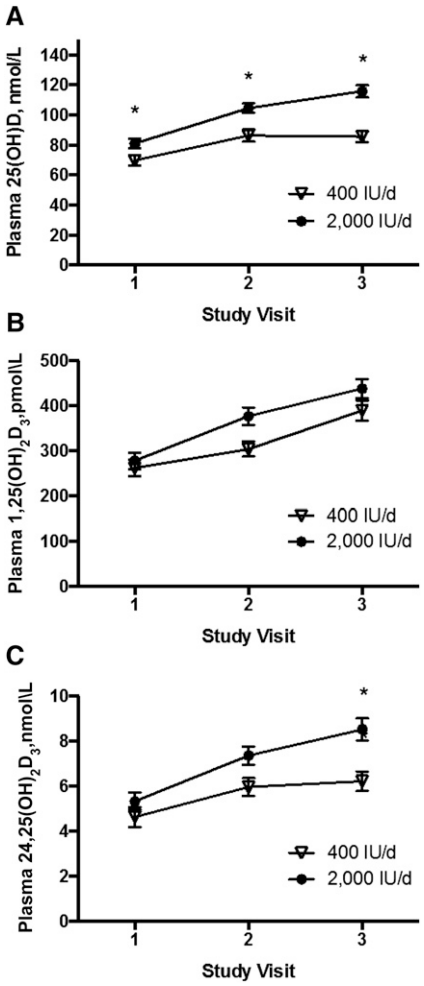

FIGURE 2 Plasma concentration of 25(OH)D (A), $1,25(OH)_2D_3$ (B), and $24,25(OH)_2D_3$ (C) in pregnant women supplemented with 400 or 2000 IU vitamin D_3/d from <20 wk gestation until delivery. Values are means ± SEMs (2000 IU/d: n = 25–28; 400 IU/d: n = 26–29). *Different from 400 IU/d, $P < 0.05$. $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; $24,25(OH)_2D_3$, 24,25-dihydroxyvitamin D_3; 25(OH)D, 25-hydroxyvitamin D.

Treatment effect on immune function: vitamin D treatment increased regulatory CD4$^+$ IL-10$^+$ T cell percentages in peripheral blood

There was a significant vitamin D treatment effect on the percentage of IL-10$^+$ CD4$^+$ T cells in peripheral blood, with the 2000-IU/d treatment group having a 36% greater percentage of this cell type at visit 3 than the 400-IU/d control group (P = 0.007; **Figure 3**A). The percentage of CD4$^+$ IL-10$^+$ T cells increased by 27% between baseline and visit 3 in the 2000-IU/d group compared with a 12% decrease in the 400-IU/d control group, corresponding to a significant treatment effect on the change in percentage CD4$^+$ IL-10$^+$ T cells (P = 0.02; **Supplemental Table 3**). The percentage of FoxP3$^+$ Treg cells in peripheral blood did not differ between the vitamin D treatment groups at any visit; the change in the percentage from baseline to visit 3 also did not differ by treatment group (Figure 3B, Supplemental Table 3).

The percentage of Th2 (CD4$^+$ IL-4$^+$) cells was marginally higher (P = 0.08; 24% difference) in the 2000-IU/d treatment than the 400-IU/d control at visit 3 (**Figure 4**C, Supplemental Table 3). No significant treatment effects were seen for other T cell subsets (Figure 4, Supplemental Table 3) for the Th1:Th2 ratio (defined as percentage CD4$^+$ IFN-γ$^+$ to percentage CD4$^+$ IL-4$^+$) (Figure 3), total T cells, CD4 T cells, CD8 T cells, or NK cells (**Supplemental Figure 1**). Similarly, there was no effect of treatment on the change in these outcomes between baseline and visit 3. Vitamin D treatment did not affect T cell cytokine production ex vivo in PBMC cultures stimulated in a T cell-specific manner with anti-CD3 and anti-CD28 antibodies (data not shown) or the change in T cell cytokines between baseline and visit 3 (data not shown). In addition, there was no effect of vitamin D treatment on plasma cytokines (**Supplemental Figure 2**), plasma inflammatory markers (**Supplemental Figure 3**), or ex vivo toll-like receptor (TLR)–stimulated cytokine concentrations in PBMC supernatants (data not shown) at visit 3 or on the change in these inflammatory markers between baseline and visit 3 (data not shown).

