Associations Between Change in Total and Free 25-Hydroxyvitamin D With 24,25-Dihydroxyvitamin D and Parathyroid Hormone

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Context: The physiologic role of free 25-hydroxyvitamin D [25(OH)D] in humans is unclear.

Objective: To assess whether rise in total vs free 25(OH)D is associated with change in downstream biomarkers of 25(OH)D entry into target cells in kidney and parathyroid: 24,25-dihyroxyvitamin D [24,25$(OH)_2$D] and PTH, respectively.

Design: 16-week randomized controlled trial.

Intervention: 60 μg (2400 IU)/d of D3 or 20 μg/d of 25(OH)D3.

Setting: Academic medical center.

Participants: 35 adults age ≥18 years with 25(OH)D levels < 20 ng/mL.

Main Outcome Measures: 24,25$(OH)_2$D, 1,25-dihyroxyvitamin D [1,25$(OH)_2$D] and PTH.

Results: At baseline, participants [D3 and 25(OH)D3 groups combined] were 35.1 ± 10.6 years. Mean total 25(OH)D, free 25(OH)D, 24,25$(OH)_2$D, and PTH were 16.6 ng/mL, 4.6 pg/mL, 1.3 ng/mL, and 37.2 pg/mL, respectively. From 0 to 4 weeks, rise in only free 25(OH)D was associated with a concurrent 24,25$(OH)_2$D increase [$P = 0.03$, adjusted for change in 1,25$(OH)_2$D and supplementation regimen] and PTH decrease ($P = 0.01$, adjusted for change in calcium and supplementation regimen). Between 4 and 8 weeks, and again from 8 to 16 weeks, rises in free and total 25(OH)D were associated with 24,25$(OH)_2$D increase; in contrast, rise in neither total nor free 25(OH)D was associated with PTH decrease during these time periods.

Conclusions: Early rise in free 25(OH)D during treatment of vitamin D deficiency was more strongly associated with changes in biomarkers of 25(OH)D entry into target kidney and parathyroid cells, suggesting a physiologic role of free 25(OH)D in humans. (***J Clin Endocrinol Metab*** **103: 3368–3375, 2018)**

Clinicians frequently measure circulating 25-hydroxyvitamin D [25(OH)D] levels to assess vitamin D nutritional status (1). In the serum of healthy individuals, >99.9% of total 25(OH)D is bound to the vitamin D-binding protein (DBP, ~85%) or albumin (~15%); <0.1% occurs in its free, unbound form (2). Controversy persists over the physiologic role of total vs free 25(OH)D in humans.

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Abbreviations: 1,25$(OH)_2$D, 1,25-dihyroxyvitamin D; 24,25$(OH)_2$ D, 24,25-dihyroxyvitamin D; 25(OH)D, 25-hydroxyvitamin D; 25(OH)D3, calcifediol; CV, coefficient of variation; D3, cholecalciferol; DBP, vitamin D-binding protein; UCLA, University of California, Los Angeles; VDR, vitamin D receptor.

The active vitamin D metabolite, 1,25-dihydroxyvitamin D [1,25$(OH)_2$D], is produced through 1-hydoxylation of 25(OH)D within a target cell that expresses the 1-α-hydroxylase, CYP27B1. Although 1,25$(OH)_2$D can be produced by many cell types (3), the classic cell type is the renal epithelial cell. Within the kidney, 25(OH)D is internalized by the renal epithelial cell (4), where it is converted to 1,25$(OH)_2$D by the CYP27B1 (4) before re-entering the general circulation and acting in an endocrine fashion at vitamin D receptor (VDR)-possessing tissues (*e.g.,* intestine and bone) to maintain organismal calcium balance (5). Another tissue that can produce 1,25$(OH)_2$D via the CYP27B1 is the parathyroid; 25(OH)D is internalized from the extracellular space by the parathyroid cell and converted to 1,25$(OH)_2$D. 1,25$(OH)_2$D then acts in a VDR-directed intracrine mode to suppress PTH production and secretion (6–10). Prior studies in mice null for the serum DBP gene ($DBP^{-/-}$) suggest that both DBP-bound and free 25(OH)D can enter kidney and parathyroid cells, but whether this is the case in humans remains unclear (6).

