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# Vitamin D kinetics in nonpregnant and pregnant women after a single oral dose of trideuterated vitamin D<sub>3</sub>

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## ABSTRACT

The plasma pool of the hormone 1,25-dihydroxyvitamin D (1,25(OH)<sub>2</sub>D) is increased throughout most of human pregnancy. Mechanisms behind this adaptation are unclear, in part due to limited data on vitamin D kinetics during pregnancy. Stable isotopes make it possible to study vitamin D kinetics in vulnerable study populations like pregnant women. We conducted a pilot study of vitamin D kinetics in nonpregnant and pregnant women. We evaluated a clinical protocol and developed analytical methods to assess the serum appearance and disappearance of trideuterated vitamin D<sub>3</sub> (d3-vitamin D<sub>3</sub>) and trideuterated 25-hydroxyvitamin D<sub>3</sub> (d3-25(OH)D<sub>3</sub>) after a single oral dose of 25 μg of [6,19,19-<sup>2</sup>H]-vitamin D<sub>3</sub> (d3-vitamin D<sub>3</sub>). Blood was collected at baseline and 2, 4, 6, 24, 168, 264, and 456 hours post-dosing. We then described the serum kinetic profiles of d3-vitamin D<sub>3</sub> and d3-25(OH)D<sub>3</sub> in nonpregnant and pregnant women. Serum kinetic profiles of d3-vitamin D<sub>3</sub> and d3-25(OH)D<sub>3</sub> followed a time course in line with previous pharmacokinetic studies. There was marked variability between participants in the area under the concentration-time curve (AUC) of d3-25(OH)D<sub>3</sub> over the 20-day study period. This AUC of d3-25(OH)D<sub>3</sub> was positively correlated with the serum vitamin D binding protein (DBP) concentration, which was higher in pregnant compared with nonpregnant women. The mean serum half-life of 25(OH)D<sub>3</sub> was longer but not significantly different in pregnant women (18.8 days) compared with nonpregnant women (13.6 days). Our pilot study demonstrated that a single oral dose of 25 μg of d3-vitamin D<sub>3</sub> can be used to study vitamin D kinetics. Serum DBP concentration is an important predictor of vitamin D kinetics, and more research is needed to fully understand the significance of elevated DBP concentration during pregnancy.

## 1. Introduction

Human pregnancy is associated with adaptations in vitamin D metabolism including a 100–150 % increase in the serum 1,25-dihydroxyvitamin D (1,25(OH)<sub>2</sub>D) concentration by the second trimester. The serum 25-hydroxyvitamin D (25(OH)D) concentration, however, remains relatively stable as a function of gestational age despite

progressive increase in plasma volume [1]. Whether expansion of the circulating 1,25(OH)<sub>2</sub>D pool is facilitated by increased hormone production, decreased clearance, or a combination of factors is unclear given the relative absence of data on vitamin D kinetics during pregnancy. There has been one prior study of vitamin D kinetics, specifically the 25(OH)D<sub>3</sub> serum half-life, during pregnancy [2]. In that study, Jones and colleagues fed Gambian women trideuterated 25(OH)D<sub>3</sub> and found

**Abbreviations:** AUC, area under the concentration-time curve; BV, blood volume; CLIA, Clinical Laboratory Improvement Amendments; C<sub>max</sub>, maximum concentration observed; CRC, Clinical Research Center; DBP, vitamin D binding protein; D1, day one; d3-25(OH)D<sub>3</sub>, trideuterated 25-hydroxyvitamin D<sub>3</sub>; d3-vitamin D<sub>3</sub>, trideuterated vitamin D<sub>3</sub>; ELISA, enzyme-linked immunosorbent assay; GFR, glomerular filtration rate; HPLC, high-performance liquid chromatography; IU, International Units; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LLOD, lower limit of detection; LLOQ, lower limit of quantitation; MRM, multiple reaction monitoring; 1,25(OH)<sub>2</sub>D, 1,25-dihydroxyvitamin D; PTAD, 4-Phenyl-1,2,4-triazoline-3,5-dione; PTH, parathyroid hormone; PV, plasma volume; t<sub>max</sub>, time to maximum concentration; 25(OH)D, 25-hydroxyvitamin D; 24,25(OH)<sub>2</sub>D, 24,25-dihydroxyvitamin D; URM, University of Rochester Medical Center.

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no significant difference in the half-life of 25(OH)<sub>2</sub>D<sub>3</sub> between pregnant and nonpregnant women, despite elevated 1,25(OH)<sub>2</sub>D levels in the pregnant group. Because participants were fed deuterated 25(OH)<sub>2</sub>D<sub>3</sub>, any potential differences in kinetics (for example in absorption or clearance) of the parent vitamin D compound by reproductive state could not be addressed.

Stable isotope studies to evaluate vitamin D absorption and turnover may fill gaps in the understanding of vitamin D metabolism not only during pregnancy but also across life stages. Early radioisotope studies showed that the rate and efficiency of hepatic conversion of radiolabeled vitamin D to 25(OH)D were inversely related to vitamin D nutritional status [3,4]. These data are still cited as a possible explanation for the nonlinear response of serum 25(OH)D concentration to vitamin D intake and for the inverse relationship between baseline serum 25(OH)D concentration and 25(OH)D response to supplementation [5]. However, the factors that influence 25(OH)D formation efficiency and underlying mechanisms have not been fully elucidated.

Previous pharmacokinetic studies of a single oral dose of vitamin D either administered radiolabeled vitamin D<sub>3</sub> [4,6–10] or assessed the change in serum vitamin D and/or 25(OH)D concentration following a large supplemental dose [11–18]. There are several limitations to these approaches. First, neither technique is suitable for use in vulnerable groups like pregnant women. In addition, large doses have the potential to perturb steady state vitamin D metabolism and thereby alter tissue distribution and elimination of vitamin D. A stable isotope technique instead may avoid perturbing normal metabolism but requires capacity to measure small quantities of vitamin D in serum or plasma.

Advances in mass spectrometry have enabled the possibility to use stable isotope techniques to safely trace vitamin D metabolism. We conducted a pilot study of vitamin D kinetics after a single oral dose of [6,19,19-<sup>2</sup>H]-vitamin D<sub>3</sub> (d3-vitamin D<sub>3</sub>). Our study objectives were to evaluate a clinical protocol and develop analytical methods to assess the serum appearance and disappearance of labeled vitamin D<sub>3</sub> and labeled 25(OH)D<sub>3</sub> after this single oral dose of d3-vitamin D<sub>3</sub> and to describe the serum kinetics of the d3-vitamin D<sub>3</sub> and d3-25(OH)D<sub>3</sub> in nonpregnant and pregnant women.

