

Vitamin D Metabolism Varies among Women in Different Reproductive States Consuming the Same Intakes of Vitamin D and Related Nutrients^{1–3}

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Abstract

Background: The impact of the reproductive state on vitamin D metabolism and requirements is uncertain in part because of a lack of studies with controlled dietary intakes of vitamin D and related nutrients.

Objective: We aimed to quantify the impact of the reproductive state on a panel of vitamin D biomarkers among women of childbearing age consuming equivalent amounts of vitamin D and related nutrients.

Methods: Nested within a feeding study providing 2 doses of choline, healthy pregnant (26–29 wk gestation; $n = 26$), lactating (5 wk postpartum; $n = 28$), and control (nonpregnant/nonlactating; $n = 21$) women consumed a single amount of vitamin D (511 ± 48 IU/d: 311 ± 48 IU/d from diet and 200 IU/d as supplemental cholecalciferol) and related nutrients (1.6 ± 0.4 g Ca/d and 1.9 ± 0.3 g P/d) for 10 wk. Vitamin D biomarkers were measured in blood obtained at baseline and study end, and differences in biomarker response among the reproductive groups were assessed with linear mixed models adjusted for influential covariates (e.g., body mass index, season, race/ethnicity).

Results: At study end, pregnant women had higher ($P < 0.01$) circulating concentrations of 25-hydroxyvitamin D [25(OH)D; 30%], 1,25-dihydroxyvitamin D [1,25(OH)₂D; 80%], vitamin D binding protein (67%), and C3 epimer of 25(OH)D₃ (100%) than control women. Pregnant women also had higher ($P \leq 0.04$) ratios of 25(OH)D to 24,25-dihydroxyvitamin D [24,25(OH)₂D; 40%] and 1,25(OH)₂D to 25(OH)D (50%) than control women. In contrast, no differences ($P \geq 0.15$) in vitamin D biomarkers were detected between the lactating and control groups. Notably, the study vitamin D dose of 511 IU/d achieved vitamin D adequacy in most participants (95%) regardless of their reproductive state.

Conclusions: The higher concentrations of vitamin D biomarkers among pregnant women than among control women suggest that metabolic adaptations, likely involving the placenta, transpire to enhance vitamin D supply during pregnancy. The study findings also support the adequacy of the current vitamin D RDA of 600 IU for achieving serum 25(OH)D concentrations ≥ 50 nmol/L among women differing in their reproductive state. This trial was registered at clinicaltrials.gov as NCT01127022. *J Nutr* 2016;146:1537–45.

Keywords: pregnancy, lactation, vitamin D, 25-hydroxyvitamin D, 1,25-dihydroxyvitamin D, 24,25-dihydroxyvitamin D, 25(OH)D:24,25(OH)₂D ratio, free 25(OH)D, Epi-25(OH)D₃, vitamin D requirements

Introduction

Worldwide vitamin D inadequacy is common among pregnant and lactating women (1–3), and it is linked in some epidemiologic

studies to adverse reproductive outcomes, including impaired fetal/neonatal growth, preeclampsia, and immune disorders (4, 5). In 2011, the Institute of Medicine (IOM)⁴ established a vitamin D

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³ Supplemental Table 1 is 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>.

⁴ Abbreviations used: CYP2R1, 25-hydroxylase gene; CYP24A1, 24-hydroxylase; DBP, vitamin D binding protein; epi-25(OH)D₃, C3 epimer of 25-hydroxyvitamin D₃; free 25(OH)D, free 25-hydroxyvitamin D; GC, vitamin D binding protein gene; HMRU, Human Metabolic Research Unit; IOM, Institute of Medicine; LMM, linear mixed-effects model; 1,25(OH)₂D, 1,25-dihydroxyvitamin D; 1,25(OH)₂D:25(OH)D, ratio of 1,25-dihydroxyvitamin D to 25-hydroxyvitamin D; 24,25(OH)₂D, 24,25-dihydroxyvitamin D; 25(OH)D, 25-hydroxyvitamin D; 25(OH)D:24,25(OH)₂D, ratio of 25-hydroxyvitamin D to 24,25-dihydroxyvitamin D.

RDA of 600 IU for women of childbearing age. This recommendation was based on the vitamin D intake projected to achieve circulating concentrations of 50 nmol/L for 25-hydroxyvitamin D [25(OH)D], the primary indicator of vitamin D status. Reproductive state was not considered in the formulation of this RDA because of an absence of 25(OH)D dose-response data among pregnant and/or lactating women (1, 6).

Interestingly, pregnancy is characterized by robust increases in circulating 1,25-dihydroxyvitamin D [1,25(OH)₂D] (7–10), the biologically active form of vitamin D, and vitamin D binding protein (DBP) (8, 11, 12), a carrier of vitamin D metabolites in blood. However, the effect of pregnancy on circulating 25(OH)D is less clear with studies reporting no effects (7, 11, 13), increases (9, 14, 15), or decreases (10, 16). These mixed findings across studies may arise from intake differences of vitamin D and relevant nutrients (e.g., calcium and phosphorus), which were either unknown or incompletely assessed. Some studies have shown that circulating concentrations of 1,25(OH)₂D, DBP, and 25(OH)D decline during lactation (9, 11, 17, 18); however, it is unclear if vitamin D status differs from that of a nonpregnant, nonlactating woman under conditions of comparable vitamin D intake. Furthermore, little is known about the effect of reproductive state on several additional biomarkers of vitamin D metabolism, including 24,25-dihydroxyvitamin D [24,25(OH)₂D], a catabolite of vitamin D; the ratio of 25(OH)D to 24,25(OH)₂D [25(OH)D:24,25(OH)₂D], a newly proposed sensitive indicator of vitamin D status (19–22); free 25(OH)D (16, 23); and the C3 epimer of 25-hydroxyvitamin D₃ [epi-25(OH)D₃], a recently identified metabolite (24, 25) with unknown biological function.