Vitamin D serum concentrations in late pregnancy were associated with plasma markers of inflammation and vascular activation and TLR cytokine production

We used regression analysis to address whether the achieved vitamin D status in late pregnancy was associated with detectable differences in the measured indicators of immune function (**Supplemental Table 4**). The percentage of IL-10$^+$ CD4 T cells was positively associated with 25(OH)D status ($P < 0.01$) in late pregnancy (**Supplemental Figure 4**). Serum 25(OH)D concentration in late pregnancy was negatively associated with plasma concentrations of TNF-α, intercellular adhesion molecule 1 (ICAM-1), and vascular cell adhesion molecule 1 (VCAM-1) ($P < 0.05$) (Supplemental Figure 4). Late-pregnancy serum 25(OH)D concentration also correlated with TLR-stimulated cytokine production ex vivo; there was a significant negative association with all TLR3 cytokines ($P < 0.05$) and a significant positive association with TLR4 cytokines ($P < 0.05$).

Vitamin D supplementation tended to decrease diastolic blood pressure and increase birth weight

Maternal pregnancy outcomes of our study population are shown in **Table 3**. No significant differences by treatment group were detected in gestational weight gain, measures of glucose tolerance, and systolic or diastolic blood pressure. With the use of mixed-model analysis, we detected an effect of visit (P = 0.004) but not the 2000-IU/d treatment group on diastolic blood pressure. Diastolic blood pressure increased between baseline and late pregnancy to a greater extent in the 400-IU/d control group than the 2000-IU/d treatment group, although the difference was not statistically significant (P = 0.06).

Fifty-one of the study participants continued on the protocol until delivery. The following clinical diagnoses were made in our study population: 1 case of preeclampsia (2000-IU/d treatment group), 4 cases of gestational diabetes (400-IU/d control group), and 2 cases of gestational hypertension (400-IU/d control group). There were no adverse events related to the supplementation

TABLE 2 Change in plasma concentrations of vitamin D metabolites from <20 wk gestation to delivery in pregnant women supplemented with 400 or 2000 IU vitamin D_3/d[1]

	400 IU/d	2000 IU/d	P^2
25(OH)D, nmol/L	13.3 ± 18.7	32.4 ± 20.6	<0.0001
$1,25(OH)_2D$, pmol/L	114 ± 148	149 ± 128	0.0007
$24,25(OH)_2D$, nmol/L	1.3 ± 2.0	3.1 ± 2.3	0.0012

[1] Values are means ± SDs, n = 51 (400 IU/d: n = 26; 2000 IU/d, n = 25). $1,25(OH)_2D$, 1,25-dihydroxyvitamin D; $24,25(OH)_2D$, 24,25-dihydroxyvitamin D; 25(OH)D, 25-hydroxyvitamin D.
[2] Difference by vitamin D treatment group.

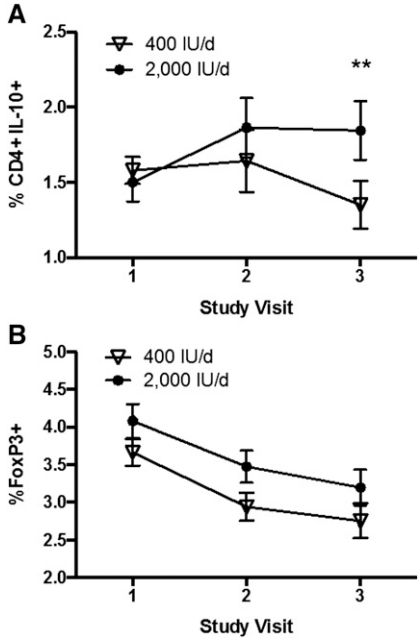

FIGURE 3 Percentage of $CD4^+$ $IL\text{-}10^+$ T cells (A) and $FoxP3^+$ $CD4^+$ regulatory T cells (B) in pregnant women supplemented with 400 or 2000 IU vitamin D_3/d from <20 wk gestation until delivery. Values are means ± SEMs (2000 IU/d: n = 22–26; 400 IU/d: n = 23–27). ** Different from 400 IU/d, $P < 0.01$.

