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Effects of cholecalciferol vs. calcifediol on total and free 25-hydroxyvitamin D and parathyroid hormone

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Context: Vitamin D deficiency (25-hydroxyvitamin D [25D] <20 ng/ml) disproportionately affects non-Caucasian individuals. Controversy persists over: 1) how to best restore low 25D levels; and 2) how to best define vitamin D status (total [protein-bound + free] vs. free 25D).
Objective: To assess: 1) the effects of vitamin D3 (cholecalciferol, D3) vs. pharmacologic 25-hydroxyvitamin D3 (calcifediol, 25D3) on total and free 25D in a multi-ethnic cohort of adults; and 2) whether change in parathyroid hormone (PTH) is more strongly associated with total vs. free 25D.
Design: 16-week randomized controlled trial. Biochemistries at 0, 4, 8, and 16 weeks.
Intervention: 60 mcg (2,400 IU)/day of D3 or 20 mcg/day of 25D3.
Setting: Academic medical center.
Participants: 35 adults ≥18 years with 25D levels <20 ng/ml.
Main Outcome Measures: Total and free 25D, PTH.
Results: Baseline total (16.2 ± 3.7 vs. 17.0 ± 2.5 ng/ml, p=0.4) and free (4.2 ± 0.8 vs. 4.7 ± 1.0 pg/ml, p=0.2) 25D were similar between D3 and 25D3 groups, respectively. 25D3 increased total (+25.5 vs. +13.8 ng/ml, p=0.001) and free (+6.6 vs. +3.5 pg/ml, p=0.03) 25D more than D3. By 4 weeks, 87.5% of 25D3 participants had total 25D levels ≥30 ng/ml, compared to 23.1% of D3 participants (p=0.001). These trends were consistent across race/ethnicity. Change in PTH was similarly associated with both total (p=0.01) and free 25D (p=0.04).
Conclusions: 25D3 increased total and free 25D levels more rapidly than D3, regardless of race/ethnicity. Free and total 25D were similarly associated with change in PTH.

We studied the effects of 20 mcg/day of calcifediol vs. 60 mcg/day of cholecalciferol, and found calcifediol to more rapidly and robustly raise total and free 25D levels in a diverse sample of adults.

INTRODUCTION

Low serum 25-hydroxyvitamin D (25D) is associated with adverse skeletal health outcomes. In particular, low 25D leads to decreased intestinal calcium absorption, increased parathyroid hormone secretion, and increased bone resorption (1). At the present time, controversy persists over: 1) how to best restore low serum 25D levels; and 2) whether vitamin D status is best assessed by measuring serum concentrations of total (protein-bound plus free) vs. free 25D.

When serum total 25D levels are low, clinicians generally recommend supplementation with either ergocalciferol (D2) or cholecalciferol (D3) (2). Most studies report that orally administered D3 raises 25D levels to a greater extent than D2 (3-7), but even supplementation with currently-recommended D3 doses may not reliably increase total 25D levels >30 ng/ml (2). While a 25D level of >20 ng/ml is likely adequate for the general population (8), a threshold of ≥30 ng/ml may be advisable for individuals with osteoporosis, especially if anti-resorptive therapy is used (2, 9). While bolus D2 and D3 are generally effective at raising total 25D levels ≥30 ng/ml, the safety of this approach has recently been called into question because of its association with increased risk of falls in elderly populations (10, 11). An alternative approach may therefore be required. Presumably owing to its ability to bypass carbon-25 hydroxylation in the liver and its relative affinity for the circulating vitamin D binding protein (DBP), pharmacologic 25-hydroxyvitamin D3 (calcefidiol, 25D3) more rapidly and robustly raises serum 25D levels than parent D3 (12-18). 25D3 administration also suppresses PTH secretion while D3 does not (18). All recent human trials comparing D3 to 25D3 were conducted in predominantly Caucasian study populations (16-18). However, since low 25D is more prevalent among individuals with increased skin pigmentation (1, 2), it is important to identify optimal approaches for restoring inadequate 25D stores in individuals of all racial/ethnic backgrounds.