The objective of this study was therefore to gain insight into the relative roles of DBP-bound vs free 25(OH)D in kidney and parathyroid cell function in humans. To operationalize this objective, we assessed whether intraindividual increases in total vs free 25(OH)D during aggressive vitamin D supplementation were associated with changes in downstream biomarkers of 25(OH)D entry into kidney and parathyroid cells. We used 24,25-dihydroxyvitamin D [24,25$(OH)_2$D] as our downstream biomarker of 25(OH)D entry into the renal epithelial cell owing to the fact that conversion of 25(OH)D to 24,25$(OH)_2$D by the 24-hydroxylase (CYP24A1) is the most robust indicator of 1,25$(OH)_2$D-VDR–directed action of 1,25$(OH)_2$D (8, 11). Here, we assume that most circulating 24,25$(OH)_2$D is produced by the kidney because (1) most circulating 1,25$(OH)_2$D is produced in renal epithelial cells (6–8) and (2) circulating 24,25$(OH)_2$D concentrations decrease with worsening renal function in CKD (9). For our downstream biomarker of 25(OH)D entry into the parathyroid cells, we used changes in the serum level PTH; a major means by which PTH production and excretion is squelched is via 25(OH)D entry into the parathyroid cell, its subsequent conversion to 1,25$(OH)_2$D, and 1,25$(OH)_2$D-VDR–mediated suppression of *PTH* expression (10, 12–15).

We previously randomly assigned 35 adults with total 25(OH)D levels $<$ 20 ng/mL to receive cholecalciferol [D3, 60 μg (2400 IU)/d] or calcifediol [25(OH)D3, 20 μg/d] for 16 weeks (16). Serum was collected at the baseline and 4-, 8-, and 16-week follow-up visits. As part of the parent study, we obtained repeated measured of total 25(OH)D (using an antibody-based assay), free 25(OH)D, and PTH (16). For this current study, we reassayed total 25(OH)D using liquid chromatography-tandem mass spectrometry and additionally measured 24,25$(OH)_2$D from stored serum. We then assessed the associations between change in total vs free 25(OH)D with dynamic changes in serum 24,25$(OH)_2$D and PTH during repletion with D3 or 25(OH)D3.

Materials and Methods

Study participants

We previously randomized 35 individuals to receive vitamin D3 [60 μg (2400 IU)/d] or 25-hydroxyvitamin D3 (20 μg/d) for 16 weeks (16). Participants were assessed at baseline, 4-, 8-, and 16-weeks follow up visits. Participants were recruited from the University of California, Los Angeles (UCLA), student body, staff, and patient population. Volunteers were eligible to participate in the study if they were ≥18 years of age; had a baseline 25(OH)D level $<$ 20 ng/mL; and did not have a history of hypercalcemia, hypercalciuria, nephrolithiasis, intestinal malabsorption, or dysregulated vitamin D metabolism. Participants agreed to maintain their typical dietary calcium intake and did not take self-prescribed calcium or vitamin D supplements during the study (16). All participants provided informed consent. The UCLA Institutional Review Board approved the study. The ClinicalTrials.gov identifier was NCT02091219.

Measurements

At each of the four study visits, serum was collected, and unused specimens were stored at −80°C. Relevant to this current study, we previously measured free 25(OH)D and intact PTH. Free 25(OH)D was assayed by using an antibody-based method from Future Diagnostics as previously described. The assay limit of detection is 1.9 pg/ml. In the range of concentrations measured, the coefficient of variation (CV) was ≤7%. PTH was measured by electrochemiluminescence immunoassay (Roche Cobas). Intra-assay and interassay CV were 0.8% to 1.5% and 1.5% to 1.8%, respectively. In addition to the previously measured analytes, we also assayed total 25(OH)D, 1,25$(OH)_2$D, and 24,25$(OH)_2$D from previously frozen serum by using liquid chromatography/tandem mass spectrometry (Heartland Assays). For total 25(OH)D, intra-assay and interassay CVs were 4.0% and 5.0%, respectively. For 1,25$(OH)_2$D, intra-assay and interassay CVs were 4.0% and 5.0%, respectively. For 24,25$(OH)_2$D, intra-assay and interassay CVs were 3.0% and 6.0%, respectively.

Statistical analyses

For this study, our goal was not to determine whether D3 vs 25(OH)D3 is a superior approach for vitamin D repletion. Rather, we aimed to gain insight into the relative importance of total vs free 25(OH)D in human physiology. We operationalized this objective by assessing how within-individual increases in total and free 25(OH)D during aggressive repletion [whether by D3 or 25(OH)D3] relate to changes in 24,25$(OH)_2$D and PTH during the same time period. We therefore analyzed our vitamin D3-treated and 25(OH)D3-treated participants as a single cohort to maximize statistical power and adjusted for supplementation regimen in all analyses.