To advance understanding of the effects of reproductive state on vitamin D status and metabolism, we used samples obtained during a feeding study that provided a single amount of vitamin D and related nutrients (e.g., calcium and phosphorus) to pregnant, lactating, and control (nonpregnant, nonlactating) women across a 10-wk time period. Our aims were as follows: 1) to quantify the impact of reproductive state on a comprehensive panel of blood vitamin D biomarkers; 2) to examine interrelations among biomarkers of vitamin D metabolism; and 3) to ascertain the adequacy of the current RDA for achieving target circulating 25(OH)D concentrations of 50 nmol/L among pregnant and lactating women.

Methods

Study participants

Pregnant women entering their third trimester (26–29 wk gestation), lactating women (5 wk postpartum), and control women of reproductive age (nonpregnant and nonlactating) were recruited from Ithaca, New York (latitude 42.4°N) between January 2009 and October 2010 as previously described (26, 27). Inclusion criteria included the following: 1) age of 21–40 y; 2) healthiness as assessed by health-related questionnaire, a blood chemistry profile, and a complete blood count; 3) normal liver and kidney function; 4) willingness to comply with the study protocol; 5) singleton pregnancy without pregnancy-associated complications (pregnant women only); and 6) willingness to breastfeed exclusively during the study period (lactating women only). Exclusion criteria included use of tobacco, drug, or alcohol and use of prescription medications known to affect liver function. The study protocol was reviewed and approved by the Institutional Review Board for Human Study Participant Use at Cornell University and the Cayuga Medical Center (the hospital where pregnant participants delivered their infants). Informed consent was obtained from all study participants before study entry. This trial was registered at clinicaltrials.gov as NCT01127022.

Study design, diet, and supplements

This study used biological samples obtained during a feeding study in which healthy pregnant ($n = 26$), lactating ($n = 28$), and control ($n = 21$) women were randomly assigned to 480 or 930 mg choline/d for ≥ 10 wk (26, 27). Throughout the feeding period, consumption of all essential micronutrients was strictly controlled and equivalent across reproductive groups.

A mean total of 511 ± 48 IU vitamin D/d was consumed by participants throughout the study period. Of this total, a mean of 311 ± 48 IU vitamin D/d was provided by the 7-d rotational menu as estimated with the use of the USDA National Nutrient Database for Standard Reference Release 28 (Supplemental Table 1), and 200 IU cholecalciferol/d came from a daily prenatal multivitamin supplement (Pregnancy Plus; Fairhaven Health LLC). For calcium and phosphorus, mean intakes were estimated to be 1.6 ± 0.4 g Ca/d and 1.9 ± 0.3 g P/d (USDA National Nutrient Database for Standard Reference Release 28). In addition to the prenatal multivitamin supplement, all women consumed a docosahexaenoic acid supplement (200 mg/d; Neuromins; Nature's Way Products) and a potassium and magnesium supplement (3 times/wk; General Nutrition Corp).

Meals were prepared in the Human Metabolic Research Unit (HMRU) at Cornell University. The pregnant and control women consumed ≥ 1 meal/d throughout the week at the HMRU, whereas the lactating women consumed 1 meal/d thrice weekly to lessen the burden of travel to and from the HMRU while caring for an infant. Supplements were consumed with the onsite meal under the supervision of study personnel. All other meals and supplements were provided as carry-out and were consumed off site. Participants were required to 1) refrain from consuming food, beverages (except water), and supplements outside of those provided by study personnel; 2) complete daily checklists of menu items and supplements; and 3) return empty food containers and disposables of their carry-out meals and supplements.

Sample collection and processing

Fasting (10-h) venous blood samples and 24-h urine samples were obtained at study baseline (week 0) and study end (defined as week 10), processed, and stored at -80°C (26).

Analytical measurements

25(OH)D. Serum 25(OH)D was quantified with an isotope dilution LC-MS/MS method (24) that was validated in part by our laboratory's achieving the performance target set by the Vitamin D External Quality Assessment Scheme for the October 2012 distribution (i.e., within 14% of All Laboratory Trimmed Mean; within 4% of the values for Vitamin D External Quality Assessment Scheme samples analyzed by the National Institute of Standards and Technology). 25(OH)D was extracted from 150- μL serum samples, calibrators, and control samples (24). The calibrators for 25(OH)D₂, 25(OH)D₃, and epi-25(OH)D₃ were made by diluting ethanol stock solutions with PBS-4% albumin. Internal standard (100 μL) that contained 75 nmol d_3 -25(OH)D₃/L and 50 nmol d_3 -25(OH)D₂/L (IsoSciences) was then added to all of the samples. Extracts (65 μL) were injected onto a PFP column (PFP Accucore $2.1 \times 100, 2.6$ mm) with matching guard column at 45°C and separated by an LC-MS/MS system that consisted of a Surveyor HPLC system (pump and autosampler) and a TSQ Quantum Ultra mass spectrometer operated with XCalibur (2.2 SP1.48) software (ThermoElectron Corp). The analytes of interest were eluted from the column at a flow rate of 150 $\mu\text{L}/\text{min}$ under the following conditions: 73% methanol and 27% water (0–9th min), linear gradient from 73% to 100% methanol (9th–11th min), 100% methanol (11th–12th min), linear gradient from 100% to 73% methanol (12th–13th min), and 73% methanol (13th–18th min). To prevent deposit build-up in the mass spectrometer, the flow was directed into the spectrometer only between the 9th and 16th min. Atmospheric pressure chemical ionization in the positive ion mode with the use of selected reaction monitoring was used for detection. Transition pairs for the analytes of interest were the same as previously described (24). Intra- and inter-assay CVs were 2.1% and 4.7%, respectively, from our in-house control duplicates that consisted of 3 human sera with 25(OH)D concentrations spanning the range of the calibration curve, a

bovine serum rich in 25(OH)D₂, and National Institute of Standards and Technology SRM 2972 (within the range of certified values). We herein refer to the sum of 25(OH)D₂ and 25(OH)D₃ as “25(OH)D” which does not include epi-25(OH)D₃.