In addition to questions relating to optimal approach to vitamin D supplementation, another area of intensive research focuses on whether total vs. free 25D represents a superior marker of vitamin D status *in vivo*. In serum, 25D is primarily bound to DBP and albumin, with less than 1% of total 25D circulating in its free (unbound) form (19). In classical vitamin D physiology, DBP-bound 25D is internalized by the renal epithelial cell via megalin (a DBP receptor), and then activated to 1,25-dihydroxyvitamin D (1,25D) by the 1-alpha hydroxylase, CYP27B1 (20). 1,25D then acts in an endocrine fashion to facilitate intestinal calcium absorption (21). Based on this, one would theorize that *total* 25D represents a better marker of vitamin D status of classical vitamin D bioactivity. In non-classical vitamin D physiology involving tissues that do not express megalin, it has been proposed that *free* 25D is internalized by the target cell via simple diffusion, and then converted to 1,25D by locally expressed CYP27B1 (19, 22). Based on this, one would hypothesize that *free* 25D represents a better marker of vitamin D status of non-classical vitamin D bioactivity.

The above paradigm, however, may be an oversimplification. In particular, it remains unclear whether 25D only enters megalin-expressing tissues bound to DBP, or if some 25D enters the target cell in free unbound form. For example, DBP -/- mice placed on a vitamin D-containing diet maintain normal serum PTH levels, and develop normal skeletons (23). If 25D only enters megalin-expressing renal epithelial cells bound to DBP, one would expect these mice to be incapable of producing adequate amounts of 1,25D, leading to secondary hyperparathyroidism and osteomalacia. This, however, was not seen, suggesting to us that, at least in mice, both DBP-bound and free 25D enters the megalin-expressing renal epithelial cell *in vivo.* In human clinical trials, PTH is frequently used as a biomarker of classical vitamin D physiology. Some cross-sectional analyses report that bioavailable or free 25D is more strongly associated with PTH (24, 25), whereas others do not (26-28). Importantly, there are limited longitudinal studies assessing the associations between total vs. free 25D and *change* in PTH. Finally, there are no data reporting the effects of 25D3 supplementation on circulating free 25D concentrations.

This study was therefore designed to address the two following questions: 1) What are the comparative effects of D3 vs. 25D3 on total and directly measured free 25D levels in a multi-ethnic cohort of healthy adults; and 2) does free 25D represent a superior *in vivo* marker of

vitamin D-mediated bioactivity in maintenance of normal calcium balance above and beyond total 25D?

MATERIALS AND METHODS

Study Subjects

We recruited a total of 35 individuals from the University of California, Los Angeles (UCLA) student body, staff, and patient population. Recruitment was carried out through e-mail advertisements, social media postings, and direct patient contact. Inclusion criteria were age ≥18 years and a baseline 25D level <20 ng/ml. Exclusion criteria included history of hypercalcemia, hypercalciuria, nephrolithiasis, intestinal malabsorption, or dysregulated vitamin D metabolism (from underlying comorbidity or medication). Participants agreed to refrain from changing their dietary calcium intake, and from taking self-prescribed calcium or vitamin D supplements for the study duration. All participants provided informed consent. The study was approved by the UCLA Institutional Review Board, and was registered on ClinicalTrials.gov under identifier NCT02091219.

Study Intervention

Study participants were randomized in blocks of four (stratified by race/ethnicity) to either D3 (60 mcg [2,400 IU]/day) or 25D3 (20 mcg/day) for 16 weeks. 20 mcg/day of 25D3 was selected because it effectively and safely raises 25D levels from <20 ng/ml to ≥30 ng/ml (16-18). 60 mcg/day of D3 was selected because it represents a bioequivalent dose (17), and corresponds to a daily intake level that, in dose-response studies, maintains 25D levels ≥30 ng/ml (29). D3 and 25D3 were obtained from DSM Nutritional Products in powder form. These were compounded by the UCLA Investigational Pharmacy into capsules for distribution to study participants. Expected D3 and 25D3 content was confirmed by liquid chromatography-mass spectrometry (LC/MS/MS) (Heartland Assays). Participants were evaluated at baseline, and at 4, 8, and 16 weeks after initiation of D3 or 25D3 (4 visits total). At each visit, participants were asked about possible adverse events. Adherence was assessed by pill count.