We generated descriptive statistics of continuous clinical covariates and biochemical measurements and confirmed their

normal distributions. Within-individual changes in each biochemical measurement between successive study visits (*i.e.*, from baseline to 4 weeks, from 4 weeks to 8 weeks, and from 8 weeks to 16 weeks) were examined by the paired *t* test. Associations between intraindividual changes in total or free 25(OH)D between successive visits (primary predictor in separate analyses) and change in 24,25(OH)$_2$D (outcome variable) during the same time period were assessed by using multivariable linear regression. Covariates included coincident change in 1,25(OH)$_2$D and supplementation regimen. Associations between intraindividual changes in total or free 25(OH)D between successive visits (primary predictor in separate analyses) and change in PTH (outcome variable) during the same time period were also assessed by using multivariable linear regression. Covariates included coincident change in calcium and supplementation regimen. To test whether supplementation regimen modified the associations between change in total or free 25(OH)D with change in 24,25(OH) $_2$D or PTH, we tested for interactions between supplementation regimen and change in total or free 25(OH)D. All analyses were performed using STATA, version 14 (StataCorp, College Station, TX).

Results

Participant characteristics

A total of 35 participants were included in this analysis; 16 and 19 received D3 and 25(OH)D3, respectively. Mean age of the entire cohort was 35.1 ± 10.6 years. Mean total and free 25(OH)D at baseline were 16.6 ± 3.0 ng/mL and 4.6 ± 1.0 pg/mL, respectively. Baseline 24,25(OH) $_2$D and PTH were 1.3 ± 0.5 ng/mL and 37.2 ± 16.3 pg/mL respectively (Table 1).

Effects of D3 or 25(OH)D3 on serum vitamin D metabolites

Figures 1 and 2, and Table 2 show within-individual changes between successive study visits in total and free 25(OH)D, 24,25(OH)$_2$D, and 1,25(OH) $_2$D after supplementation with D3 or 25(OH)D3. Overall, during the 16-week study period, supplementation with D3 or 25(OH)D3 (all participants pooled) increased total 25(OH)D by 22.7 ± 13.6 ng/mL ($P < 0.0001$), free 25(OH)D by 5.2 ± 4.4 pg/mL ($P < 0.0001$), and 24,25(OH)$_2$D by 2.9 ± 2.1 ng/mL ($P < 0.0001$). Total 25(OH)D, free 25(OH)D, and 24,25(OH)$_2$D increased most rapidly during the first 8 weeks of supplementation regardless of regimen and plateaued after 8 weeks (Figs. 1 and 2; Table 2). 1,25(OH)$_2$D did not change significantly during the study period. The ratio of increase in 24,25(OH)$_2$D to concurrent increase in total or free 25(OH)D was greater than the ratio of increase in 1,25(OH)$_2$D to concurrent increase in total or free 25(OH)D during all study periods (0 to 4 weeks, 4 to 8 weeks, and 8 to 16 weeks).

Table 1. Baseline Characteristics

Characteristic	Value
Age, y	35.1 ± 10.6
Race/ethnicity, n (%)	
White	5 (14.2)
African American	11 (31.4)
Asian American	12 (34.3)
Hispanic/Latino	7 (20.0)
BMI, kg/m^2	26.6 ± 6.8
Total 25(OH)D, ng/mL	16.6 ± 3.0
Free 25(OH)D, pg/mL	4.6 ± 1.0
1,25(OH)$_2$D, pg/mL	55.6 ± 16.3
24,25(OH)$_2$D, ng/mL	1.3 ± 0.5
Calcium, mg/dL	9.5 ± 0.4
Urinary calcium:creatinine ratio	0.06 ± 0.04
PTH, pg/mL	37.2 ± 16.3

Data are presented as mean ± SD unless otherwise indicated.

Association between total vs free 25(OH)D and 24,25(OH)$_2$D

From baseline to the 4-week follow-up visit, rise in free 25(OH)D (P = 0.03), but not total 24,25(OH)$_2$D (P = 0.3), was significantly associated with increase in 24,25(OH)2D. Between weeks 4 and 8, rise in both total (P = 0.01) and free ($P < 0.001$) 25(OH)D were associated with increase in 24,25(OH)$_2$D, but the association was stronger with free 25(OH)D. Finally, from 8 to 16 weeks, rise in total 25(OH)D was more strongly associated with increase in 24,25(OH) $_2$D (P = 0.007) than rise in free 25(OH)D (P = 0.05). All analyses were adjusted for change in 1,25(OH)$_2$D and supplementation regimen (Table 3). There were no significant interactions between supplementation regimen and change in total or free 25(OH)D, suggesting that supplementation regimen did not modify the associations between rises in total or free 25(OH)D with increase in 24,25(OH)$_2$D.