noted in our study. There was no significant difference by treatment group in gestational age at delivery, delivery mode, or Apgar scores. Infants born to mothers in the 2000-IU/d treatment group had an 8.6% greater birth weight than those in the 400-IU/d control group, but the difference was not statistically significant ($P = 0.06$) (**Table 4**).

Pregnancy progression is associated with decreased Th1 and Treg cells and T cell cytokines and increased plasma markers of inflammation and TLR-stimulated cytokines

Pregnancy progression was associated with a significant decrease in the percentage of peripheral blood Th1 ($CD4^+$ $IFN\text{-}\gamma^+$) and Th17 ($CD4^+$ $IL\text{-}17^+$) cells between baseline and late pregnancy ($P < 0.05$) (**Supplemental Table 5**). The percentage of $FoxP3^+$ Treg cells decreased significantly between baseline and mid-pregnancy (from 3.9% to 3.2%; $P < 0.01$) and between baseline and late pregnancy (from 3.9% to 3.0%; $P < 0.01$) (Figure 3B, Supplemental Table 5). Pregnancy progression was also associated with decreased ex vivo production of the Th2 cytokines IL-4 and IL-5 and regulatory cytokine IL-10, but no changes were seen for Th1 cytokines (Supplemental Table 5). In contrast, the mean plasma concentrations of IL-6, TNF-α, and neopterin increased significantly over the course of pregnancy (**Supplemental Table 6**). Cytokines from TLR agonist-stimulated PBMC cultures were measured as an indicator of innate immune function. Cytokines that increased significantly from baseline to late pregnancy included IL-6 (TLRs 3 and 7/8), IL-8 (TLRs 2, 3, 4, and 7/8), and IL-1 (TLR7/8) (**Supplemental Table 7**).

Discussion

In our study population, 2000 IU/d was more effective than 400 IU/d at increasing 25(OH)D to above the target serum concentration of 75 nmol/L, which was similar to the effect of supplementation in the randomized control trial by Hollis et al. (37). The mean 25(OH)D status of all women at 36 wk gestation was 100 nmol/L, and 8 women, all in the 2000-IU/d group, had values >125 nmol/L at 36 wk, the concentration to be avoided according to the Institute of Medicine Dietary Reference Intake report (34). No women in our study had values >225 nmol/L, a concentration that has been suggested as an alternative cutoff for hypervitaminosis D (44).

The serum concentration of $1,25(OH)_2D$ increases during the first trimester and remains 100–200% above nonpregnancy concentrations, indicating a role for this hormone in the maintenance of pregnancy (45–48). In our study population, the serum concentration of $1,25(OH)_2D$ increased in both groups, but the women in the 2000-IU/d group had greater concentrations in late pregnancy. These data are consistent with the notion that, within the serum concentrations seen herein, increased concentrations of serum 25(OH)D will increase $1,25(OH)_2D$ synthesis, which may be important for the effects of vitamin D on placental function and the modulation of immune response (47).