## 2. Material and methods

### 2.1. Participants

Six nonpregnant, nonlactating women and 4 pregnant Caucasian women, aged 19–35 y, were recruited from the University of Rochester Medical Center (Rochester, NY) and surrounding community. Study visits were conducted between November 2015 and June 2016. Eligible participants had a current or pre-pregnancy BMI ≤ 28 kg/m<sup>2</sup>. Pregnant participants were carrying a single fetus and were 20 to < 36 weeks pregnant at entry into the 20-day study. Participants agreed to refrain from travel and use of tanning salons during the study. Nonpregnant participants agreed to discontinue use of any dietary supplements during the study, whereas pregnant participants continued to ingest their prenatal supplement as recommended except on the day of isotope dose administration. Participants were ineligible if they had diabetes, current gestational diabetes, other endocrine disorders, malabsorption disease, eating disorders, HIV infection, pregnancy hypertension, elevated diastolic blood pressure (> 110 mm/Hg), history of substance abuse, or were using steroids or medications that influence vitamin D or calcium homeostasis. The study was approved by the Institutional Review Boards at the University of Rochester and Cornell University before registration with clinicaltrials.gov (NCT02705287). All participants provided written informed consent.

### 2.2. Dose preparation

Thirty-five μg of trideuterated [6,19,19-<sup>2</sup>H]-vitamin D<sub>3</sub> (d3-vitamin D<sub>3</sub>) (Sigma-Aldrich) was added to 700 μL of commercial soybean oil in

an amber glass vial and vortexed to mix thoroughly. Each dose of d3-vitamin D<sub>3</sub> was prepared individually in a research kitchen at Cornell University on the day before dosing. The dose was stored at –20 °C and transported on ice the next morning from Ithaca, NY to Rochester, NY.

### 2.3. Study protocol

Participants came to the Clinical Research Center (CRC) at the University of Rochester Medical Center (URMC) on the morning of study day 1 (D1) in the fasted state. Height, weight, and blood pressure were obtained. A urine sample was collected from nonpregnant participants for a urine pregnancy test. A saline lock intravenous catheter was placed, and baseline blood was obtained. Participants were served a standardized breakfast of toast with unfortified margarine, sliced fruit, and a selection of beverages. Immediately prior to administration, the dose was brought to room temperature and vortexed to mix. Using a pipette, a study investigator dispensed 500 μL of the 0.05 μg/μL isotope solution (a total dose of 25 μg (1000 International Units (IU)) of d3-vitamin D<sub>3</sub>) in two aliquots onto the toast. The entire dose was consumed under direct observation. To rinse the pipette tip, an additional 500 μL of unlabeled soybean oil was drawn up then dispensed onto the toast. The remainder of the breakfast was consumed under direct observation.

Participants spent the following 6 h post-dosing in their exam room at the CRC. Lunch and optional mid-morning and afternoon snacks were served at set times from a standardized menu. Dietary intake while in the CRC was estimated by weighing the foods before and after snack and lunch times. Total nutrient intakes were determined using the Nutrition Data System for Research (NDSR 2015, University of Minnesota). A non-fasted blood sample was obtained at 2 h, 4 h, 6 h, 24 h, 168 h, 264 h, and 456 h post-dosing. At two points during the study (D1 and D20), resistance and reactance were measured by a bioelectrical impedance analyzer (Quantum X, RJL Systems).

### 2.4. Laboratory analysis

Total serum 25(OH)D concentrations at baseline, 2 h, 4 h, 6 h, 24 h, 168 h, 264 h, and 456 h were measured by liquid chromatography-tandem mass spectrometry (LC–MS/MS) at a Clinical Laboratory Improvement Amendments (CLIA)-certified lab at the URMC. Total serum 1,25(OH)<sub>2</sub>D concentrations at baseline, 168 h, and 456 h were measured by LC–MS/MS at a URMC reference lab (ARUP Laboratories), and the molar ratio of 1,25(OH)<sub>2</sub>D to 25(OH)D was calculated for each of these time points. A serum comprehensive metabolic panel at baseline and 456 h was measured at the URMC clinical lab. Blood was collected in an EDTA tube or heparin tube and analyzed for intact PTH or whole blood ionized calcium, respectively, at baseline and 456 h at the URMC lab. Serum vitamin D binding protein (DBP) at baseline and 456 h was measured using a commercial ELISA (Immundiagnostik AG), and the molar ratio of 1,25(OH)<sub>2</sub>D to DBP was calculated for each of these time points. Free serum 25(OH)D at baseline was measured by ELISA (Future Diagnostics B.V.). The percent free 25(OH)D at baseline was calculated by dividing the measured free 25(OH)D concentration by total 25(OH)D concentration (quantified by LC–MS/MS in the clinical lab) and multiplying by 100. Serum 24,25(OH)<sub>2</sub>D<sub>3</sub> at baseline was measured at an external laboratory with an LC–MS/MS method that simultaneously quantifies 25(OH)D<sub>3</sub> [19]. The molar ratio of 24,25(OH)<sub>2</sub>D<sub>3</sub> to 25(OH)D<sub>3</sub> at baseline was calculated from these data.

### 2.5. Preparation of calibrators and sample pretreatment

Serum was isolated from each post-dosing blood sample and kept at –80 °C or on dry ice during transport until preparation and analysis at the Cornell Proteomics and Metabolomics Core Facility. For sample pretreatment, 100 μL of serum in a polypropylene microcentrifuge tube was spiked with internal standards, 1.25 ng of d6-vitamin D<sub>3</sub> ([26,26,26,27,27,27-<sup>2</sup>H]-vitamin D<sub>3</sub>, Chemaphor) and 0.3125 ng of d6-

25(OH)D<sub>3</sub> ([26,26,26,27,27,27-<sup>2</sup>H]-25(OH)D<sub>3</sub>, Chemaphor). Samples were vortexed to mix, spun down in a centrifuge, incubated for 30 min in the dark at room temperature, and stored overnight at -20 °C.

Samples were thawed at room temperature the following morning. All solvents were LC-MS grade unless specified otherwise. Serum proteins were precipitated with 200 µL of acetonitrile (Fisher-Scientific) and vortexed for 1 min at 1800 rpm. This was followed by sequential addition of 100 µL of Milli-Q deionized water (Millipore), 400 µL of methanol (Fisher Scientific), and 100 µL of chloroform (ACS grade, Fisher Scientific). Samples were vortexed for 5 min at 1200 rpm, centrifuged for 2 min at 16,000 g, and the supernatant was collected in a clean polypropylene microcentrifuge tube. To rinse the protein pellet, 400 µL of methanol was added to the original sample, which was vortexed for 5 min at 1200 rpm and centrifuged for 2 min at 16,000 g. The supernatants were pooled and evaporated to dryness in a vacuum concentrator.