Measurements

Biochemical assessment was performed at all four study visits. Serum measurements included total 25D; free 25D; total 1,25-dihydroxyvitamin D (1,25D); calcium; and intact parathyroid hormone (PTH). Urinary measurements included a calcium:creatinine excretion ratio on an early morning fasting sample. Total 25D was measured by chemiluminescence immunoassay (Diasorin, Liaison) in the UCLA Department of Pathology and Laboratory Medicine; this laboratory participates in the College of American Pathologists Accuracy-Based Vitamin D Survey. Intra- and inter-assay CV were 2.1-2.2% and 4.0-4.5%, respectively. Total 1,25D was measured by chemiluminescence immunoassay (Diasorin, Liaison). Intra- and inter-assay CV were 2.4-3.9% and 4.5-7.8%, respectively. While LC/MS/MS represents the gold standard for measuring vitamin D metabolites, the Diasorin, Liaison assay has acceptable performance for measuring D3 metabolites (relevant to this study) when compared to LC/MS/MS (30). Free 25D was measured using an antibody-based assay from Future Diagnostics as previously described (28). The assay limit of detection is 1.9 pg/ml. In the range of concentrations measured, the CV was ≤7%. PTH was measured by electrochemiluminescence immunoassay (Roche Cobas). Intra- and inter-assay CV were 0.8-1.5% and 1.5-1.8%, respectively.

Statistical analyses

Descriptive statistics of relevant continuous clinical covariates and biochemical measurements were generated and assessed for normality. Differences in baseline characteristics between D3 vs. 25D3 groups were assessed by the independent samples t-test (continuous variables) or Chi-square test (categorical variables). Changes in biochemical measurements within D3 and 25D3 treatment groups were examined by the paired t-test. Differences in biochemical measurements at weeks 4, 8, and 16 between D3 vs. 25D3 groups were assessed by the independent samples t-test. Associations between total or free 25D (primary predictor in separate analyses) and change in PTH from time of 25D measurement to next follow-up visit (outcome variable) were assessed using repeated-measures, mixed-effects regression. All models were adjusted for factors that may influence change in PTH, and include serum calcium, age, BMI, race/ethnicity, and supplementation regimen.

RESULTS

Patient characteristics

A total of 16 and 19 subjects were randomized to receive D3 and 25D3, respectively. Age, race/ethnicity, and BMI were not significantly different between D3 and 25D3 groups. Baseline total 25D (16.2 $\pm$ 3.7 [D3] vs. 17.0 $\pm$ 2.5 [25D3], p=0.5), free 25D (4.2 $\pm$ 0.8 [D3] vs. 4.7 $\pm$ 1.0 [25D3], p=0.2), 1,25D, calcium, and PTH were also similar between groups (Table 1).

Effects of D3 vs. 25D3 on serum vitamin D metabolites

At 16-week follow-up, total 25D increased to a greater extent with 25D3 (+25.5 ng/ml) than with D3 (+13.8 ng/ml) (p=0.008). Final total 25D was 42.4 $\pm$ 15.9 ng/ml with 25D3 supplementation, compared to 29.6 $\pm$ 4.1 ng/ml with D3 (p=0.007). Along these lines, free 25D also increased to a greater extent with 25D3 (+6.9 pg/ml) than with D3 (+3.6 pg/ml) (p=0.03). Final free 25D was 11.6 $\pm$ 5.6 pg/ml with 25D3 supplementation, compared to 7.8 $\pm$ 1.9 pg/ml with D3 (p=0.02) (Figure 1). Total and free 25D were highly correlated (Figure 2). Highlighting the rapidity with which 25D3 restores 25D levels, total and free 25D were already significantly higher in the 25D3 group by 4 weeks (p=0.004 for total 25D, p=0.02 for free 25D). Further, by 4 weeks, mean total 25D was greater than 30 ng/ml in the 25D3 group (34.5 $\pm$ 10.4 ng/ml), but not in the D3 group (25.4 $\pm$ 4.3 ng/ml). In fact, by week 4, 14 of 16 25D3 participants had achieved total 25D levels $\geq$30 ng/ml, compared to only 3 of 19 D3 participants (p=0.001). Of note, at 16 weeks, mean total 25D remained $\leq$30 ng/ml in the D3 group (29.6 $\pm$ 4.1 ng/ml). All trends were similar across race/ethnic groups.

From baseline to 16 weeks, total 1,25 increased with both D3 (+15 pg/ml, p=0.005) and 25D3 (+11.5 pg/ml, p=0.09). Final 1,25D concentrations were similar between groups (66.8 $\pm$ 13.9 [D3] vs. 70.3 $\pm$ 23.4 [25D3] pg/ml, p=0.6).