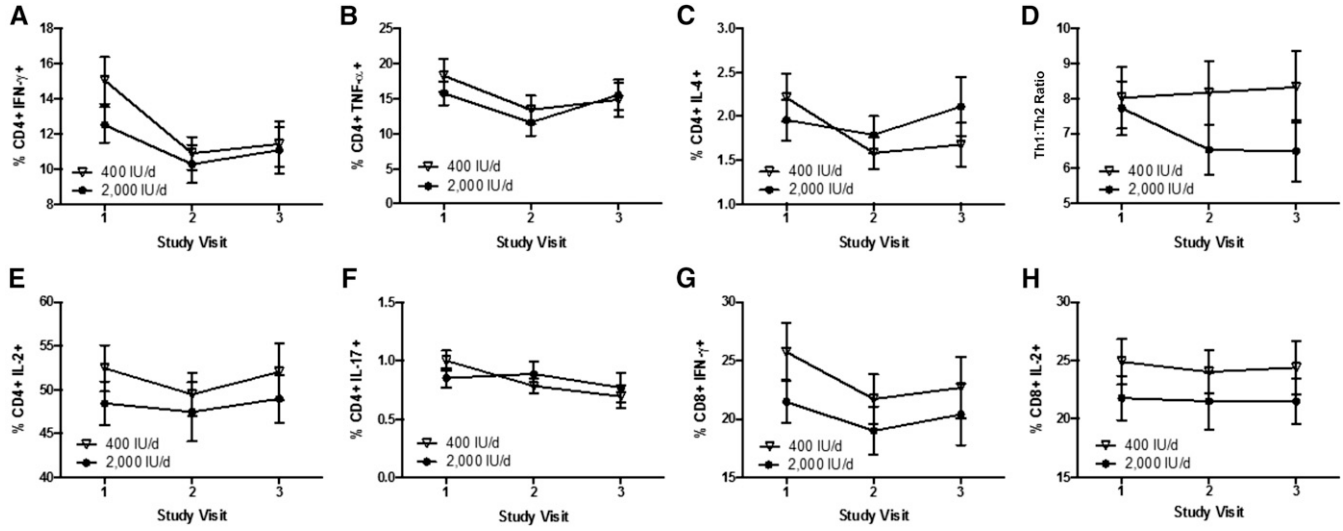

FIGURE 4 Percentage of $CD4^+$ $IFN\text{-}\gamma^+$ (A), $CD4^+$ $TNF\text{-}\alpha^+$ (B), $CD4^+$ $IL\text{-}4^+$ (C), Th1:Th2 ratio ($CD4^+$ $IFN\text{-}\gamma^+$:$CD4^+$ $IL\text{-}10^+$) (D), $CD4^+$ $IL\text{-}2^+$ (E), $CD4^+$ $IL\text{-}17^+$ (F), $CD8^+$ $IFN\text{-}\gamma^+$ (G), and $CD8^+$ $IL\text{-}2^+$ (H) T cell subsets in pregnant women supplemented with 400 or 2000 IU vitamin D_3/d from <20 wk gestation until delivery. Values are means ± SEMs (2000 IU/d: n = 25–28; 400 IU/d: n = 25–29).

TABLE 3 Clinical characteristics of pregnant women supplemented with 400 or 2000 IU vitamin D_3/d from <20 wk gestation until delivery[1]

	All subjects		400 IU/d		2000 IU/d		
	Values	*n*	Values	*n*	Values	*n*	P^2
Gestational weight gain, kg	14.2 ± 5.7[3]	50	13.3 ± 6.0	25	15.2 ± 4.9	25	0.49
Systolic BP, mm Hg							
Visit 1 (<20 wk)	111 ± 14.0	55	111 ± 12.2	28	111 ± 15.9	27	0.85
Visit 2 (26–28 wk)	111 ± 10.9	51	113 ± 11.3	26	110 ± 10.6	25	0.47
Visit 3 (35–36 wk)	113 ± 13.0	50	114 ± 13.2	25	111 ± 12.9	25	0.12
Diastolic BP, mm Hg							
Visit 1 (<20 wk)	63.5 ± 7.0	55	63.5 ± 7.5	28	63.6 ± 6.5	27	0.90
Visit 2 (26–28 wk)	64.7 ± 7.7	51	66.2 ± 7.9	26	63.2 ± 7.4	25	0.37
Visit 3 (35–36 wk)	68.1 ± 11.0	49	70.0 ± 13.0	24	66.0 ± 8.1	25	0.07
Change in systolic BP[4]	1 (−3, 8)[5]	50	5 (−1, 9)	25	−1 (−5, 6)	25	0.16
Change in diastolic BP[4]	4 (1, 9)	49	7 (0.5, 12)	24	3 (1, 6)	25	0.06
Fasting plasma glucose,[6] mg/dL	79.1 ± 5.0	31	78.4 ± 5.7	16	79.7 ± 4.3	15	0.46
OGTT,[6] mg/dL							
60 min plasma glucose	126 ± 27.9	36	132 ± 32.6	18	120 ± 21.4	18	0.30
120 min plasma glucose	110 ± 20.2	33	115 ± 19.8	16	106 ± 20.2	17	0.21
Hemoglobin A1c,[6] %	5.2 ± 0.3	26	5.2 ± 0.3	13	5.1 ± 0.3	13	0.45