## 2.6. Liquid-liquid extraction and derivatization

Dried sample residues were reconstituted with 200 µL of ethyl acetate (Sequencing grade, Fisher Scientific), vortexed to mix, and spun down before addition of 100 µL each of water and 0.4 M potassium phosphate dibasic (ACS grade, Sigma-Aldrich) in water. Samples were vortexed for 5 min at 1800 rpm, centrifuged for 2 min at 16,000 g, and the organic layer was collected. After removing the organic layer, 200 µL of ethyl acetate was added to the original sample, which was vortexed and centrifuged under the same conditions. The organic layers were pooled and centrifuged for 1 min at 16,000 g. Any aqueous layer was removed by pipette and discarded, and the extracts were then dried in a vacuum concentrator. Extracts were then reconstituted and redried from 50 µL of acetonitrile (Fisher-Scientific) two times prior to derivatization.

A solution of 4-Phenyl-1,2,4-triazoline-3,5-dione (PTAD) (Sigma-Aldrich) was prepared to 0.75 mg/mL (w/v) in anhydrous acetonitrile (DNA synthesis grade, Applied Biosystems). 75 µL was added to extracts, which were vortexed for 1 min at 1600 rpm, spun down, and incubated at room temperature in the dark in a desiccator for 45 min. An additional 25 µL of the PTAD solution was added (no mixing) after 25 min of incubation. The reaction was quenched at 45 min by addition of 100 µL of methanol, and extracts were vortexed to mix, spun down, and dried in a vacuum concentrator.

## 2.7. LC-MS/MS and quantitation

The extracts were reconstituted in 60 µL of methanol, vortexed for 1 min at 1600 rpm, and centrifuged to spin down. 50 µL was transferred to polypropylene autosampler vials (SUN-SRI) for analysis. Analytes were separated by HPLC using a Dionex UltiMate3000 with an Ultra C8 column (5 µm, 100 Å, 1 × 150 mm, Restek) and binary solvent system using an injection volume of 15 µL (25 % of the original sample). Solvent A was 10 % methanol / 0.2 % glacial acetic acid (HPLC grade, Fisher-Scientific) in water, and solvent B was 0.2 % glacial acetic acid in methanol. The linear binary gradient elution program was as follows: 65–65–100–100–65–65 % solvent B, 0–3–20–25–26–36 min, entire column effluent to mass spectrometer. MS/MS quantitation (QTRAP 4000, Sciex) was performed in multiple reaction monitoring

**Table 1**  
MRM precursor/product ion transitions for analytes and internal standards.

PTAD derivative	Precursor ion (m/z) <sup>a</sup>	Product ion (m/z)	Collision energy (V)
d3-vitamin D <sub>3</sub>	563.6	301.2	26
d3-25(OH)D <sub>3</sub>	579.6	301.2	27
d6-vitamin D <sub>3</sub>	566.6	298.2	25
d6-25(OH)D <sub>3</sub>	582.6	298.2	23

<sup>a</sup> [M+H].

(MRM) mode (Table 1).

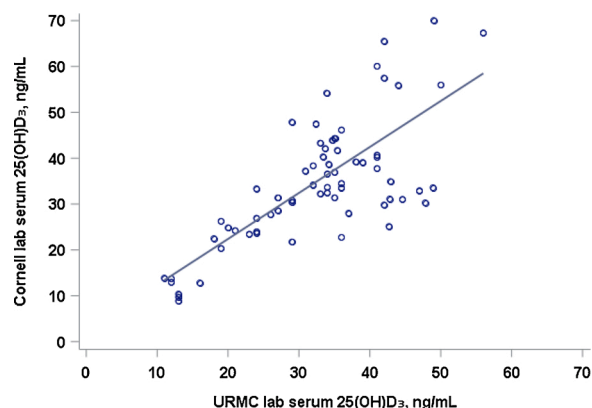
Analyte concentrations were quantified using 6-point calibration curves generated with each run. The calibration curves included a blank (matrix control) and 5 calibrators with calibration points at 0.0, 0.4, 0.8, 1.6, 2.4, and 4.0 ng/mL for d3-vitamin D<sub>3</sub> and 0.0, 0.2, 0.6, 1.2, 2.4, and 4.0 ng/mL for d3-25(OH)D<sub>3</sub>. The lower limit of detection (LLOD) was defined as the lowest amount/concentration with an analytical signal to noise ratio of 3:1. The LLOD was 1.5 pg (0.06 ng/mL) for d3-vitamin D<sub>3</sub> and 0.5 pg (0.02 ng/mL) for d3-25(OH)D<sub>3</sub>. All observations below the LLOD were set to zero. The lower limit of quantitation (LLOQ) was defined by a signal to noise ratio of 10:1. The LLOQ was 5.0 pg (0.2 ng/mL) for d3-vitamin D<sub>3</sub> and 1.7 pg (0.07 ng/mL) for d3-25(OH)D<sub>3</sub>. For d3-vitamin D<sub>3</sub>, four observations were above the LLOD but below the LLOQ, so these values were set to half of the LLOQ. Prior to analyses, fresh serum from a healthy nonpregnant nonlactating Caucasian woman of reproductive age was spiked with the analytes and frozen in aliquots to be used as an in-house control. Based on this control, at a concentration of 3-4 ng/mL, the between run coefficient of variation was 8.6 % for d3-vitamin D<sub>3</sub> and 15.6 % for d3-25(OH)D<sub>3</sub>. Unlabeled, native vitamin D<sub>3</sub> and 25(OH)D<sub>3</sub> were also quantified using the same calibration curves. Although native serum 25(OH)D<sub>3</sub> concentrations exceeded the calibration range, there was good agreement between this serum 25(OH)D<sub>3</sub> concentration and the result from the URM clinical lab ( $r = 0.74$ ; Fig. 1). Only the clinical lab results were used for statistical analyses.

## 2.8. Pharmacokinetic parameters

To correct for variability in compartment size, concentrations of serum d3-vitamin D<sub>3</sub> and d3-25(OH)D<sub>3</sub> were normalized to mean plasma volume using the equation:

$$\frac{\text{Concentration} \times \text{Plasma Volume}}{\text{Sample Mean Plasma Volume}}$$

Plasma volume was estimated from height, weight, and, when applicable, gestational age at study baseline using published equations [20]. However, since there is no widely accepted method for estimating plasma volume during pregnancy, we also normalized the tracer concentrations to mean blood volume. Blood volume was estimated as 65 mL/kg for nonpregnant participants and 70 mL/kg for pregnant participants [20]. Finally, because plasma and blood volumes could not be measured directly, calculation of pharmacokinetic parameters and statistical analyses were conducted with both the uncorrected and corrected concentrations.



**Fig. 1.** Serum 25(OH)D<sub>3</sub> concentration was measured by LC-MS/MS in each post-dosing sample at both the University of Rochester Medical Center (URMC) CLIA-certified clinical lab and the Cornell University Proteomics and Mass Spectrometry Core Facility. The plot and regression line ( $y = 1.0x + 2.3$ ) show positive correlation ( $r = 0.74$ ) between these two measurement methods ( $n = 10$  participants, 69 observations).