Associations between total vs free 25(OH)D and PTH

From baseline to the 4-week follow-up visit, rise in free 25(OH)D (P = 0.01), but not total 25(OH)D (P = 0.5), was associated with a decrease in PTH. Between weeks 4 and 8, and then again from weeks 8 to 16, rises in neither total nor free 25(OH)D were related to decline in PTH ($P >$ 0.5 for all). All analyses were adjusted for change in serum calcium and supplementation regimen (Table 4). In addition, supplementation regimen did not modify the associations between rises in total or free 25(OH)D with decrease in PTH.

Adherence

Adherence, assessed by pill count, was 91.0% for the entire cohort after 16 weeks of supplementation.

Safety

Hypercalcemia, hypercalciuria, or nephrolithiasis did not occur during the study. Serum and urinary

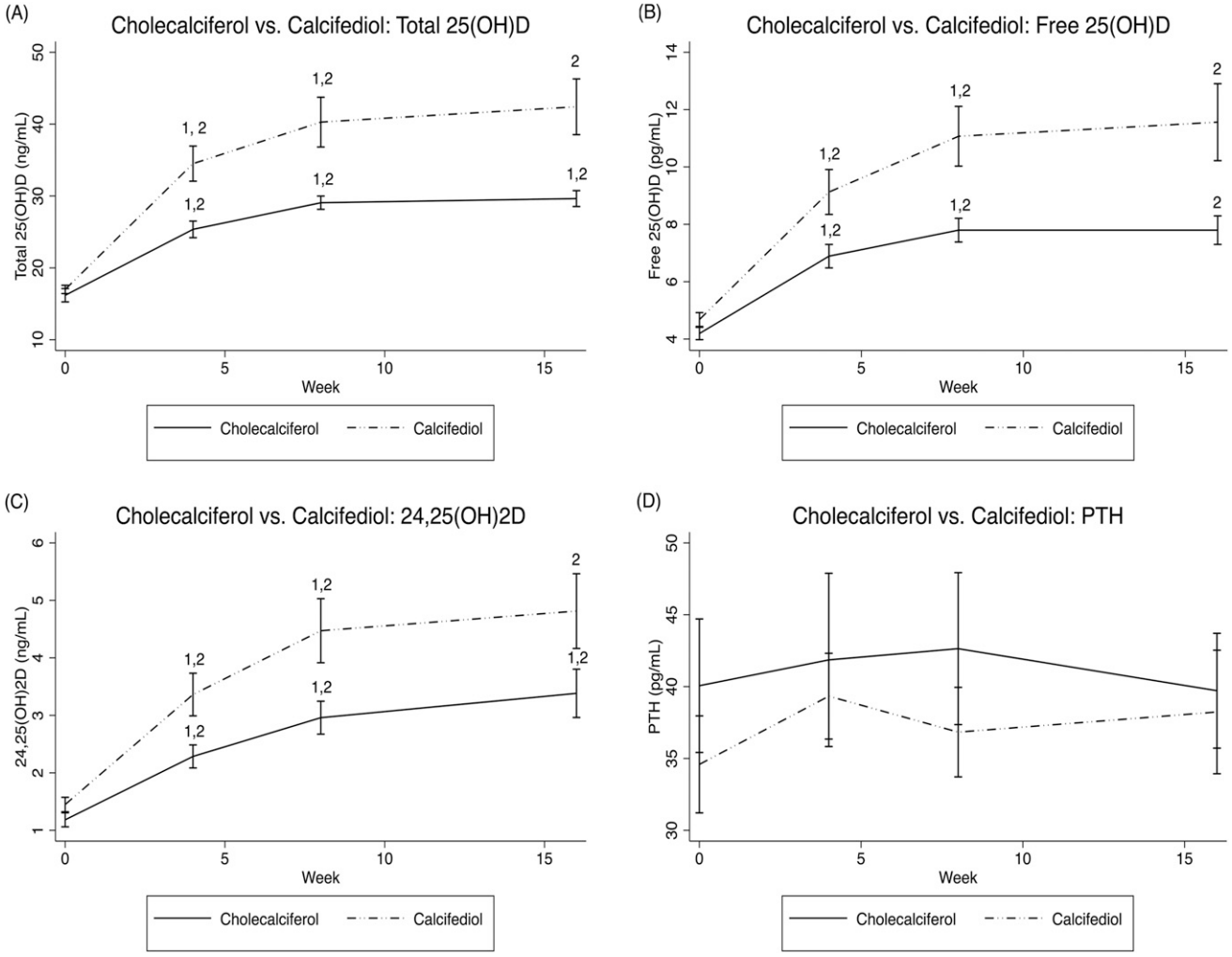

Figure 1. Change in vitamin D metabolites and PTH with cholecalciferol (vitamin D3) or calcifediol (25-hydroxyvitamin D3). (A) Total 25(OH)D. (B) Free 25(OH)D. (C) 24,25$(OH)_2$D. (D) PTH. 1, $P < 0.05$ for within-group change from prior visit; 2, $P < 0.05$ for between-group differences at specified time point.

calcium did not change significantly with D3 or 25(OH)D3.