Association between change in PTH and vitamin D metabolites

The associations between higher total vs. free 25D and subsequent decrease in PTH were examined in adjusted mixed-effects regression models (Table 2). After adjusting for covariates that may influence PTH level (i.e., age, BMI, race/ethnicity, serum calcium, and supplementation regimen), we found that both higher total and free 25D were significantly associated with declines in PTH from time of 25D measurement to next follow-up visit. More specifically, for every ng/ml increase in total 25D, PTH decreased by 0.8% over the ensuing four weeks (p=0.01). Along the same lines, for every pg/ml increase in free 25D, PTH decreased by 2.5% over the ensuing four weeks (p=0.04). Of note, PTH did not decrease significantly over the course of the study with either supplementation regimen (p>0.6 for all). However, PTH was

already relatively low at study baseline (40.1 ± 18.6 [D3] vs. 34.6 ± 13.9 [25D3] pg/ml, p=0.4). Higher 1,25D was not significantly associated with decrease in PTH.

Adherence

Adherence to supplementation by pill count. Adherence was 90.1% and 91.9% in the D3 and 25D3 groups, respectively.

Safety

Serum calcium and urinary calcium excretion did not change significantly from baseline to 16 weeks with either D3 or 25D3 (p>0.4 for all). There were no reports of hypercalcemia, hypercalciuria, or nephrolithiasis.

DISCUSSION

The aims of this 16-week study were to: 1) compare the effects of D3 vs. 25D3 on circulating levels of total and free 25D in a multi-ethnic cohort of healthy adults; and 2) determine if increases in total or free 25D are more strongly associated with decrease in PTH, a marker of vitamin D bioactivity. As hypothesized, we found that 25D3 more rapidly and robustly increases total and free 25D levels than D3; this was true regardless of race/ethnicity. Among those who received 25D3, mean total 25D increased to ≥30 ng/ml by 4 weeks. In contrast, among those who received D3, mean total 25D remained <30 ng/ml for the entire study. We also found that higher levels of both total and free 25D were significantly associated with future decline in serum PTH.

Our first key finding was that 20 mcg/day of 25D3 increases total 25D levels more rapidly and robustly than 60 mcg/day of D3. These data are consistent with prior studies that have compared the effects of D3 and 25D3 on total serum 25D levels. In a 4-month trial of 20 postmenopausal women, 20 mcg/day of 25D3 increased mean serum total 25D levels to ≥30 ng/ml by ~35 days. Final total 25D at study completion was 69.5 ng/ml. In contrast, 20 mcg (800 IU)/day of D3 did not reliably increase total 25D levels to ≥30 ng/ml, as ~50% of participants remained below this threshold at the end of 16 weeks; final total 25D was 30.1 ng/ml (16). Another 10-week trial of 56 adults ≥50 years compared placebo against 20 mcg (800 IU)/day of D3, 7 mcg/day of 25D3, and 20 mcg/day of 25D3. Again, 20 mcg/day of 25D3 increased mean total 25D to ≥30 ng/ml by 5 weeks; final total 25D at 10 weeks was 53.6 ng/ml. As was the case in the prior study (18), 20 mcg/day of D3 did not raise mean total 25D levels ≥30 ng/ml even by the completion of the study presented here.

Our second key finding was that 25D3 increases free 25D levels more quickly and to a greater extent than D3, and that both free and total 25D were similarly associated with subsequent decline in serum PTH. To our knowledge, free 25D response to 25D3 supplementation has not been previously reported. Our data are consistent with prior cross-sectional and longitudinal studies demonstrating that free and total 25D levels are strongly correlated, such that changes in both track closely together (7, 26, 28, 31-33). With respect to the question of whether free vs. total 25D is a superior marker of vitamin D bioactivity, multiple studies have assessed the cross-sectional association between total vs. free 25D and markers of skeletal health, e.g., serum PTH, bone turnover markers, and bone mineral density (24-27, 34, 35). Some studies have reported a stronger association with free 25D levels (24, 25, 34), but others have not (26, 35). These inconsistencies may result from differences in study populations and methodologies for determining bioavailable or free 25D levels. In particular, free 25D levels can be directly measured by antibody-based assays (as we have done here) or indirectly

calculated from total 25D, albumin, DBP quantity, and DBP isoform (which affects DBP affinity for 25D) (19). Of note, recent studies have questioned both the accuracy of a commonly used monoclonal antibody-based assay for measuring DBP levels, as well as the frequent practice of using a single DBP affinity constant when calculating free 25D concentrations (31, 33, 36). Given their cross-sectional nature, heterogeneity in methodology for assessing free 25D levels, and possibly biased approach for calculating free 25D levels, these studies do not allow us to draw cause-and-effect conclusions.