[1] BP, blood pressure; OGTT, oral glucose tolerance test; 25(OH)D, 25-hydroxyvitamin D.
[2] Difference by treatment group determined by ANCOVA and adjusted for baseline 25(OH)D and baseline variable value (systolic and diastolic BP).
[3] Mean ± SD (all such values).
[4] The change in systolic BP and diastolic BP is the difference between baseline and 35–36-wk gestation measurements.
[5] Median; IQR in parentheses (all such values).
[6] Measured at 26–28 wk gestation.

Treg cells, including FoxP3$^+$ Treg and IL-10$^+$ CD4 T cells, play an important role in maintaining fetal tolerance during pregnancy (24, 25, 49–51). Vitamin D treatment ex vivo can increase the development of both FoxP3$^+$ Treg cells and IL-10$^+$ CD4 T cells (27, 29, 30), suggesting that supplementation may increase these cell types in vivo, including during pregnancy (26). In support of this idea, a recent observational study in pregnant women found a positive association of maternal plasma 25(OH)D with FoxP3$^+$ Treg cells and with serum IL-10, although IL-10$^+$ CD4$^+$ T cells were not examined (52). However, no intervention studies to our knowledge have been conducted in pregnancy to examine this question; thus, to our knowledge, our study is the first to report that vitamin D supplementation in pregnancy affects regulatory immunity, increasing the concentration of the IL-10$^+$ CD4$^+$ T cells subset but without affecting FoxP3$^+$ Treg cells. Aside from pregnancy, 2 intervention trials used a high dose of vitamin D_3 (140,000 IU/mo) in healthy adults. Both found an increase in FoxP3$^+$ Treg cells, although IL-10$^+$ CD4$^+$ T cells were not examined (42, 53). One uncontrolled intervention with a very high dose of vitamin D_3 (20,000 IU/d) for 12 wk in multiple sclerosis patients, in which vitamin D deficiency has been associated with disease risk (54), found an increase in IL-10$^+$ CD4$^+$ T cells but no effect on FoxP3$^+$ Treg cells (28). Our results suggest that future studies should be conducted in pregnant women to examine the effect of vitamin D interventions on regulatory immunity because such changes could affect the risk of preeclampsia and other inflammation-related conditions in pregnancy.

Independent of vitamin D status, we noted numerous changes in immune function during pregnancy. In particular, we noted decreases in both Th1 and Th17 cell populations and T cell cytokines but increases in numerous plasma markers of inflammation and TLR-stimulated cytokines. These results contribute to our understanding of how immunity changes during pregnancy, but our ability to interpret these results is limited because of the fact that the vitamin D treatment groups, with known differences in vitamin D status, were combined in this analysis.

In our post hoc regression analysis, we found a significant inverse association between plasma 25(OH)D concentration in late pregnancy and plasma TNF-α, a marker of systemic immune activation, as well as plasma ICAM-1 and VCAM-1, markers of endothelial activation. High concentrations of circulating inflammatory cytokines and markers of endothelial cell activation have been shown to be associated with preeclampsia; thus, vitamin D supplementation may play a role in the maintenance of healthy vascular function by decreasing this type of inflammation. Vitamin D deficiency might be a risk factor for preeclampsia (4, 55), presumably because of the effects of vitamin D on dampening inflammation and improving vascular function (56). In a clinical trial in patients with chronic kidney disease, high-dose vitamin D supplementation decreased