Pharmacokinetic parameters were obtained for each participant individually. Parameters included maximum concentration observed ( $C_{max}$ ), the time to maximum concentration ( $t_{max}$ ), and the area under the concentration-time curve (AUC) of serum d3-vitamin D<sub>3</sub> and d3-25(OH)D<sub>3</sub>. The AUC of d3-vitamin D<sub>3</sub> from 0 to 24 h (AUC<sub>0–24h</sub> of d3-D<sub>3</sub>) and the AUC of d3-25(OH)D<sub>3</sub> from 0 to 456 h (AUC<sub>0–456h</sub> of d3-25(OH)D<sub>3</sub>) were approximated using the linear trapezoidal method. The serum half-life of 25(OH)D<sub>3</sub> was estimated from the slope of the line ( $-k_e$ ) fit to the plot of the natural log concentrations of d3-25(OH)D<sub>3</sub> at 168 h, 264 h, and 456 h and the following equation, assuming first-order reaction kinetics [5,21]:

$$t_{1/2} = \frac{\ln(2)}{k_e}$$

Percent of dose recovered was calculated for each participant individually. The serum concentrations of d3-vitamin D<sub>3</sub> and d3-25(OH)D<sub>3</sub> were multiplied by the participant's plasma volume to give the total mass of each circulating at each time point. Mass values were converted to nmol and then were used to calculate fraction of dose recovered from the molar equivalent of the dose.

## 2.9. Statistical analysis

Data were analyzed with SAS 9.4 and JMP Pro 13 (SAS Institute Inc.). Differences in participant characteristics and kinetic parameters by reproductive state were tested with 2-sample *t*-tests. Potential predictors of the AUC<sub>0–24h</sub> of d3-D<sub>3</sub>, AUC<sub>0–456h</sub> of d3-25(OH)D<sub>3</sub>, and the 25(OH)D<sub>3</sub> half-life including serum vitamin D<sub>3</sub>, total 25(OH)D, free 25(OH)D, percent free 25(OH)D, DBP, PTH, 1,25(OH)<sub>2</sub>D, the molar ratio of 1,25(OH)<sub>2</sub>D to DBP, 24,25(OH)<sub>2</sub>D, and ionized calcium, dietary calcium intake, GFR, current or pre-pregnancy BMI, and body fat percentage (in nonpregnant participants only) were explored using linear regression. A two-sided alpha = 0.05 was used to define statistical significance.

## 3. Results

### 3.1. Participant characteristics

Mean age was 26.4 years and was not different between reproductive groups (Table 2). Three nonpregnant participants reported current use of hormonal birth control pills. Two were using a combination birth control with estrogen and progestin, and 1 was using a progestin-only pill. The other 3 nonpregnant participants were not on birth control or other medications. Two pregnant women were studied during the 2nd trimester (D1 gestational age: 22 wk/1 d and 22 wk/3 d), and two were studied during the 3rd trimester (D1 gestational age: 29 wk/6 d and 30 wk/0 d). The pregnant participants did not report taking any medications, but all reported using a daily prenatal supplement containing either 400 IU ( $n = 2$ ), 600 IU, or 1000 IU of vitamin D<sub>3</sub>, which they did not ingest on the day of isotope dose administration.

No laboratory indicators changed significantly within participants during the 20-day study. For this reason, the average of all the repeated measures is shown in Table 2 and was used in statistical analyses. Total serum 25(OH)D concentration tended to be higher in pregnant participants, and the difference between groups approached significance ( $P = 0.07$ ). In addition, pregnant participants had significantly higher serum 1,25(OH)<sub>2</sub>D and DBP, whereas their PTH and percent free 25(OH)D were significantly lower. Mean serum 24,25(OH)<sub>2</sub>D<sub>3</sub> was lower in pregnant participants despite higher 25(OH)D<sub>3</sub>, and, as a result, the molar ratio of 24,25(OH)<sub>2</sub>D<sub>3</sub> to 25(OH)D<sub>3</sub> was significantly lower in the pregnant group. As expected, glomerular filtration rate (GFR) tended to be higher in pregnant participants ( $P = 0.06$ ).

**Table 2**  
Participant characteristics and laboratory indicators.

	All participants 10	Nonpregnant 6	Pregnant 4
<i>n</i>	10	6	4
Age at baseline, y	26.4 ± 5.5	26.0 ± 6.9	27.0 ± 3.6
Height at baseline, cm	170 ± 5.2	170 ± 3.2	162 ± 4.1
Weight at baseline, kg	65.8 ± 8.7	67.1 ± 9.3	64.0 ± 8.9
Baseline or pre-pregnancy BMI, kg/m <sup>2</sup>	NA	23.3 ± 3.9	21.1 ± 2.7
Week gestation on D1	NA	NA	26.1 ± 4.5
Blood volume, mL <sup>a</sup>	4408 ± 578	4358 ± 603	4482 ± 620
Plasma volume, mL <sup>b</sup>	2905 ± 368	2641 ± 102	3300 ± 202*
Laboratory indicators <sup>c</sup>			
Serum vitamin D <sub>3</sub> , ng/mL	5.3 ± 4.4	4.3 ± 5.3	6.8 ± 2.4
Total serum 25(OH)D, ng/mL <sup>d</sup>	32.4 ± 10.6	27.5 ± 9.9	39.8 ± 7.3
Free serum 25(OH)D, pg/mL	8.0 ± 2.3	8.5 ± 2.8	7.2 ± 1.6
Percent free 25(OH)D, %	0.027 ± 0.01	0.033 ± 0.01	0.018 ± 0.001*
Serum 1,25(OH) <sub>2</sub> D, pg/mL	75.5 ± 28	59.1 ± 15	100.0 ± 24*
1,25(OH) <sub>2</sub> D:25(OH)D (10 <sup>-3</sup> ) <sup>e</sup>	2.4 ± 0.8	2.2 ± 0.6	2.6 ± 1.0
Serum 24,25(OH) <sub>2</sub> D <sub>3</sub> , ng/mL	2.9 ± 1.1	3.1 ± 1.2	2.7 ± 1.2
24,25(OH) <sub>2</sub> D <sub>3</sub> :25(OH)D <sub>3</sub> <sup>e</sup>	0.078 ± 0.023	0.090 ± 0.019	0.059 ± 0.014*
Serum intact PTH, pg/mL	36.5 ± 12	42.8 ± 11	27.2 ± 5.9*
Serum DBP, mg/L	484 ± 120	423 ± 110	575 ± 72*
Serum albumin, g/dL	4.1 ± 0.5	4.5 ± 0.2	3.5 ± 0.2*
Serum ionized calcium, mg/dL	4.7 ± 0.1	4.7 ± 0.08	4.6 ± 0.1
GFR, mL/min/1.73 m <sup>2</sup>	116 ± 15	109 ± 15	127 ± 8
Usual calcium intake, mg/d <sup>f</sup>	1028 ± 250	1080 ± 222	950 ± 302

Abbreviations: D1, day 1; GFR, glomerular filtration rate; 1,25(OH)<sub>2</sub>D, 1,25-dihydroxyvitamin D; PTH, parathyroid hormone; 25(OH)D, 25-hydroxyvitamin D; 24,25(OH)<sub>2</sub>D, 24,25-dihydroxyvitamin D. Values are mean ± SD.