Discussion

The aim of this study was to gain insight into whether free 25(OH)D may have a physiologic role in humans. We operationalized this objective by examining the associations between increases in total vs free 25(OH)D during 16 weeks of aggressive vitamin D repletion with concurrent change in downstream biomarkers of 25(OH)D entry into kidney [24,25$(OH)_2$D] and parathyroid (PTH) cells. We found that, after we accounted for supplementation regimen, increase in free but not total 25(OH)D was significantly associated with increase in 24,25$(OH)_2$D and decrease in PTH, early in the repletion course (baseline to 4 weeks) when 25(OH)D levels increase most rapidly. Between 4 and 16 weeks, the association between increase in total 25(OH)D and increase in 24,25$(OH)_2$D strengthened, whereas the opposite was true for free 25(OH)D. For PTH during this same time period, rise in neither total nor free 25(OH)D was related to decline in PTH. These findings suggest that when total and free 25(OH)D levels change rapidly during the early course of vitamin D repletion, entry of free 25(OH)D into target kidney and parathyroid cells may be physiologically relevant.

The physiologic importance of free 25(OH)D is unknown; some investigators suggest that free 25(OH)D levels in the range observed in human studies are too small to carry out any biological function (2, 17). Previously published studies have evaluated the associations between total vs free 25(OH)D with various biomarkers of vitamin D bioactivity, including PTH, bone turnover markers, bone mineral density, and immune cell function (18–26). Some studies report stronger associations between free or bioavailable 25(OH)D and these measures (18, 19, 23, 26), whereas others do not (20, 21, 24, 25). There are limited data on whether dynamic, within-individual changes in total vs free 25(OH)D are more