Here, our *longitudinal* data show that higher total and free 25D levels were similarly associated with future decline in PTH, and that free 25D did not provide any additional information above and beyond total 25D. PTH secretion is regulated by two principle mechanisms, the calcium sensing receptor (CaSR) and vitamin D receptor (VDR) (37-39). CaSR detects changes in circulating calcium concentrations, which are partly determined by 1,25D-mediated intestinal calcium absorption (37). Under normal physiologic conditions, circulating 1,25D is principally produced by megalin-expressing renal epithelial cells (40). One would therefore expect total 25D to be more strongly associated with decline in PTH. The parathyroid cell also expresses CYP27B1 and VDR (38), suggesting that it possesses the cellular machinery necessary to convert internalized 25D to 1,25D; this locally-produced 1,25D then engages the VDR, leading to suppression of PTH production. Since parathyroid cells also express megalin (19, 40), one would similarly expect total 25D to be the physiologically relevant metabolite. There is evidence, however, to suggest that megalin-expressing tissues internalize not only DBP-bound, but free 25D as well (23). For example, DBP-null mice placed on a vitamin D-containing diet have a normal serum PTH level and develop normal skeletons (23). If the renal epithelial cell exclusively internalizes DBP-bound 25D, one would expect these mice to exhibit secondary hyperparathyroidism and osteomalacia. Since this was not seen, we presume that, at least in mice, both DBP-bound and free 25D enters megalin-expressing renal epithelial and/or parathyroid cells *in vivo.* Our current data do not definitively allow us to discern whether this is similarly the case in humans. Of note, some investigators suggest that free 25D levels in the range observed in this study are too minuscule to carry out meaningful biological function (19, 41). This may be true for megalin-expressing target cells, since DBP-bound 25D concentrations far exceed free 25D. However, in non-megalin-expressing cells, free 25D may be more biologically active because DBP-bound 25D does not have a mechanism for cellular entry. Future studies will be required to determine whether concentrations of circulating free 25D achieved *in vivo* are adequate to influence either skeletal and/or non-skeletal vitamin D physiology.

Our study has several clinical implications. First, 25D3 is more reliable at increasing total 25D levels $\geq$30 ng/ml. While a 25D level $\geq$20 ng/ml is likely sufficient to maintain skeletal health in the generally healthy population (8), a 25D level $\geq$30 ng/ml may be preferable among those with osteoporosis, especially if anti-resorptive therapy is prescribed (2, 9). In fact, some even recommend restoring 25D levels to this threshold prior to initiating osteoporosis pharmacotherapy (9, 42). To this end, identifying an approach for raising inadequate 25D levels to target threshold levels reliably, and quickly, is important for preventing delay of therapy and possibly optimizing response to therapy. While one could argue that bolus D2 or D3 fills this role adequately, this approach has recently been called into question given its association with increased risk of falls (10, 11). In this setting, daily 25D3 may be advantageous to D3. Despite its more potent effects on raising serum 25D levels, 25D3 increased 1,25D concentrations to a similar extent as D3. This suggests that pharmacologic 25D3 does not overwhelm the body's

compensatory mechanisms for maintaining normal calcium homeostasis, and risk of developing hypercalcemia may not be increased with 25D3. It does warrant mention, however, that parent vitamin D may have important physiological effects that were not previously recognized (e.g., endothelial stabilization) (41, 43). When targeting these tissues, D3 may be preferable. Future studies are necessary to assess when D3 versus 25D3 is indicated. Finally, our data suggests that with respect to skeletal health, measuring free 25D is likely not superior to measuring total 25D in instances where DBP and/or albumin synthesis is not altered (36).