<sup>a</sup> Blood volume (mL) = weight (kg) × 65 mL if nonpregnant; weight (kg) × 70 mL if pregnant.

\* Different significantly from nonpregnant,  $P < 0.05$ .

<sup>b</sup> Plasma volume (mL) = (height (cm) × B1) + (weight (kg) × B2) + A. Nonpregnant: B1 = 20.2, B2 = 14.9, and A = -1793; ~20 weeks since last menstrual period: B1 = 27.9, B2 = 1.7, and A = -1557; ~28 weeks since last menstrual period: B1 = 25.6, B2 = 5.8, and A = -1040.

<sup>c</sup> Values for laboratory indicators are the means of all available time points.

<sup>d</sup> Results from URMC clinical laboratory.

<sup>e</sup> Molar ratio.

<sup>f</sup> Average of two 24-h dietary recalls. Includes calcium from prenatal supplement.

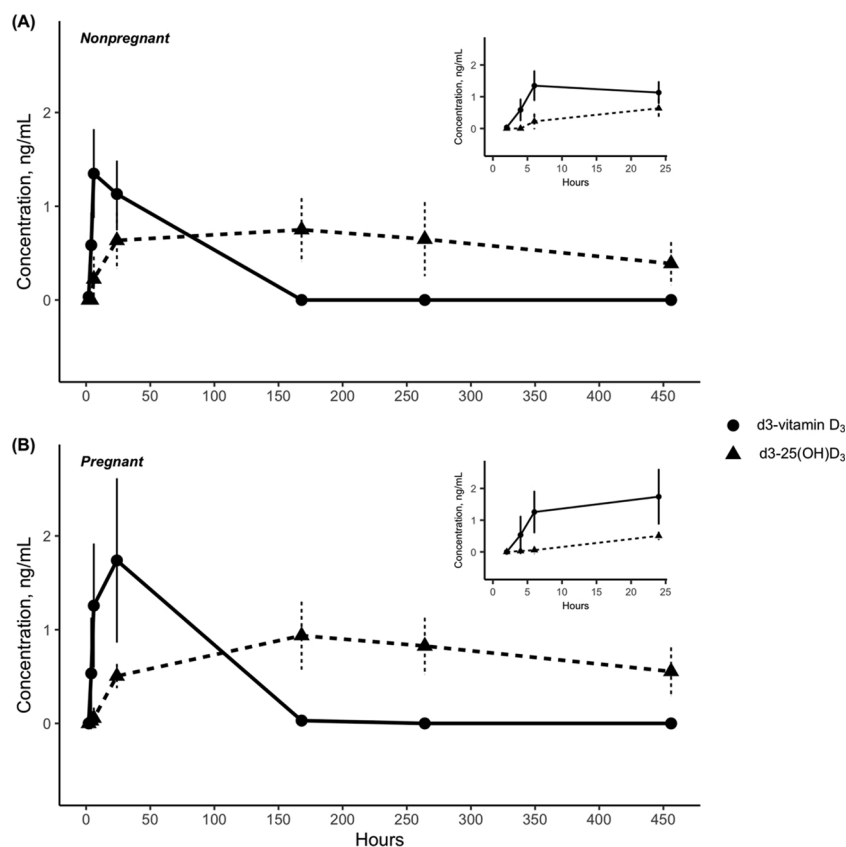
### 3.2. Serum kinetic profiles of d3-vitamin D<sub>3</sub> and d3-25(OH)D<sub>3</sub>

#### 3.2.1. Time course of serum kinetic profiles

Fig. 2 shows the mean ± SD blood volume (BV) corrected concentrations of d3-D<sub>3</sub> and d3-25(OH)D<sub>3</sub> over time in each reproductive group, and Fig. 3 shows the serum kinetic profiles for each participant. In 70 % of participants ( $n = 7$ ), d3-D<sub>3</sub> was first detected at 4 h post-dosing. It was evident in the 2 h sample in two nonpregnant participants and not observed until 6 h in one pregnant participant. The observed  $t_{max}$  of d3-D<sub>3</sub> occurred at either 6 h or 24 h post-dosing with no apparent relationship between  $t_{max}$  and reproductive state. The d3-D<sub>3</sub> was no longer detectable by 168 h (D8) except in one pregnant participant in whom a small amount (above the LLOD but below the LLOQ) remained.

Trideuterated 25(OH)D<sub>3</sub> (d3-25(OH)D<sub>3</sub>) was first observed in the 6 h post-dosing sample in all nonpregnant participants. In contrast, it was first detected in the 24 h sample in all pregnant participants except one in whom it was detected earlier at the 4 h time point. The  $t_{max}$  of d3-25(OH)D<sub>3</sub> occurred at 168 h (D8) in all but 2 participants and did not appear to vary by reproductive state. In nonpregnant participant 6





**Fig. 2.** Mean  $\pm$  SD blood volume corrected serum concentrations of trideuterated vitamin D<sub>3</sub> (d3-vitamin D<sub>3</sub>) (circles with solid line) and d3-25(OH)D<sub>3</sub> (triangles with dashed line) in nonpregnant ( $n = 6$ ) (A) and pregnant ( $n = 4$ ) (B) women from 2 to 456 h after single oral dosing with 25  $\mu$ g of d3-vitamin D<sub>3</sub>. Insets show the magnified view of the concentrations between 2 and 24 h post-dosing.

(NP6), the  $t_{\max}$  of d3-25(OH)D<sub>3</sub> occurred at 264 h (D12), and, as a result, we had an especially limited view of d3-25(OH)D<sub>3</sub> elimination in this participant. We estimated the half-life with the same methods for all participants and, when relevant, conducted sensitivity data analysis excluding this participant. The d3-25(OH)D<sub>3</sub> was quantifiable ( $>0.07$  ng/mL) in the final post-dosing sample at 456 h (D20) in all participants.

### 3.2.2. Maximum concentrations observed ( $C_{\max}$ )

Correcting for blood volume (BV) or plasma volume (PV) magnified the differences in tracer concentrations between nonpregnant and pregnant women, with PV having the greatest effect. For this reason, BV corrected concentrations were considered the primary results. There was significant between-person variability in the BV corrected  $C_{\max}$  of d3-vitamin D<sub>3</sub>, which ranged from 1.03 to 2.98 ng/mL. The mean was 1.62 ng/mL with no significant difference between groups (Table 3). When corrected for plasma volume, this  $C_{\max}$  of d3-vitamin D<sub>3</sub> was statistically significantly higher in pregnant women (Table 3). The BV corrected  $C_{\max}$  of d3-25(OH)D<sub>3</sub> ranged from 0.374 to 1.29 ng/mL. The mean was 0.846 ng/mL, and there was no significant difference between group means regardless of whether concentrations were uncorrected or corrected (Table 3).