DBP and 1,25(OH)₂D. DBP and 1,25(OH)₂D in plasma samples were quantified with ELISA kits [R&D Systems for DBP; Immunodiagnostic Systems, Inc. for 1,25(OH)₂D], according to the manufacturers’ instructions. Intra- and inter-assay CVs for the DBP assays were 5.7% and 5.6%, respectively, from our in-house controls of human plasma with 3 different DBP concentrations. In the 1,25(OH)₂D assays, both intra- and inter-assay CVs were <10% (5.2% and 7.8%, respectively) from kit controls of low and high concentrations. In addition, all measured concentrations of the kit controls fell within the acceptable ranges provided by the manufacturer.

24,25(OH)₂D. Quantification of circulating 24,25(OH)₂D was performed with the LC-MS/MS method (20) with modifications from our instrumentation. Briefly, 24,25(OH)₂D₃ calibration curves were created by diluting ethanol stock solutions of 24,25(OH)₂D₃ with PBS-4% albumin. All plasma samples, calibrators, and control samples were mixed with 5 μL of internal standard solution that contained 1.5 pmol *d*₆-24,25(OH)₂D₃/L (Toronto Research Chemicals Inc.). Next, 24,25(OH)₂D₃ was extracted from 200-μL samples, calibrators, and controls with the use of a liquid-liquid extraction, and the upper layer was dried under nitrogen before derivatization with DMEQ-TAD (20). Extracts (65 μL) were resuspended in 60:40 methanol/water solution and injected onto a PFP column (PFP Accucore 2.1 × 100, 2.6 mm) with matching guard column at 45°C and separated by the LC-MS/MS system described for serum 25(OH)D quantification. 24,25(OH)₂D₃ was eluted from the column at a flow rate of 200 μL/min under the following conditions: 40% acetonitrile and 60% water (0–3rd min), linear gradient from 40% to 60% acetonitrile (3rd–5th min), 60% acetonitrile (5th–10th min), linear gradient from 50% to 70% acetonitrile (10th–12th min), linear gradient 70–80% acetonitrile (12th–15th min), returning to 40% acetonitrile (15th–17th min). To prevent deposit build-up, the flow directed into the mass spectrometer was limited from the 2nd to 10th min. Atmospheric pressure chemical ionization in the positive ion mode with selected reaction monitoring was used for 24,25(OH)₂D₃ detection. Two transition pairs were used for 24,25(OH)₂D₃ [*m/z* 762.5 > 468.2 (762.5 > 247.1 qualifier)], whereas a single transition pair was used for *d*₆-24,25(OH)₂D₃ (*m/z* 768.5 > 247.1). Intra- and inter-assay CVs were 5.7% and 4.9%, respectively, from duplicate measurements of our in-house control samples (human plasma with 2 different 24,25(OH)₂D₃ concentrations).

Free 25(OH)D. Free 25(OH)D concentrations were estimated with a previously described equation (28, 29) in which quantified circulating concentrations of 25(OH)D [i.e., 25(OH)D₂ and 25(OH)D₃], albumin, and DBP were computed. Because of limited availability of serum samples, plasma DBP concentrations were measured and then entered in the equation.

Total calcium. Serum total calcium was quantified by an automated chemistry analyzer (Dimension Xpand Plus; Siemens Healthcare Diagnostics).

Genotyping. The *GC* (vitamin D binding protein gene) rs7041 G > T, *CYP2R1* (25-hydroxylase gene) rs12794714 A > G, and *CYP2R1* rs10741657 A > G genotypes were determined by Endpoint Genotyping on a Roche LightCycler 480 with the use of the Applied Biosystems TaqMan Genotyping Assays (Life Technologies) after DNA extraction and purification with the Qiagen DNeasy kit.

Statistical analysis

To test for differences in baseline characteristics among the reproductive groups, we used 1-factor ANOVA for normally distributed continuous variables, Kruskal-Wallis test for non-normally distributed continuous variables, and χ^2 tests or Fisher exact tests for categorical variables. To examine the effect of reproductive state on biomarkers of vitamin D

metabolism at both study baseline and study end, we used linear mixed-effects models (LMMs). For each biomarker, the LMM included reproductive state, time, and their interaction term as fixed effects, and a random participant identifier factor. In addition, each LMM considered the following covariates: age, ethnicity/race, prepregnancy/baseline BMI (in kg/m²), education, season of study entry, multivitamin supplement use before study entry, genetic variants in vitamin D metabolism, and choline intake (480 or 930 mg/d). Covariates that achieved statistical significance ($P < 0.05$) were retained in the final models, and the Bonferroni correction was used for post hoc comparisons. Finally, relations among the biomarkers of vitamin D were assessed with Pearson correlation analysis.

All analyses were performed with JMP Pro 11 (SAS Institute, Inc.). Data that did not meet the normality and homogeneity of variance criteria were ln-transformed. Two influential outliers with studentized residuals >3 were excluded from free 25(OH)D analysis. Because epi-25(OH)D₃ had values below the limit of detection of 1.0 nmol/L, the limit of detection was used in place of “not detectable” among 47% of control, 31% of pregnant, and 54% of lactating women in the statistical models. Data are presented as arithmetic means ± SDs or geometric means (95% CI), unless otherwise specified. *P* values were 2-tailed and considered significant at <0.05. Data derived from the LMMs are “predicted mean concentrations (or ratios)” of the vitamin D biomarkers and account for influential covariates.

Results

Participant characteristics and study baseline measurements (ANOVA)

The characteristics of the study participants (26 pregnant, 28 lactating, and 21 control women) and their study baseline (week 0) measures are shown in Table 1. No differences in age and prepregnancy/baseline BMI were observed among the reproductive groups. In addition, the distributions of ethnicity/race, season of study entry, and education were balanced across the groups as were the distributions of the vitamin D-related genetic variants, *GC* rs7041 G > T and *CYP2R1* rs12794714 A > G polymorphisms. In contrast, *CYP2R1* rs10741657 A > G polymorphism distribution differed ($P = 0.03$) among the groups with the pregnant women having a lower prevalence of the variant GG genotype than the control and lactating women. Multivitamin supplement use before study entry also differed ($P < 0.001$) among the reproductive groups, with higher use in the pregnant and lactating women than in the control women.