Several weaknesses warrant mention. First, the study sample size was relatively small. This however, would bias our results towards null. Therefore, the differences between D3 and 25D3, and the associations seen between change in PTH and change in total and free 25D would likely only be strengthened with increased sample size. Second, our study participants were not severely vitamin D deficient, as suggested by the relatively low PTH levels observed at baseline. This may be one reason that PTH did not decrease significantly even with 25D3, as was previously reported (18). Perhaps if we had exclusively included patients with both low 25D levels and frank secondary hyperparathyroidism, a more pronounced biomarker benefit would have been seen. Finally, our study focused on the association between total vs. free 25D and a marker of skeletal health/calcium homeostasis. Since non-skeletal vitamin D-mediated bioactivity is generally carried out by cells that do not express megalin, free 25D may be a superior marker of these physiologic functions.

To conclude, this is the first study to compare the effects of D3 to 25D3 on both total and directly measured free 25D levels in a multi-ethnic cohort of vitamin D deficient healthy adults. Compared to D3, 25D3 more rapidly and robustly increases serum concentrations of total and free 25D, and more reliably increases total 25D to levels $\geq$30 ng/ml by week 4 of supplementation. These findings may have clinical implications for both skeletal and non-skeletal health outcomes. With respect to osteoporosis, 25D3 may have an important therapeutic role as higher 25D thresholds may be warranted, especially among those receiving pharmacologic therapy. In terms of non-skeletal health outcomes, 25D3 may be similarly beneficial as higher 25D levels are likely necessary to optimize local vitamin D bioactivity (22, 44). We also found that the association between higher total and free 25D levels and subsequent decline in PTH were similar, adding to the emerging consensus that free 25D may not provide additional information above and beyond total 25D for skeletal health measures. Future studies will be necessary to determine if free 25D is a superior marker of non-skeletal vitamin-mediated bioactivity.

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DISCLOSURE SUMMARY

Disclosure statement: The authors have nothing to disclose.

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Figure 1. Changes in vitamin D metabolites with D3 vs. 25D3

Figure 2. Relationship between free and total 25D levels stratified by supplementation regimen

Table 1. Baseline Characteristics[1]

	Cholecalciferol (D3) N=16	Calcifediol (25D3) N=19	p-value
Age (years)	36.9 (12.7)	34.8 (8.6)	0.6
Race/Ethnicity			
Caucasian	2 (12.5)	3 (15.8)	0.8
African American	6 (37.5)	5 (26.3)	0.5
Asian American	6 (37.5)	6 (31.6)	0.7
Hispanic/Latino	2 (12.5)	5 (26.3)	0.3
BMI (kg/m^2)	25.7 (6.1)	27.4 (7.4)	0.5
Total 25-hydroxyvitamin D (ng/ml)	16.2 (3.7)	17.0 (2.5)	0.4
Free 25-hydroxyvitamin D (pg/ml)	4.2 (0.8)	4.7 (1.0)	0.2
1,25-dihydroxyvitamin D (pg/ml)	51.8 (14.2)	58.8 (17.6)	0.2
Calcium (mg/dl)	9.3 (0.4)	9.6 (0.3)	0.1
Urinary calcium:creatinine	0.06 (0.04)	0.05 (0.04)	0.6
Parathyroid hormone (pg/ml)	40.1 (18.6)	34.6 (13.9)	0.3