### 3.2.3. Percent of dose recovered

The percent of dose recovered for the maximum observed combined concentration of d3-D<sub>3</sub> and d3-25(OH)D<sub>3</sub> was  $20.8 \pm 4.1$  for pregnant participants and  $29.0 \pm 8.5$  for nonpregnant participants (mean  $\pm$  SD). The percent of dose recovered ranged from 17.0 to 28.5 for pregnant participants and from 18.1 to 38.6 for nonpregnant participants. Calculation of the percent of dose recovered provides a low-end estimate of bioavailability but tends to underestimate bioavailability. The percent of dose recovered as vitamin D<sub>3</sub> ranged from 11.2 to 20.8 for pregnant

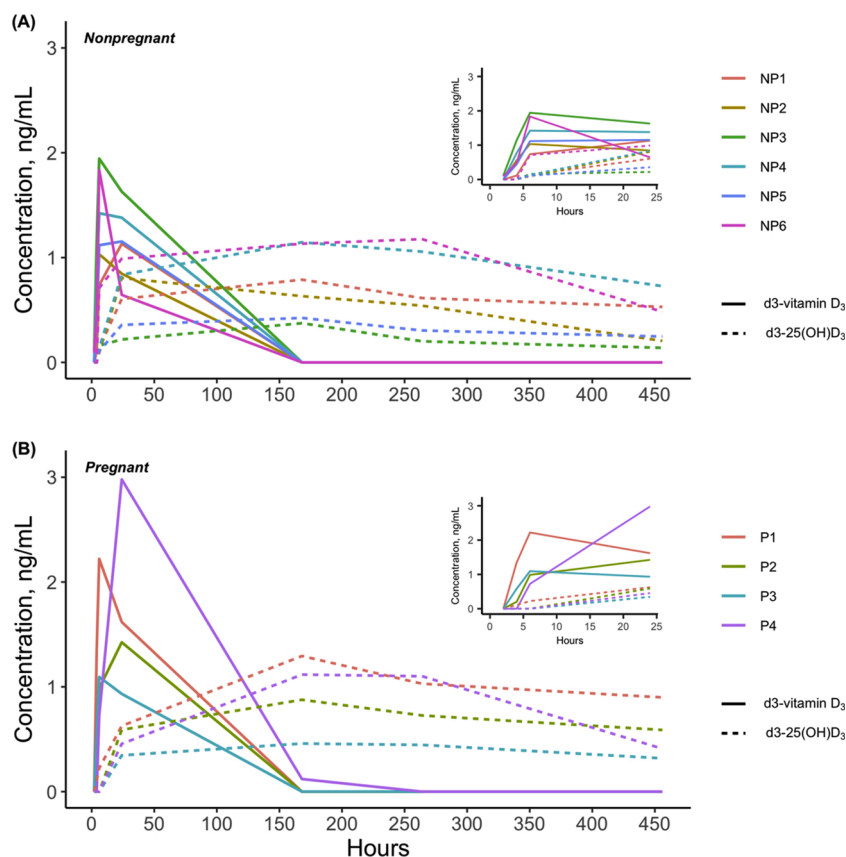
and 15.6 to 33.7 for nonpregnant, while the percent of dose recovered as 25(OH)D<sub>3</sub> ranged from 3.5 to 12.8 for pregnant and 6.3 to 16.0 for nonpregnant.

### 3.2.4. Area under the concentration-time curve (AUC)

The mean AUC of each analyte in all participants combined and by reproductive state is shown in Table 3. BV corrected  $AUC_{0-24h}$  of d3-vitamin D<sub>3</sub> ranged from 17.8 to 39.5 ng/mL $\cdot$ h. The mean was 26.7 ng/mL $\cdot$ h with no difference between groups. The BV corrected  $AUC_{0-456h}$  of d3-25(OH)D<sub>3</sub> was more variable than the AUC of d3-vitamin D<sub>3</sub>. It ranged from 107 to 443 ng/mL $\cdot$ h, with a mean of 295 ng/mL $\cdot$ h that did not differ between groups. Because a more complete serum concentration-time curve was obtained for d3-25(OH)D<sub>3</sub> (rather than d3-vitamin D<sub>3</sub>), we explored potential predictors of the  $AUC_{0-456h}$  of d3-25(OH)D<sub>3</sub>. This outcome was not different between groups, so all observations were combined to maximize statistical power. In simple linear regression models, the serum DBP concentration was the only statistically significant predictor of the  $AUC_{0-456h}$  of d3-25(OH)D<sub>3</sub>. Each 1 SD (123 mg/L) increase in serum DBP was associated with an 85 (95 % CI: 10–159) ng/mL $\cdot$ h greater  $AUC_{0-456h}$  of d3-25(OH)D<sub>3</sub> ( $R^2 = 0.39$ ).

### 3.2.5. Serum half-life of 25(OH)D<sub>3</sub>

Mean  $\pm$  SD serum half-life of 25(OH)D<sub>3</sub> was  $13.6 \pm 6.1$  days in nonpregnant women and  $18.8 \pm 7.5$  days in pregnant women ( $P$ -for-difference = 0.26). The statistically significant predictors of the 25(OH)D<sub>3</sub> half-life in simple linear regression models were total serum 25(OH)D concentration ( $\beta = 0.42$ ; SE = 0.17;  $P = 0.041$ ) and PTH concentration ( $\beta = -0.36$ ; SE = 0.15;  $P = 0.047$ ). When both were included in a multivariable linear regression model, only total 25(OH)D remained significant because these biomarkers were inversely correlated. These results were not materially different when nonpregnant participant 6



**Fig. 3.** Observed trajectories of blood volume corrected serum concentrations of trideuterated vitamin D<sub>3</sub> (d3-vitamin D<sub>3</sub>) (solid lines) and d3-25(OH)D<sub>3</sub> (dashed lines) in nonpregnant (A) and pregnant (B) women from 2 to 456 h after single oral dosing with 25 µg of d3-vitamin D<sub>3</sub>. Insets show the magnified view of the concentrations between 2 and 24 h post-dosing. NP, nonpregnant; P, pregnant.