Most vitamin D metabolites varied by reproductive state at baseline (Table 1). The pregnant women exhibited 40% higher ($P = 0.01$) serum 25(OH)D concentrations than the control women, and 100–150% higher ($P < 0.001$) DBP concentrations than the control and lactating women. Similarly, the pregnant women had 90% and 130% higher ($P < 0.001$) 1,25(OH)₂D concentrations than the control and lactating women, respectively. Although 24,25(OH)₂D concentrations did not differ across the groups at baseline, 25(OH)D:24,25(OH)₂D was higher in the pregnant women than in the control (30%; $P = 0.03$) and lactating (61%; $P < 0.001$) women, which did not differ from each other. The pregnant women also had 70% higher ($P = 0.048$) serum epi-25(OH)D₃ concentrations than those of the control women and almost double ($P = 0.004$) the concentrations of the lactating women.

The lactating women differed from the control and pregnant women in several variables. The 1,25(OH)₂D:25(OH)D was lower in the lactating women than in the control (−31%; $P = 0.04$) and pregnant (−47%; $P < 0.001$) women, which did not differ from each other. In addition, the lactating women showed higher concentrations of free 25(OH)D than the control

TABLE 1 Baseline characteristics and concentrations of vitamin D metabolites among the pregnant, lactating, and control women¹

	Pregnant (n = 26)	Lactating (n = 28)	Control (n = 21)	P
Age, y	28 ± 3	29 ± 5	29 ± 5	0.82
Ethnicity, n				0.81
White	16	20	14	
African American	1	1	2	
Hispanic	4	3	2	
Asian	4	1	1	
Other	1	3	2	
BMI, ² kg/m ²	23 [21, 26]	25 [21, 32]	24 [21, 25]	0.45
Season at study entry, n				0.81
April–September	14	17	11	
October–March	12	11	10	
Multivitamin supplement use before study entry, n	22	21	7	<0.001
Education, n				0.30
≤High school	4	8	6	
College	11	13	5	
>College	11	17	10	
GC rs7041 G > T polymorphism, n				0.35
GG	9	3	6	
GT	11	16	9	
TT	6	8	6	
CYP2R1 rs10741657 A > G polymorphism, n				0.03
AA	2	3	2	
AG	20	12	8	
GG	4	13	11	
CYP2R1 rs12794714 A > G polymorphism, n				0.15
AA	1	6	4	
AG	20	13	11	
GG	5	9	6	
Serum 25(OH)D, nmol/L	89 ± 29 ^a	73 ± 23 ^{a,b}	64 ± 25 ^b	0.006
Plasma DBP, µg/mL	405 (319, 515) ^a	165 (136, 199) ^b	204 (164, 254) ^b	<0.001
Plasma 1,25(OH) ₂ D, pmol/L	283 (232, 344) ^a	125 (104, 152) ^b	151 (129, 178) ^b	<0.001
Plasma 24,25(OH) ₂ D, nmol/L	9.6 (7.5, 12.4)	12.7 (10.2, 15.9)	9.1 (6.7, 12.5)	0.13
25(OH)D:24,25(OH) ₂ D	8.7 (7.5, 10.1) ^a	5.4 (4.8, 6.1) ^b	6.7 (5.8, 7.8) ^b	<0.001
1,25(OH) ₂ D:25(OH)D	3.4 (2.7, 4.2) ^a	1.8 (1.5, 2.2) ^b	2.6 (2.1, 3.2) ^b	<0.001
Serum epi-25(OH)D ₃ , nmol/L	3.2 ± 2.1 ^a	1.5 ± 0.9 ^b	1.9 ± 1.2 ^b	0.003
Free 25(OH)D, pmol/L	17.3 (13.8, 21.5) ^b	28.8 (24.1, 34.4) ^a	19.5 (14.9, 25.5) ^b	0.002
Serum total calcium, mg/dL	8.7 ± 0.4 ^c	9.4 ± 0.3 ^a	9.2 ± 0.3 ^b	<0.001

¹ Values are geometric means (95% CIs), means ± SDs, or medians (IQRs). Values in a row with a superscript letter indicate significant differences in the vitamin D metabolite among the reproductive groups (i.e., a>b>c), $P < 0.05$. *CYP2R1*, 25-hydroxylase gene; DBP, vitamin D binding protein; epi-25(OH)D₃, C3 epimer of 25-hydroxyvitamin D₃; GC, vitamin D binding protein gene; 1,25(OH)₂D, 1,25-dihydroxyvitamin D; 1,25(OH)₂D:25(OH)D, ratio of 1,25-dihydroxyvitamin D to 25-hydroxyvitamin D; 24,25(OH)₂D, 24,25-dihydroxyvitamin D; 25(OH)D, 25-hydroxyvitamin D; 25(OH)D:24,25(OH)₂D, ratio of 25-hydroxyvitamin D to 24,25-dihydroxyvitamin D.

² Self-reported prepregnancy BMI of the pregnant and lactating women and baseline BMI of the control women.

(47%; $P = 0.03$) and pregnant (67%; $P = 0.002$) women, which did not differ from each other. Finally, the lactating women had higher ($P < 0.03$) serum calcium concentrations than the control and pregnant women, which differed from each other with lower ($P < 0.001$) serum calcium concentrations among the pregnant women.

Effect of reproductive state on blood biomarkers of vitamin D metabolism (covariate-adjusted LMM)

Serum 25(OH)D. Reproductive state did not interact with time ($P = 0.48$) to affect serum 25(OH)D concentrations. However, a 25% increase ($P = 0.02$) in 25(OH)D concentrations was observed among the control women but not among the pregnant and lactating women whose concentrations remained stable ($P \geq 0.21$) throughout the study (Figure 1A). Similar to study

baseline, the pregnant women had ~30% higher ($P < 0.01$) concentrations of 25(OH)D (89 nmol/L) than the control women (69 nmol/L) at study end (Figure 1A), whereas serum 25(OH)D concentrations in the lactating women (75 nmol/L) did not differ ($P \geq 0.15$) from the pregnant and control women. At study end, predicted mean serum concentrations for all reproductive groups were above the estimated average requirement and RDA target values of 40 nmol/L and 50 nmol/L, respectively (Figure 1A). In addition, all participants, except for 2 pregnant and 2 control women, had unadjusted 25(OH)D concentrations >50 nmol/L.