TABLE 4 Birth characteristics of infants born to pregnant women supplemented with 400 or 2000 IU vitamin D_3/d from <20 wk gestation until delivery[1]

	All subjects		400 IU/d		2000 IU/d		
	Values	*n*	Values	*n*	Values	*n*	P^2
Birth weight, g	3521 ± 515	49	3379 ± 557	25	3668 ± 431	24	0.06
Gestational age at delivery, wk	39.3 ± 1.5	46	39.0 ± 1.6	24	39.5 ± 1.2	22	0.20
Mode of delivery, %		49		25		24	0.40
Vaginal	83.7		88		79.2		
Caesarean	16.3		12		20.8		
Apgar score							
At 1 min	7.9 ± 1.7	46	8.1 ± 1.4	23	7.6 ± 2.1	23	0.20
At 5 min	9.0 ± 0.3	44	9.0 ± 0.4	21	9.0 ± 0.3	23	0.72

[1] Values are means ± SDs unless otherwise indicated.
[2] Group differences determined by ANCOVA (adjusted for baseline 25-hydroxyvitamin D) or chi-squared test.

ICAM-1 and VCAM-1 (57), whereas another study in pregnant women found that better vitamin D status at 12–18 wk gestation was associated with lower ICAM-1 at 24–26 wk gestation (58). Taken together, our data and previous studies suggest benefits of vitamin D supplementation in decreasing systemic and vascular inflammation in pregnancy.

In our study, plasma 25(OH)D concentration was negatively associated with cytokine responses to TLR3 stimulation but positively associated with cytokine responses to TLR4 stimulation. These are the first such observations to our knowledge from a study in pregnant women. Results from previous studies in infants (59) and healthy adults (60) are inconsistent. Given the differences in immune regulation that exist during pregnancy, further work is needed to better characterize the effects of vitamin D on innate immunity in this population.

Our study suggests a dampening effect of the 2000-IU/d dose on the increase in blood pressure that is seen in pregnancy, although the difference with the 400-IU/d group was not statistically significant. This possible benefit warrants further examination because vitamin D deficiency has previously been associated with elevated blood pressure, and $1,25(OH)_2D$ affects the renin-angiotensin system (61). One vitamin D supplementation trial in pregnant women demonstrated a significant decrease in both systolic and diastolic blood pressure at 36 wk gestation (62).

We found no association between vitamin D treatment and response to oral glucose challenge or gestational weight gain. This result is similar to the results of the combined analysis of 2 large interventional trials in pregnancy (9).

Better vitamin D status in late pregnancy may increase birth weight. Maternal dietary intake of vitamin D during pregnancy is positively associated with infant birth weight (63, 64), higher maternal 25(OH)D status is positively correlated with infant size at birth (65), and cord blood 25(OH)D status is positively associated with infant birth weight (66, 67). However, other studies showed no such associations (68, 69). A recent meta-analysis (70) of vitamin D supplementation trials in pregnancy found a significant effect of vitamin D supplementation on increased birth weight, although another meta-analysis (71) only found this effect in trials that were supplemented with both vitamin D and calcium. Our data suggest that there may be a positive effect of maternal vitamin D supplementation on infant birth weight, but further investigation is necessary to determine the mechanism of this effect.

This study provides evidence in a randomized controlled trial of a potentially beneficial effect of vitamin D supplementation on the proportion of $CD4^+$ $IL\text{-}10^+$ T cells in pregnant women. However, the relatively small sample size of the study limits its power to detect effects on other outcomes. Because there is much interindividual variation in T cell cytokine expression and plasma inflammatory markers, a larger sample size may have increased our ability to detect group differences. Our study population was, on average, of sufficient vitamin D status at baseline. We suspect that we may have demonstrated immune changes of greater magnitude among women with serum 25(OH)D concentrations <50 nmol/L at baseline who were supplemented with 2000 IU/d. Further investigations in larger populations of pregnant women, including those with lower starting plasma 25(OH)D concentrations, are necessary to elucidate the effects of vitamin D status during pregnancy on clinical outcomes.

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