**Table 3**  
Pharmacokinetic parameters of d3-vitamin D<sub>3</sub> and d3-25(OH)D<sub>3</sub>.

	All participants	Nonpregnant	Pregnant
<i>n</i>	10	6	4
<i>C</i> <sub>max</sub> d3-D <sub>3</sub> , ng/mL	1.61 ± 0.50	1.45 ± 0.38	1.85 ± 0.60
PV corrected <i>C</i> <sub>max</sub> d3-D <sub>3</sub> , ng/mL	1.63 ± 0.63	1.31 ± 0.33	2.12 ± 0.70*
BV corrected <i>C</i> <sub>max</sub> d3-D <sub>3</sub> , ng/mL	1.62 ± 0.63	1.42 ± 0.39	1.93 ± 0.85
d3-D <sub>3</sub> AUC <sub>0–24h</sub> , ng/mL•h	26.7 ± 6.8	25.4 ± 6.3	28.7 ± 7.9
PV corrected d3-D <sub>3</sub> AUC <sub>0–24h</sub> , ng/mL•h	26.8 ± 7.9	23.0 ± 5.4	32.5 ± 8.2
BV corrected d3-D <sub>3</sub> AUC <sub>0–24h</sub> , ng/mL•h	26.7 ± 7.7	24.9 ± 6.9	29.3 ± 9.0
<i>C</i> <sub>max</sub> d3-25(OH)D <sub>3</sub> , ng/mL	0.844 ± 0.32	0.800 ± 0.34	0.910 ± 0.32
PV corrected <i>C</i> <sub>max</sub> d3-25(OH)D <sub>3</sub> , ng/mL	0.850 ± 0.35	0.727 ± 0.31	1.04 ± 0.36
BV corrected <i>C</i> <sub>max</sub> d3-25(OH)D <sub>3</sub> , ng/mL	0.846 ± 0.34	0.785 ± 0.35	0.938 ± 0.36
d3-25(OH)D <sub>3</sub> AUC <sub>0–456h</sub> , ng/mL•h	295 ± 120	279 ± 139	318 ± 99.8
PV corrected d3-25(OH)D <sub>3</sub> AUC <sub>0–456h</sub> , ng/mL•h	297 ± 127	254 ± 126	362 ± 111
BV corrected d3-25(OH)D <sub>3</sub> AUC <sub>0–456h</sub> , ng/mL•h	295 ± 125	274 ± 139	326 ± 112
25(OH)D <sub>3</sub> serum half-life, days	15.7 ± 6.8	13.6 ± 6.1	18.8 ± 7.5

Abbreviations: AUC, area under the concentration-time curve; BV, blood volume; *C*<sub>max</sub>, maximum concentration observed; d3-D<sub>3</sub>, trideuterated vitamin D<sub>3</sub>; d3-25(OH)D<sub>3</sub>, trideuterated 25-hydroxyvitamin D<sub>3</sub>; PV, plasma volume. Values are mean ± SD. \* Different from nonpregnant, *P* < 0.05.

was excluded from analyses.

#### 4. Discussion

This pilot study demonstrated that a single oral dose of 25 µg (1000 IU) of trideuterated vitamin D<sub>3</sub> (d3-vitamin D<sub>3</sub>) can be used to assess the serum appearance and disappearance of both d3-vitamin D<sub>3</sub> and d3-25(OH)D<sub>3</sub> in nonpregnant and pregnant women. The serum kinetic profiles of d3-vitamin D<sub>3</sub> and d3-25(OH)D<sub>3</sub> followed a time course consistent with previous pharmacokinetic studies. There was marked variability between participants in the AUC of d3-25(OH)D<sub>3</sub> over the 20-day study period. The primary predictor of this AUC of d3-25(OH)D<sub>3</sub> was the serum DBP concentration, which was higher in pregnant compared with nonpregnant women. The mean serum half-life of 25(OH)D<sub>3</sub> was longer but not significantly different in the pregnant group.

The analytical methods developed for this pilot study provided capacity to use a relatively small, 25 µg oral dose of trideuterated vitamin D<sub>3</sub>. We know of one other published method that used liquid-liquid extraction and LC-MS/MS to simultaneously quantify vitamin D<sub>3</sub> and 25(OH)D<sub>3</sub> in serum, and the reported lower limit of quantitation was 2 ng/mL for vitamin D<sub>3</sub> and 1 ng/mL for 25(OH)D<sub>3</sub> [22]. Dimitris et al. adapted this method and reported a lower limit of quantitation of 1.0 ng/mL for vitamin D<sub>3</sub> [23]. Based on initial evaluation, our method has high analytical sensitivity. The lower limit of quantitation was 5.0 pg (0.2 ng/mL) for d3-vitamin D<sub>3</sub> and 1.7 pg (0.07 ng/mL) for d3-25(OH)D<sub>3</sub>.

The *t*<sub>max</sub> of d3-vitamin D<sub>3</sub> occurred at 6 h or 24 h post-dosing and seemed to be unrelated to reproductive state. While there was significant between-person variability in the *C*<sub>max</sub> of d3-vitamin D<sub>3</sub>, this should be interpreted with caution, as it may indicate variability in the rate rather

than efficiency of absorption. Several previous studies of a single oral dose of vitamin D<sub>3</sub> reported the  $t_{\max}$  of vitamin D<sub>3</sub> was between 6 and 16 h, regardless of the amount of radiotracer [6,9,10,24] or supplement [11,14] administered. Thus, it is likely that, due to the blood sampling protocol, we did not observe the true  $C_{\max}$  of d3-vitamin D<sub>3</sub> in many participants. To ensure observation of the  $C_{\max}$  of d3-vitamin D<sub>3</sub> in future research will require frequent serial blood sampling during a dosing visit with a duration of longer than 12 h.

In these pregnant and nonpregnant women, d3-25(OH)D<sub>3</sub> peaked in the Day 8 sample following consumption of d3-vitamin D<sub>3</sub>, and this  $t_{\max}$  is consistent with results from previous pharmacokinetic studies of oral vitamin D in both nonpregnant [12,13,18] and pregnant individuals [18]. Upon administering a single oral dose of 70,000 IU of vitamin D<sub>3</sub>, Roth and colleagues observed subtle differences by reproductive state in the serum 25(OH)D response [18]. Serum 25(OH)D concentration rose more rapidly and peaked slightly earlier in nonpregnant relative to pregnant women. Likewise, in our study, the d3-25(OH)D<sub>3</sub> first appeared at 6 h post-dosing in all nonpregnant participants but was only detected in 1 pregnant participant at that early time point. This may indicate that delivery of vitamin D<sub>3</sub> to the liver is slower in pregnant women, perhaps due to higher serum DBP concentration during pregnancy. Note that estrogen-containing contraception is associated with modestly elevated serum DBP concentration [25,26], and 3 nonpregnant participants were using hormonal birth control pills [combination estrogen-progestin ( $n = 2$ ) and progestin only ( $n = 1$ )]. This may have attenuated differences in the pharmacokinetic parameters between the reproductive groups.