Plasma DBP. Reproductive state did not interact with time ($P = 0.13$) to influence circulating DBP concentrations that did not change ($P > 0.9$) among the lactating and control women but tended to decrease (−9%; $P = 0.07$) among the pregnant women

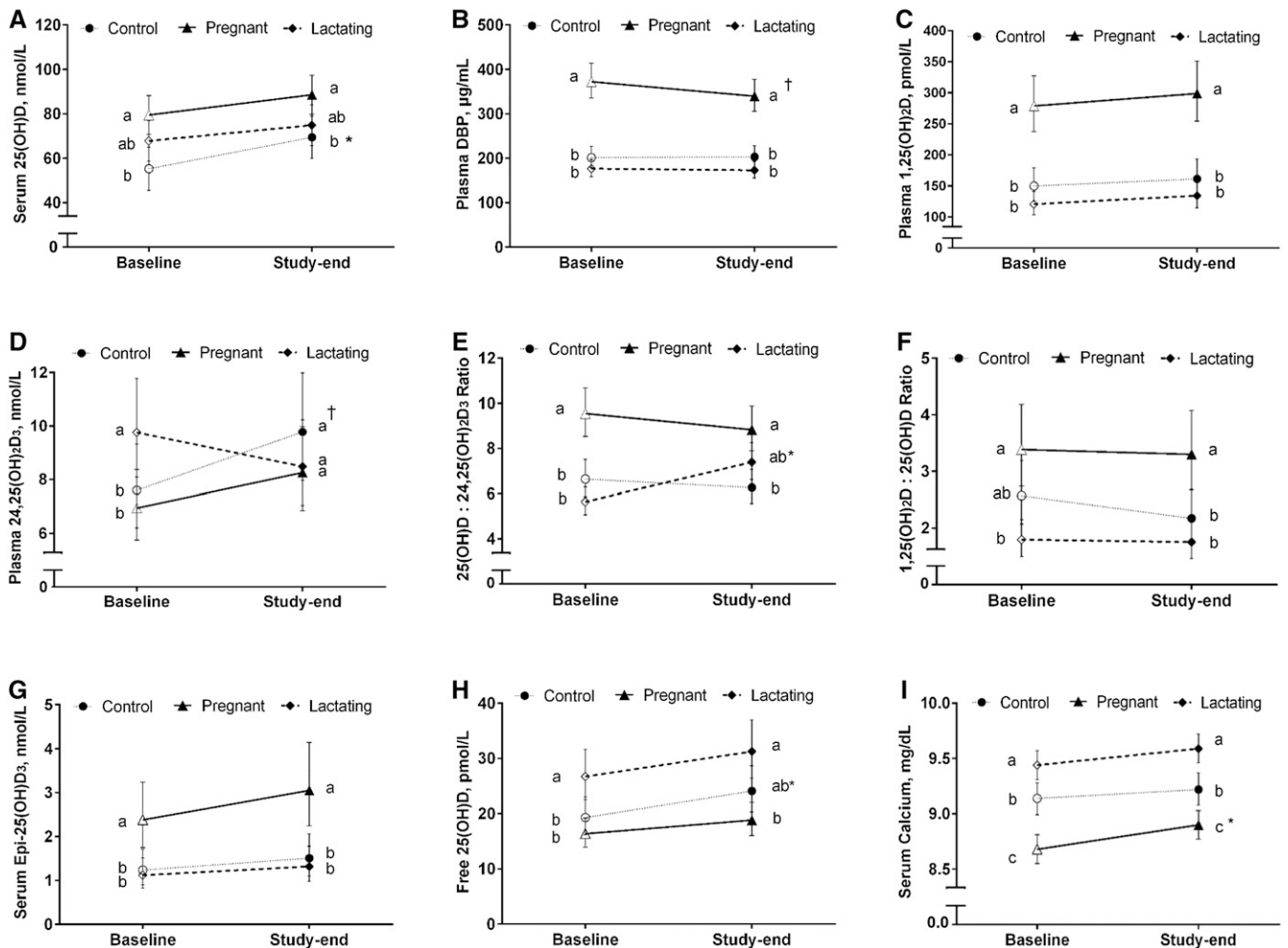


FIGURE 1 Circulating vitamin D metabolites [25(OH)D (A), DBP (B), 1,25(OH)₂D (C), 24,25(OH)₂D (D), 25(OH)D:24,25(OH)₂D (E), 1,25(OH)₂D:25(OH)D (F), epi-25(OH)D₃ (G), free 25(OH)D (H), and total calcium (I)] among the pregnant (*n* = 26), lactating (*n* = 28), and control (*n* = 21) women who consumed equivalent amounts of vitamin D and related nutrients (e.g., calcium and phosphorus) for 10 wk. All concentrations are predicted geometric means (95% CIs), except 25(OH)D (predicted arithmetic mean), derived from the covariate-adjusted linear mixed models. Means at a time without a common letter differ, *P* < 0.05. *, †Significantly different from baseline: **P* < 0.05; †*P* ≤ 0.07. DBP, vitamin D binding protein; epi-25(OH)D₃, C3 epimer of 25-hydroxyvitamin D₃; 1,25(OH)₂D, 1,25-dihydroxyvitamin D; 1,25(OH)₂D:25(OH)D, ratio of 1,25-dihydroxyvitamin D to 25-hydroxyvitamin D; 24,25(OH)₂D, 24,25-dihydroxyvitamin D; 25(OH)D, 25-hydroxyvitamin D; 25(OH)D:24,25(OH)₂D, ratio of 25-hydroxyvitamin D to 24,25-dihydroxyvitamin D.

(Figure 1B). Similar to study baseline, the pregnant women had 67% higher (*P* < 0.001) DBP concentrations (340 µg/mL) than the control women (203 µg/mL) and almost 100% higher (*P* < 0.001) concentrations than the lactating women (173 µg/mL) at study end. DBP concentrations in the lactating and control women did not differ (*P* = 0.42) from each other (Figure 1B).