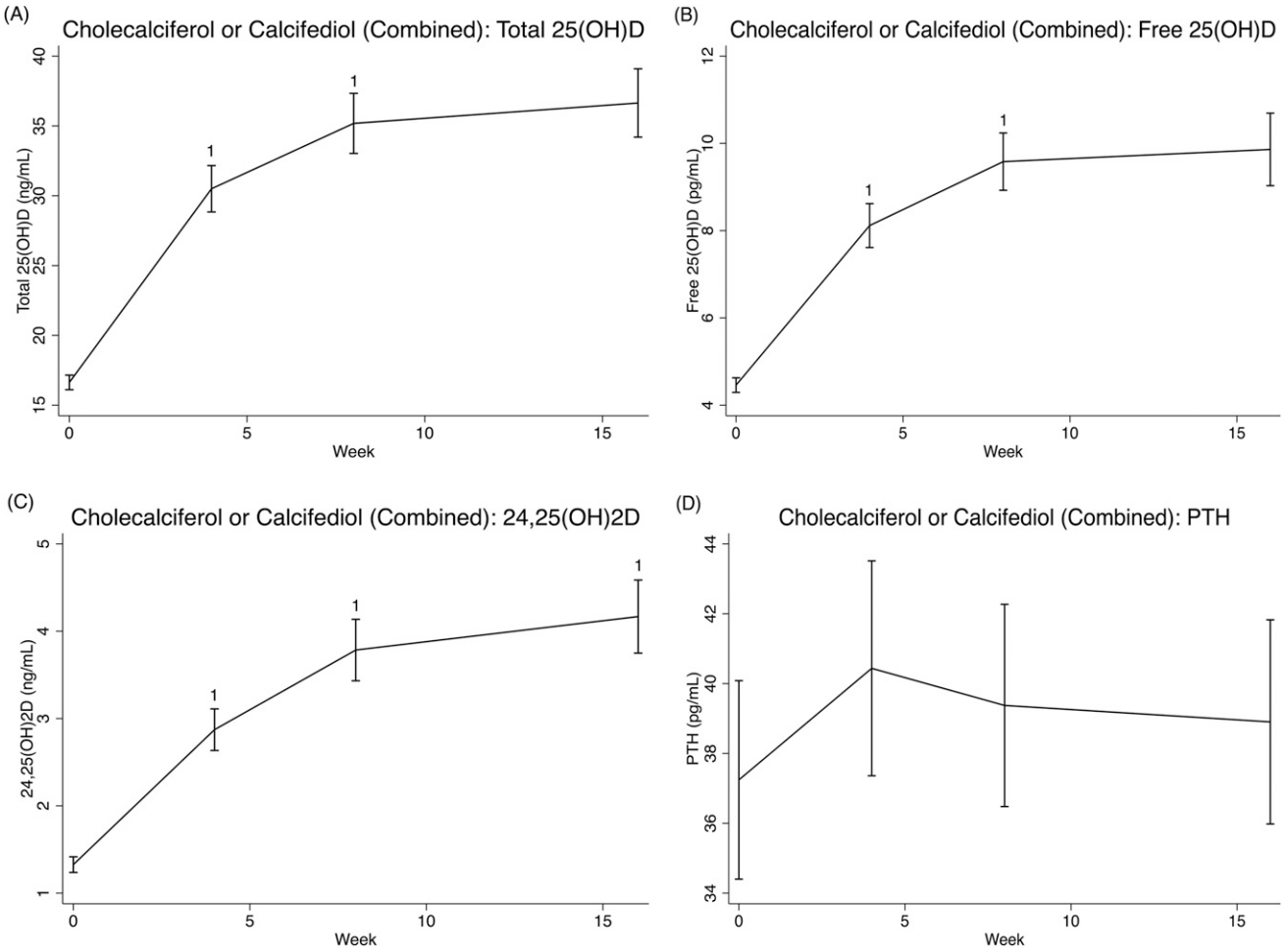

Figure 2. Change in vitamin D metabolites and PTH with cholecalciferol (vitamin D3) and calcifediol (25-hydroxyvitamin D3) (both groups combined). (A) Total 25(OH)D. (B) Free 25(OH)D. (C) 24,25(OH)$_2$D. (D) PTH. 1, $P < 0.05$ for within-group change from prior visit.

strongly related to downstream biomarkers of 25(OH)D entry into kidney and parathyroid cells, specifically (16). We believe that determining the relative importance of DBP-bound vs free 25(OH)D is better answered by examining the associations between dynamic changes in downstream biomarkers of vitamin D bioactivity with change in total vs free 25(OH)D after provoking the human system with vitamin D supplementation.

Our key finding is that during the initial 4 weeks of supplementation with D3 or 25(OH)D3, only rise in free 25(OH)D was significantly associated with increase in 24,25(OH)$_2$D and decrease in PTH. We used circulating 24,25(OH)$_2$ as our downstream biomarker of 25(OH)D entry into the renal epithelial cell because conversion of 25(OH)D to 24,25(OH)$_2$D by the CYP24A1 is the most robust indicator of 1,25(OH)$_2$D-VDR–directed action of 1,25(OH)$_2$D. More specifically, after 25(OH)D enters the renal epithelial cell, it is converted to 1,25(OH)$_2$D. 1,25(OH)$_2$D then engages to VDR to induce expression of CYP24A1, which catabolizes 25(OH)D to 24,25(OH)$_2$D to prevent tissue level 1,25(OH)$_2$D toxicity (4, 8, 27). Here, we assume that most circulating 24,25(OH)$_2$D is produced by the kidney because (1) most circulating 1,25(OH)$_2$D is produced in renal epithelial cells (6–8) and (2) circulating 24,25(OH)$_2$D concentrations decrease with worsening renal function in chronic kidney disease (9). We used PTH as a biomarker of 25(OH)D entry into the parathyroid cell. Parathyroid cells express CYP27B1 and the VDR and thus possess the necessary machinery to locally covert 25(OH)D to 1,25(OH)$_2$D. One major mechanism by which PTH production and excretion are suppressed is via 25(OH)D entry into the parathyroid cell, its subsequent conversion 1,25(OH)$_2$D, and 1,25(OH)$_2$D-VDR–mediated suppression of *PTH* expression (13–15).

Our results suggest that early in the course of vitamin D repletion, when 25(OH)D levels rise rapidly, rapid movement of free 25(OH)D from the extracellular space into target kidney and parathyroid cells is physiologically relevant. These results were unexpected because kidney and parathyroid cells express megalin (2, 4, 12); one would therefore expect change in total 25(OH)D [which included DBP-bound 25(OH)D] to be more strongly associated with changes in 24,25(OH)$_2$D or PTH. One possible explanation is that production of 24,25(OH)$_2$D

Table 2. Absolute Change in Vitamin D Metabolites Between Successive Visits During 16 Weeks of Supplementation With Vitamin D3 or 25-Hydroxyvitamin D3