The maximum percent of dose recovered in serum at any time point ranged from 17–39 %, when accounting for both d3-vitamin D<sub>3</sub> and d3-25(OH)D<sub>3</sub>. This range is in good accord with the findings of Davies et al. [9]. After administering a <sup>14</sup>C-labeled dose of vitamin D<sub>3</sub> orally, these authors recovered approximately 3–5.4 % of the dose per liter of serum for healthy control subjects, which would be equivalent to 9–25 % of dose if corrected for total serum volume. The percent of the dose recovered is an underestimation that can provide a low-end estimate of bioavailability. Davies et al. determined net absorption of the <sup>14</sup>C-vitamin D<sub>3</sub> dose by recovering radioactivity in the feces to find that net absorption ranged from 70–99 % of the dose, which is much higher than the serum recovery of 9–25 % of dose. This contrast between the serum recovery and actual net absorption values based on fecal recovery illustrates the tendency for serum or plasma peak values to underestimate bioavailability.

The  $AUC_{0-456h}$  of d3-25(OH)D<sub>3</sub> was highly variable between individuals and was primarily predicted by the serum DBP concentration. Higher DBP concentration was associated with greater AUC of d3-25(OH)D<sub>3</sub>, and there was also a positive association between DBP and the  $AUC_{0-24h}$  of d3-vitamin D<sub>3</sub> that approached significance (data not shown). These findings align with the known role of DBP in maintaining a circulating reservoir of vitamin D and its metabolites. DBP prevents plasma loss of vitamin D and 25(OH)D due to metabolism and excretion. The first study of DBP-null mice showed that relative to *WT*, mice lacking DBP experienced more rapid yet less efficient hepatic clearance of intravenous radiolabeled vitamin D<sub>3</sub> [27]. Serum DBP concentration would be expected to influence the kinetics of not only injected but also dietary vitamin D<sub>3</sub>, as a large portion of vitamin D<sub>3</sub> that enters the circulation on chylomicrons is transferred to DBP prior to reaching the liver [5,28]. The serum half-life of intravenous radiolabeled 25(OH)D<sub>3</sub> also was significantly shorter in the DBP-nulls. Overall, our results indicate that higher DBP concentration is associated with increased total plasma exposure to 25(OH)D, a finding that corroborates vitamin D metabolic studies in animals and supports the validity of our stable isotope method for investigating vitamin D metabolism in humans.

We estimated the serum half-life of 25(OH)D<sub>3</sub>, and the mean half-life (overall and within reproductive group) fell within the expected range of 2–3 weeks [29,30]. The 25(OH)D<sub>3</sub> half-life was longer but not significantly different in pregnant women, which corroborates the one prior

study of this outcome during pregnancy [2]. However, the difference we observed between reproductive groups disappeared after adjustment for total serum 25(OH)D concentration, which tended to be higher in the pregnant women who were taking prenatal supplements containing vitamin D<sub>3</sub>. In our study, 25(OH)D<sub>3</sub> half-life was positively associated with total serum 25(OH)D, and this association has been observed in some [2,3,31] but not all [30,32] previous studies. Disagreement in the literature may be due to methodology, including study duration. For example, in our study, it would have been preferable to have more data points to confidently define the terminal slope of disappearance in each participant. Thus, future studies should follow the labeled 25(OH)D for longer than 19 days to allow for accurate estimation of the terminal half-life. Our pilot data suggest this will be feasible when using a 25 µg oral dose of trideuterated vitamin D<sub>3</sub>. In addition, a larger study with sufficient power is required. Based on our current results, we calculate that a sample size of 28 women in each group will be required to detect a statistically significant difference in 25(OH)D<sub>3</sub> half-life between pregnant and nonpregnant women. To better match the groups according to vitamin D exposure, future studies may consider enrolling nonpregnant participants who are consuming a low dose vitamin D<sub>3</sub> supplement or embedding a tracer study within a controlled feeding study. Future studies should also consider conducting all individual pharmacokinetic studies during the months when sun exposure is minimal.

Emerging data indicate that pregnancy is a state of reduced 25(OH)D catabolism [2,33,34]. Serum 25(OH)D is catabolized by the 24-hydroxylase enzyme to 24,25(OH)<sub>2</sub>D, and we detected a statistically significantly lower molar ratio of 24,25(OH)<sub>2</sub>D<sub>3</sub> to 25(OH)D<sub>3</sub> in pregnant compared with nonpregnant women. Two recent studies and one earlier study also observed that serum 24,25(OH)<sub>2</sub>D concentration is low in the pregnant compared with the nonpregnant state [2,33,35]. Reduced 25(OH)D catabolism during pregnancy may help to meet the increased demand for 1,25(OH)<sub>2</sub>D and calcium and may explain why the 25(OH)D half-life is either longer or not different during pregnancy despite increased 1,25(OH)<sub>2</sub>D concentration throughout most of gestation. In addition to the possible buffering effect of elevated DBP, reduced 25(OH)D catabolism during pregnancy may stem from suppression of the 24-hydroxylase by feedback regulation or reproductive hormones such as PTH-related peptide and estrogen [36].

The conclusions that can be drawn from our pilot study have limitations, many of which relate to the blood sampling protocol. However, one of the objectives of the pilot to determine an optimal protocol for larger follow up studies was achieved. Future studies of a single oral dose of labeled vitamin D should collect blood several times during the first day and the first week post-dosing and follow labeled 25(OH)D for longer than 19 days to obtain complete serum concentration-time curves and terminal slopes for vitamin D and 25(OH)D. In addition, a future study should more comprehensively evaluate the sensitivity and precision of the mass spectrometry assay developed for this pilot. Whereas an oral dose of labeled vitamin D enables evaluation of vitamin D turnover, a potential limitation of this design is that slow release of labeled vitamin D from extravascular storage depots during the study period might contribute to the labeled 25(OH)D pool and distort the estimate of the 25(OH)D half-life. However, we detected no d3-vitamin D<sub>3</sub> in the serum between days 8 and 20, which suggests that minimal labeled vitamin D<sub>3</sub> was released from extrahepatic storage during the study period. Regarding hepatic storage, autopsy studies indicate that vitamin D does not reside for long in the liver, and only ~1 % of a dose of radiolabeled vitamin D remains in the liver after 1 week [37].

#### 4.1. Conclusions

To summarize, this pilot study demonstrated the feasibility of tracing vitamin D<sub>3</sub> turnover with a stable isotope, and our technique can be applied to learn more about vitamin D<sub>3</sub> metabolism across physiological conditions. Future research should include a more diverse study population and further investigate the influence of serum DBP concentration

and related variables on vitamin D kinetics in nonpregnant and pregnant women.

### Author Statement

**Cora Best:** Data curation, Formal analysis, Investigation, Methodology, Project administration, Visualization, Writing – original draft.

**Robert Sherwood:** Formal analysis, Investigation, Methodology, Validation, Writing – review & editing.

**Janet Novotny:** Formal analysis, Writing – review & editing.

**Sheng Zhang:** Methodology, Resources, Writing – review & editing.

**Eva Pressman:** Conceptualization, Funding acquisition, Resources, Supervision, Writing – review & editing.

**Kimberly O'Brien:** Conceptualization, Funding acquisition, Methodology, Resources, Supervision, Writing – original draft.

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### Declaration of Competing Interest

None.

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