Plasma 1,25(OH)₂D. Reproductive state did not interact with time (*P* = 0.88) to affect circulating 1,25(OH)₂D response with stable (*P* ≥ 0.52) concentrations of 1,25(OH)₂D among all the reproductive groups throughout the study (Figure 1C). Similar to study baseline, the pregnant women had 80% and 120% higher (*P* < 0.001) 1,25(OH)₂D concentrations (299 pmol/L) than the control (162 pmol/L) and lactating (134 pmol/L) women at study end, respectively (Figure 1C). 1,25(OH)₂D concentrations in the lactating and control women did not differ from each other (*P* > 0.9).

Plasma 24,25(OH)₂D. Reproductive state interacted with time (*P* = 0.003) to affect circulating 24,25(OH)₂D concentrations.

Although no changes (*P* ≥ 0.31) in 24,25(OH)₂D concentrations were observed within the pregnant and lactating women throughout the study, 24,25(OH)₂D tended to increase (22%; *P* = 0.06) among the control women (Figure 1D). Interestingly, despite 42% higher (*P* < 0.03) concentrations of 24,25(OH)₂D in the lactating women than the pregnant women at baseline, no differences (*P* > 0.9) were detected among the pregnant (8.3 nmol/L), lactating (8.5 nmol/L), and control (9.8 nmol/L) groups at study end (Figure 1D).

25(OH)D:24,25(OH)₂D. Reproductive state and time interacted (*P* < 0.001) to influence 25(OH)D:24,25(OH)₂D, with increases (*P* < 0.001) observed among the lactating women (Figure 1E) but not among the pregnant and control women (*P* > 0.9). Similar to study baseline, the pregnant women exhibited a 40% higher ratio (*P* < 0.001) than the control women (8.8 compared with 6.3) at study end. However, in contrast to the higher ratio (*P* < 0.001) among the pregnant (compared with lactating) women at study baseline, no difference (*P* = 0.17) between the 2 groups (8.8 compared with 7.4) was detected at study end (Figure 1E).

1,25(OH)₂D:25(OH)D. Reproductive state did not interact with time ($P = 0.36$) to influence 1,25(OH)₂D:25(OH)D that remained stable ($P \geq 0.56$) among all the reproductive groups across study time points. At study end, the pregnant women had a 50% higher ratio ($P = 0.04$) than the control women (3.3 compared with 2.2), despite no differences ($P = 0.53$) in this ratio between the 2 groups at study baseline (Figure 1F). The pregnant women also had an 83% higher ($P < 0.001$) 1,25(OH)₂D:25(OH)D than the lactating women (3.3 compared with 1.8) at study end, which was similar to study baseline (Figure 1F).

Serum epi-25(OH)D₃. Reproductive state did not interact with time ($P = 0.92$) to influence serum epi-25(OH)D₃ concentrations that did not change ($P > 0.9$) among all reproductive states throughout the study. Similar to study baseline, epi-25(OH)D₃ concentrations in the pregnant group (3.05 nmol/L) were twice ($P < 0.004$) those of the control (1.51 nmol/L) and lactating (1.31 nmol/L) groups at study end (Figure 1G), which did not differ from each other ($P > 0.9$).

Free 25(OH)D. Reproductive state did not interact with time ($P = 0.65$) to affect free 25(OH)D concentrations that remained stable ($P \geq 0.1$) among the lactating and control women over the course of the study and increased slightly (10%; $P = 0.02$) among the pregnant women (Figure 1H). Although the lactating women tended to have higher ($P = 0.05$) concentrations of free 25(OH)D than the control women at study baseline, no difference ($P = 0.15$) was observed among these groups at study end (31.3 and 24.1 pmol/L for lactating and control women, respectively). Similar to study baseline, the lactating women had 66% higher ($P < 0.001$) concentrations of free 25(OH)D than those of the pregnant women (18.8 pmol/L) at study end, which did not differ from the control women ($P = 0.18$).

Serum total calcium. Reproductive state did not interact with time ($P = 0.46$) to influence serum calcium concentrations that was stable ($P \geq 0.5$) among the lactating and control women but increased slightly (3%; $P = 0.04$) in the pregnant women throughout the study (Figure 1I). Similar to study baseline, the lactating women (9.6 mg/dL) had higher calcium concentrations than those of the control (9.2 mg/dL; $P = 0.003$) and pregnant women (8.9 mg/dL; $P < 0.001$) at study end. In addition, the pregnant women showed lower ($P = 0.01$) serum calcium concentrations than the control women at study end.

Associations among circulating vitamin D metabolites in all participants throughout the study

Among all study participants, circulating 25(OH)D correlated positively with 24,25(OH)₂D (Figure 2A), 1,25(OH)₂D (Figure 2B), DBP (Figure 2C), and free 25(OH)D (Figure 2D) at both baseline and study end. 1,25(OH)₂D and 24,25(OH)₂D were correlated throughout the study (Figure 2E) as was 1,25(OH)₂D with DBP (Figure 2F). No correlations were detected between 1,25(OH)₂D and free 25(OH)D at either baseline ($P = 0.68$) or study end ($P = 0.29$); however, free 25(OH)D correlated positively with 24,25(OH)₂D at both study time points (Figure 2G).

Associations among circulating vitamin D metabolites by reproductive state at study end

At study end, circulating 25(OH)D correlated positively with 1,25(OH)₂D among the control women (Table 2) and tended to correlate among the pregnant women ($r = 0.36$, $P = 0.07$). 25(OH)D was also highly correlated with 24,25(OH)₂D, and

with epi-25(OH)D₃ in all the reproductive groups (Table 2). Although 1,25(OH)₂D correlated positively with 24,25(OH)₂D among the control women, no relation between these metabolites was observed among the pregnant or lactating women.

Epi-25(OH)D₃ correlated positively with 24,25(OH)₂D in all the reproductive groups and with DBP in the pregnant women at study end (Table 2). In addition, free 25(OH)D correlated with epi-25(OH)D₃ and with 24,25(OH)₂D among the pregnant and control women at study end (Table 2). However, only the pregnant women showed correlations of free 25(OH)D with 25(OH)D ($r = 0.38$, $P = 0.057$) and 1,25(OH)₂D (Table 2).