	D3		25(OH)D3		D3 + 25(OH)D3 Combined	
Variable	**Absolute Change**	***P* Value**	**Absolute Change**	***P* Value**	**Absolute Change**	***P* Value**
Baseline to 4-wk follow-up						
Total 25(OH)D, ng/mL	11.5 ± 6.6	<0.0001	20.4 ± 12.6	<0.0001	16.3 ± 11.1	<0.0001
Free 25(OH)D, pg/mL	2.5 ± 1.2	<0.0001	4.1 ± 3.0	<0.0001	3.3 ± 2.5	<0.0001
$1,25(OH)_2D$, pg/mL	6.9 ± 48.4	0.6	17.3 ± 21.6	0.08	12.1 ± 37.0	0.1
$24,25(OH)_2D$, ng/mL	1.1 ± 0.7	<0.0001	1.9 ± 1.4	<0.0001	1.6 ± 1.2	<0.0001
PTH, pg/mL	1.9 ± 13.4	0.6	1.3 ± 11.1	0.7	1.5 ± 12.0	0.5
4-wk follow- up to 8-wk follow-up						
Total 25(OH)D, ng/mL	3.3 ± 4.6	0.02	3.9 ± 9.8	0.1	3.6 ± 7.7	0.01
Free 25(OH)D, pg/mL	1.1 ± 0.6	<0.0001	1.9 ± 2.0	0.002	1.5 ± 1.5	<0.0001
$1,25(OH)_2D$, pg/mL	3.8 ± 14.1	0.4	−7.1 ± 18.8	0.2	−2.2 ± 17.4	0.6
$24,25(OH)_2D$, ng/mL	0.7 ± 0.7	0.002	1.0 ± 1.1	0.001	0.9 ± 0.9	<0.01
PTH, pg/ml	0.7 ± 15.6	0.9	−2.1 ± 14.5	0.6	−0.9 ± 14.8	0.8
8-wk follow-up to 16-wk follow-up						
Total 25(OH)D, ng/mL	3.1 ± 4.7	0.03	1.4 ± 8.8	0.5	2.2 ± 7.2	0.1
Free 25(OH)D, pg/mL	0.1 ± 0.7	0.7	0.5 ± 2.3	0.4	0.3 ± 1.8	0.4
$1,25(OH)_2D$, pg/mL	1.7 ± 23.8	0.8	4.9 ± 17.8	0.4	3.9 ± 19.3	0.4
$24,25(OH)_2D$, ng/mL	0.4 ± 0.7	0.04	0.3 ± 1.1	0.2	0.4 ± 0.9	0.04
PTH, pg/mL	−2.4 ± 16.1	0.6	1.2 ± 13.8	0.7	−0.3 ± 14.7	0.9

Data are expressed as mean ± SD unless otherwise indicated.

is more efficient in parts of the kidney (*e.g.*, the distal tubule) that do not express megalin (4). Another possible explanation is that free 25(OH)D is indeed physiologically relevant *in vivo*, even in megalin-expressing target tissues. This notion is supported by evidence that free 25(OH)D enters the monocyte (which also expresses megalin) to modulate VDR-directed expression of antimicrobial peptides (28, 29). This is also supported by animal data confirming that $DBP^{-/-}$ mice, in which megalin-mediated entry of 25(OH)D into target cells is not possible, do not develop osteomalacia or secondary hyperparathyroidism when placed on a vitamin D–replete diet (6). Our results are also consistent with recent human data showing that during high-dose supplementation with vitamin D3, increases in free, but not total, 25(OH)D during the first 7 to 14 days of supplementation were associated with increase in immune cell (*i.e.*, another extrarenal target cell) expression of antimicrobial peptides (26).

Between weeks 4 and 16, the associations between increase in total 25(OH)D and increase in $24,25(OH)_2D$ strengthened, whereas the opposite was true for free 25(OH)D. During this same time period, rise in neither total nor free 25(OH)D was related to decline in PTH. Our $24,25(OH)_2D$ findings suggest that once 25(OH)D levels begin to stabilize (*i.e.*, after week 4), entry of 25(OH)D from the extracellular space into target kidney cells may rely on more deliberate megalin-mediated uptake of DBP-bound 25(OH)D instead of rapid movement of free 25(OH)D. Our PTH findings suggest that after 25(OH)D levels begin

Table 3. Adjusted Associations Between Increase in Total vs Free 25(OH)D and Percentage Increase in $24,25(OH)_2D$ Between Successive Visits During 16 Weeks of Supplementation with Vitamin D3 or 25-Hydroxyvitamin D3

Variable	**Increase in $24,25(OH)_2D$ [β (95% CI)] per Unit Increase in Total vs Free 25(OH)D Between Successive Time Points, %**	***P* Value**
Baseline to 4-wk follow-up		
Total 25(OH)D, ng/mL	3.19 (−3.30 to 9.67)	0.3
Free 25(OH)D, pg/mL	32.1 (2.99–61.29)	0.03
4-wk follow-up to 8-wk follow-up		
Total 25(OH)D, ng/mL	1.92 (0.38–3.36)	0.01
Free 25(OH)D, pg/mL	12.08 (5.14–19.01)	0.001
8-wk follow-up to 16-wk follow-up		
Total 25(OH)D, ng/mL	2.59 (0.78–4.40)	0.007
Free 25(OH)D, pg/mL	8.36 (−0.32 to 17.04)	0.05

Associations are adjusted for change in $1,25(OH)_2D$ and supplementation regimen. The β coefficient should be interpreted as follows: For each unit increase in total or free 25(OH)D, $24,25(OH)_2D$ increases by "β" percent during the listed time interval.