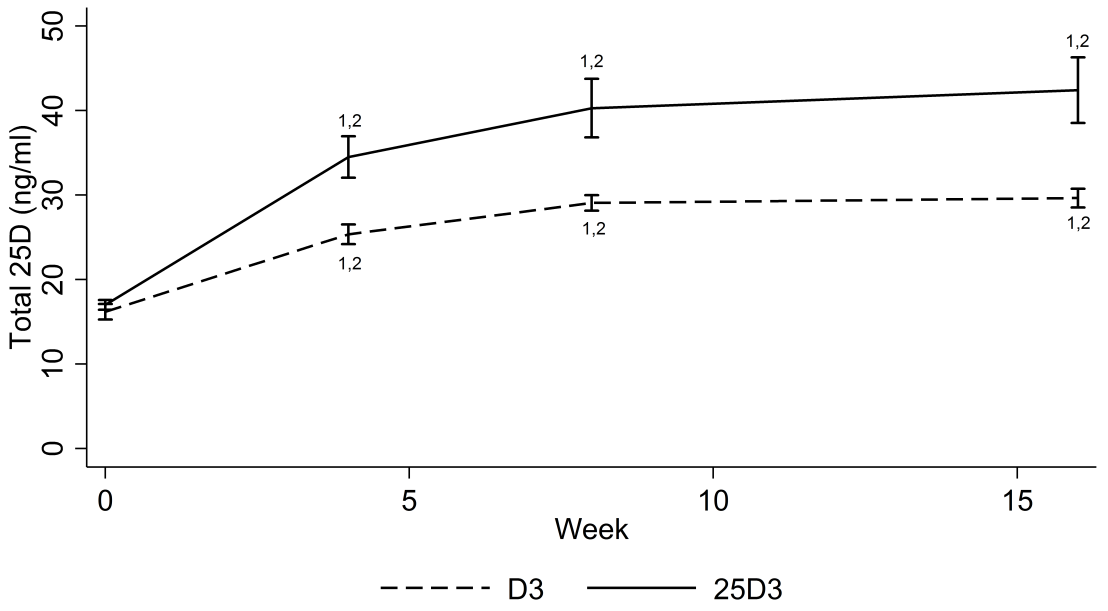
1 Continuous variables presented as mean (SD). Categorical variables presented as count (percentage).

Table 2. Adjusted[1] associations between total vs. free 25D and percent decrease in PTH from time of 25D measurement to next follow-up visit

	Percent decrease in PTH (95% CI) (from time of 25D measurement to next follow-up visit) per unit increase in total vs. free 25D	
	β coefficient[2] (95% CI)	p-value
Total 25D	0.804 (1.475, 0.134)	0.01
Free 25D	2.512 (4.976, 0.048)	0.04

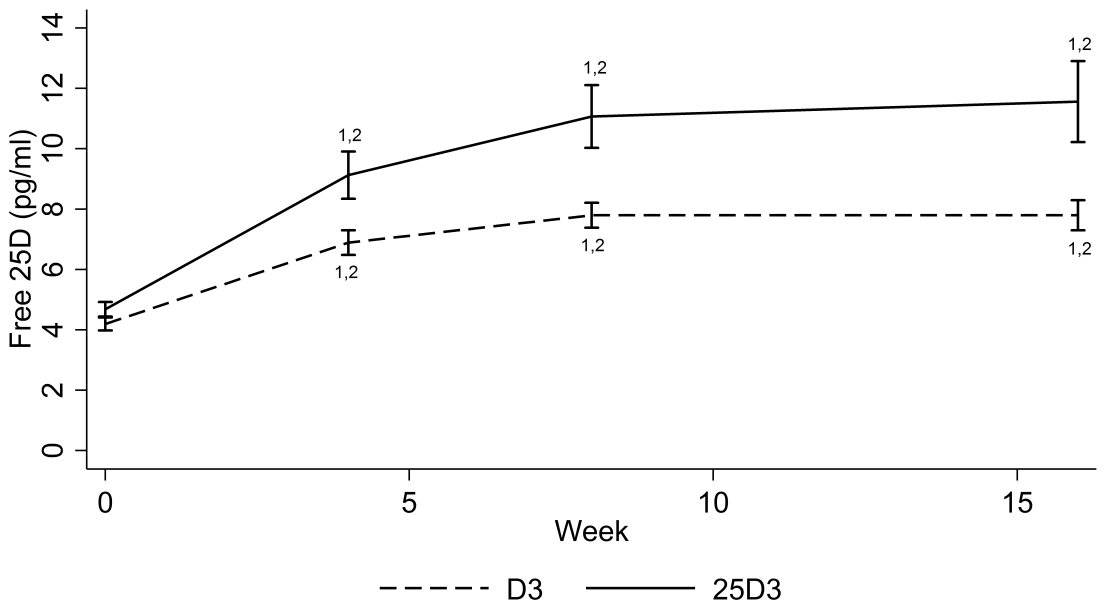
1 Adjusted for serum calcium, age, BMI, race/ethnicity and supplementation regimen
2 The β coefficient should be interpreted as follows: For each ng/ml increase in total 25D or pg/ml increase in free 25D, PTH decreases by "β" percent.

A



1: p<0.05 for within-group change from baseline
2: p<0.05 for between-group difference at given time point

B



1: p<0.05 for within-group change from baseline
2: p<0.05 for between-group difference at given time point