Discussion

To the best of our knowledge, this is the first feeding study to control intakes of vitamin D and related nutrients such as calcium and phosphorus that can affect vitamin D status and metabolism. Three main findings emerged as follows: 1) pregnancy induces alterations in vitamin D metabolism, including increases in 25(OH)D, 25(OH)D:24,25(OH)₂D, and epi-25(OH)D₃; 2) reproductive state modulates the interrelations among circulating vitamin D metabolites; and 3) the RDA for vitamin D is likely adequate for most women of reproductive age, including pregnant and lactating women.

Pregnancy increases the circulating pool of vitamin D.

Circulating concentrations of 1,25(OH)₂D, DBP, and 1,25(OH)₂D:25(OH)D were significantly higher among third-trimester pregnant women than the control women, confirming previous reports (7–12). The pregnant women also exhibited significantly higher circulating concentrations of 25(OH)D than the control women even after adjusting for season, prestudy supplement use, ethnicity/race, BMI, and genetic variants that influence 25(OH)D concentrations. This elevation in 25(OH)D paralleled the rise in DBP among the pregnant women, suggesting that DBP-bound 25(OH)D, rather than free 25(OH)D (which did not differ between the pregnant and control women), contributed to the higher total circulating concentrations of this metabolite. DBP-bound 25(OH)D may be favored over the free form because it is more stable and is taken up in a regulated manner by tissues that express megalin-cubilin receptors such as the placenta (30).

No differences in circulating concentrations of 24,25(OH)₂D, a major catabolite of 25(OH)D (22), were detected between the pregnant and control women. However, 25(OH)D:24,25(OH)₂D was significantly higher among the pregnant women than the control women, indicating attenuation of vitamin D catabolism in this reproductive state. In addition, the pregnant women had higher concentrations of epi-25(OH)D₃ than the control women which may be a consequence of the elevated maternal vitamin D pool or could imply a possible role for this metabolite in maternal and fetal health.

In contrast to the robust effects of pregnancy on biomarkers of vitamin D metabolism, no differences were detected at study end between the lactating (15 wk postpartum) and control women. These findings are consistent with previous reports that examined calcium homeostasis during lactation (9, 11, 31). Of note, circulating 25(OH)D:24,25(OH)₂D increased throughout the study among lactating women because of a decrease in their 24,25(OH)₂D. Thus, lactating women may achieve vitamin D pools that are similar to nonlactating women by reducing 24-hydroxylase (CYP24A1) activity.

Reproductive state influenced the interrelations among circulating vitamin D metabolites. Data from the present study showed strong positive correlations between 25(OH)D

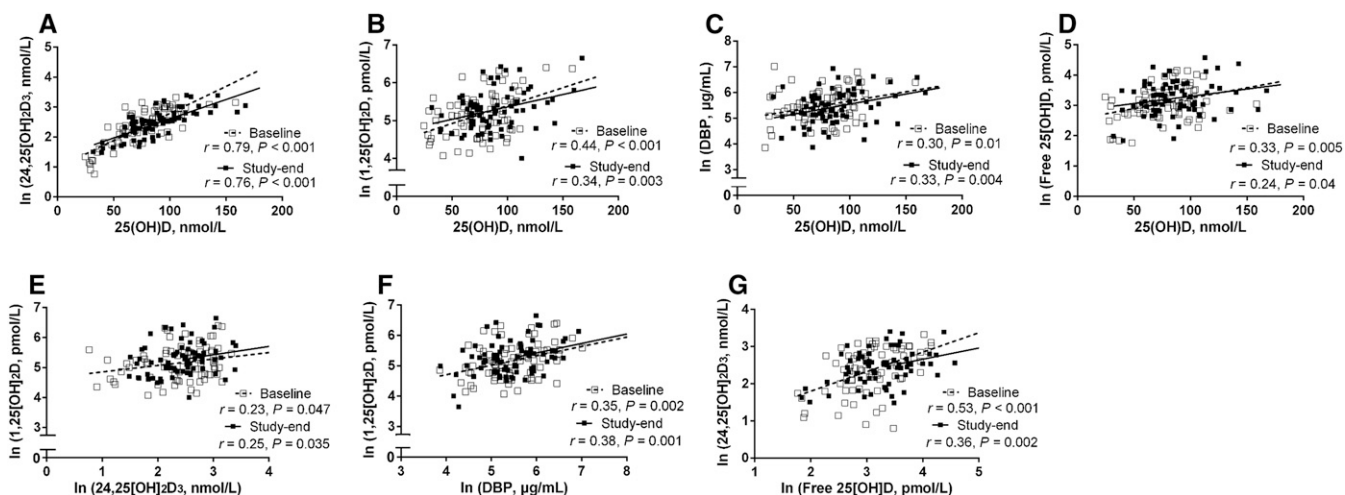


FIGURE 2 Relations among circulating vitamin D metabolites in all participants ($n = 75$) by study time point: 25(OH)D and 24,25(OH)₂D (A), 25(OH)D and 1,25(OH)₂D (B), 25(OH)D and DBP (C), free 25(OH)D and 25(OH)D (D), 1,25(OH)₂D and 24,25(OH)₂D (E), 1,25(OH)₂D and DBP (F), and free 25(OH)D and 24,25(OH)₂D (G). All metabolites were ln-transformed, except for 25(OH)D. DBP, vitamin D binding protein; 1,25(OH)₂D, 1,25-dihydroxyvitamin D; 24,25(OH)₂D, 24,25-dihydroxyvitamin D; 25(OH)D, 25-hydroxyvitamin D.