Table 4. Adjusted Associations Between Increase in Total vs Free 25(OH)D and Percentage Decrease in PTH Between Successive Visits During 16 Weeks of Supplementation With Vitamin D3 or 25-Hydroxyvitamin D3

Variable	Increase in PTH [β (95% CI)] per Unit Increase in Total vs Free 25(OH)D Between Successive Time Points, %	*P* Value
Baseline to 4-wk follow-up		
Total 25(OH)D, ng/mL	1.11 (−2.24 to 4.45)	0.5
Free 25(OH)D, pg/mL	15.43 (3.79–27.05)	0.01
4-wk follow up to 8-wk follow-up		
Total 25(OH)D, ng/mL	0.80 (−1.25 to 1.42)	0.9
Free 25(OH)D, pg/mL	−1.30 (−7.35 to 4.75)	0.6
8-wk follow-up to 16-wk follow-up		
Total 25(OH)D, ng/mL	1.11 (−0.15 to 2.37)	0.1
Free 25(OH)D, pg/mL	3.08 (−1.22 to 7.37)	0.1

Associations are adjusted for change in serum calcium and supplementation regimen. The β coefficient should be interpreted as follows: For each unit increase in total or free 25(OH)D, PTH decreases by "β" percent during the listed time interval.

to plateau, entry of DBP-bound and free 25(OH)D is not physiologically significant, and PTH expression may be principally regulated by the calcium-sensing receptor (30).

The clinical implication of our findings is that early in the course of vitamin D repletion, entry of free 25(OH)D from the extracellular compartment into target kidney and parathyroid cells may be physiologically important. This suggests that a vitamin D supplementation regimen that more rapidly and robustly raises free 25(OH)D levels may be preferable. In this regard, we previously found that 25(OH)D3 is superior to a bioequivalent dose of D3 (16). In vitamin D-deficient individuals, it may therefore be reasonable to consider using 25(OH)D3 as an initial option, followed by maintenance with parent vitamin D (16). The fact that 1,25(OH)$_2$D levels did not increase significantly due to diversion of 1,25(OH)$_2$D down the CYP24A1 pathway suggests that this approach would not expose patients to higher risk for vitamin D toxicity.

Several weaknesses warrant mention. First, the study sample size was relatively small. As a result, we pooled our D3 and 25(OH)D3 study participants together for all analyses. Given the different pharmacokinetics between D3 vs 25(OH)D3, we ideally would have analyzed these participants separately. However, the pattern of change in the various vitamin D metabolites was similar between D3 and 25(OH)D3 (16, 31). To maximize statistical power, we pooled all participants and confirmed that supplementation regimen did not modify the association between rises in total or free 25(OH)D with increase in 24,25(OH)$_2$D or decrease in PTH. Second, we were not able to model entry of 25(OH)D into nonskeletal target cells (*e.g.,* immune cells). Because these target cells generally do not express the DBP receptor, megalin, it is possible that the difference between free and total 25(OH)D would have been more pronounced. However, such a biomarker is not readily available. Third, although we contend that most circulating 24,25(OH)$_2$D was produced by the kidney, we cannot rule out the possibility that some 24,25(OH)$_2$D was produced by extrarenal sources, and that the strength of the association seen between free 25(OH)D and 24,25(OH)$_2$D during the initial 4 weeks of supplementation was driven by 24,25(OH)$_2$D produced by cells that did not express megalin. Finally, our study was relatively short, which may, in part, explain why PTH did not decrease significantly during the 16-week supplementation regimen (32).

To conclude, we aimed to assess whether entry of free 25(OH)D from the extracellular compartment into target kidney and parathyroid cells may have a physiologic role in humans. We examined the associations between increase in total vs free 25(OH)D with downstream biomarkers of 25(OH)D entry into kidney [24,25(OH)$_2$D] and parathyroid (PTH) cells during aggressive vitamin D supplementation for 25(OH)D deficiency. We found that early in the course of aggressive vitamin D repletion, rise in free 25(OH)D was associated with increase in 24,25(OH)$_2$D and decrease in PTH, whereas rise in total 25(OH)D was not. These findings suggest that when 25(OH)D levels rise rapidly and robustly, rapid movement of free 25(OH)D into renal epithelial and parathyroid cells may be physiologically important.

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