and 24,25(OH)₂D at study end among the pregnant, lactating, and control women, supporting the catabolism of 25(OH)D to 24,25(OH)₂D as a means to maintain homeostasis. These findings are consistent with previous reports in healthy adults (19, 20, 22) and pregnant women at term (7), but they deviate somewhat from a study in lactating women which reported significant correlations of 25(OH)D and 24,25(OH)₂D at 1 wk postpartum but not at later time points (18). In addition, although 25(OH)D tended ($n = 26$; $r = 0.36$; $P = 0.07$) to be correlated with the bioactive 1,25(OH)₂D metabolite among the pregnant women at study end, the linear relation between these 2 metabolites became significant when the pregnant women with serum 25(OH)D concentrations <100 nmol/L were examined separately ($n = 33$; $r = 0.39$; $P = 0.02$). This finding supports prior work to suggest that substrate-dependent 1,25(OH)₂D production from 25(OH)D reaches a plateau at ~ 100 nmol 25(OH)D/L

in pregnant women (32). Pregnant women also exhibited a higher number of significant correlations between free 25(OH)D, a metabolite recently linked to bone health (33, 34), and other metabolites. Specifically, free 25(OH)D was associated with 4 metabolites among pregnant women [i.e., total 25(OH)D, 1,25(OH)₂D, 24,25(OH)₂D, and epi-25(OH)D₃], 2 metabolites among control women [i.e., 24,25(OH)₂D and epi-25(OH)D₃], and zero metabolites among lactating women. This suggests that free 25(OH)D may be a useful indicator of vitamin D status during pregnancy but not during lactation. Notably, the bioactive metabolite 1,25(OH)₂D, was associated with 24,25(OH)₂D concentrations in control women but not in pregnant and lactating women. This finding supports the notion that feedback inhibition of 1,25(OH)₂D production (by CYP24A1) is uncoupled during pregnancy (35, 36) and lactation, possibly because of a higher demand for 1,25(OH)₂D in these reproductive states.

TABLE 2 Correlations among circulating vitamin D metabolites in pregnant, lactating, and control women at study end¹

Correlations between vitamin D metabolites	Pregnant ($n = 26$)		Lactating ($n = 28$)		Control ($n = 21$)	
	r	P	r	P	r	P
25(OH)D, 1,25(OH) ₂ D	—	NS	—	NS	0.48	0.03
25(OH)D, 24,25(OH) ₂ D	0.85	<0.001	0.67	<0.001	0.89	<0.001
25(OH)D, DBP	—	NS	—	NS	—	NS
25(OH)D, Epi-25(OH)D ₃	0.72	<0.001	0.63	<0.001	0.59	0.007
25(OH)D, Free 25(OH)D	—	NS	—	NS	—	NS
1,25(OH) ₂ D, 24,25(OH) ₂ D	—	NS	—	NS	0.64	0.002
1,25(OH) ₂ D, DBP	—	NS	—	NS	—	NS
1,25(OH) ₂ D, Free 25(OH)D	0.47	0.02	—	NS	—	NS
1,25(OH) ₂ D, Epi-25(OH)D ₃	—	NS	—	NS	—	NS
Free 25(OH)D, 24,25(OH) ₂ D	0.46	0.02	—	NS	0.48	0.03
Free 25(OH)D, Epi-25(OH)D ₃	0.49	0.03	—	NS	0.42	0.03
Epi-25(OH)D ₃ , DBP	0.40	0.04	—	NS	—	NS
Epi-25(OH)D ₃ , 24,25(OH) ₂ D	0.56	0.003	0.58	0.001	0.49	0.03

¹ Metabolites were ln-transformed, except 25(OH)D and epi-25(OH)D₃. NS, $P \geq 0.05$. DBP, vitamin D binding protein; epi-25(OH)D₃, C3 epimer of 25-hydroxyvitamin D₃; 1,25(OH)₂D, 1,25-dihydroxyvitamin D; 24,25(OH)₂D, 24,25-dihydroxyvitamin D; 25(OH)D, 25-hydroxyvitamin D.

Vitamin D intake approximating the current RDA achieved adequacy in $\geq 90\%$ of the study participants within each reproductive group. This study provided a mean of 511 IU vitamin D/d, falling between the current estimated average requirement (400 IU/d) and the RDA (600 IU/d), through a mixed diet and a prenatal supplement. Although a simulated dose-response curve of serum 25(OH)D concentrations generated by the IOM did not include pregnant and lactating populations (6), it is notable that the study dose readily achieved 25(OH)D concentrations above both the RDA-targeted value and the IOM cutoff for vitamin D adequacy (≥ 50 nmol/L) in 95% of the participants: 92% in pregnant, 100% in lactating, and 90% in control women after a 10-wk period of controlled feeding. In addition, on examining 25(OH)D:24,25(OH)₂D, all participants had values <20 (unadjusted ratios) at study end, corresponding to vitamin D sufficiency (20). As such, the RDA of 600 IU/d, which is higher than the vitamin D dose of the present study, would be expected to meet vitamin D requirements of these populations as defined by the IOM (5), although additional dose-response studies are warranted.

Study limitations. The provision of a single dose of vitamin D is the main limitation of this study. We cannot exclude the possibility that the effect of reproductive state on vitamin D

biomarkers and their relations might differ under conditions of lower or higher vitamin D intakes. In addition, oral contraceptive use of the control women was not considered in our statistical models. However, inclusion of this variable would be expected to accentuate the difference between the pregnant and control women (8 of whom used oral contraceptives), secondary to the positive relation between oral contraceptive use and serum 25(OH)D concentrations (37).

Conclusions. Pregnancy increases circulating pools of vitamin D metabolites in a manner that is independent of dietary intake, supplement use, season, ethnicity/race, and BMI. The factors contributing to this increase are unclear, but they may involve the placenta that expresses the vitamin D machinery required for the synthesis of 25(OH)D and 1,25(OH)₂D (30, 38, 39). The study findings also show that interrelations among vitamin D metabolites are modified by reproductive state, particularly during pregnancy. Finally, our data support the adequacy of the vitamin D RDA (600 IU) for achieving serum 25(OH)D concentrations of 50 nmol/L among women of childbearing age, including those who are pregnant or breastfeeding.

Acknowledgments

HP, PMB, and MAC designed the research; HP, AAW, JY, XJ, CAP, and OVM conducted the research; HP analyzed the data; HP and MAC wrote the manuscript; PMB and SM contributed to the methodology development and data interpretation; and MAC had primary responsibility for the final content. All authors read and approved the final manuscript.

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