

SPECIAL GUEST EDITOR SECTION

High-Dose Intramuscular Vitamin D Provides Long-Lasting Moderate Increases in Serum 25-Hydroxyvitamin D Levels and Shorter-Term Changes in Plasma Calcium

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The best management of vitamin D deficiency, defined as a 25-hydroxyvitamin D [(25(OH)D] level <50 nM, is unclear. Intramuscular (IM) injection of a large bolus of vitamin D (≥100 000 IU) is used, but its safety is uncertain. In 10 adults given an IM injection of 600 000 IU vitamin D₃, we measured at baseline and at 1, 2, 3, and 4 weeks postinjection the serum levels of vitamin D₃, 25(OH)D₃, 25(OH)D₂, total 25(OH)D, 3-epi-25(OH)D₃, and 24,25-dihydroxyvitamin D₃ [24,25(OH)₂D₃] using a standardized LC with tandem MS (MS/MS) assay; serum levels of 25(OH)D using the Abbott ARCHITECT i2000 immunoassay; and markers of bone metabolism. Bone markers and 25(OH)D (immunoassay) were remeasured at 24 weeks. All participants had baseline total 25(OH)D levels >50 nM. Serum 25(OH)D levels increased at 3, 4, and 24 weeks postinjection, peaking at 4 weeks

[mean ± SEM of 126 ± 7.9 nM (immunoassay) and 100 ± 5.5 nM (LC-MS/MS)] but generally remained <125 nM, the upper limit recommended by the U.S. Institute of Medicine. Serum 24,25(OH)₂D₃ levels increased at 3 and 4 weeks postinjection. Serum ionized calcium levels were higher than baseline at 1, 3, and 4 weeks postinjection but remained within the clinically normal range. Other biochemical parameters, including other vitamin D metabolites, plasma alkaline phosphatase, and parathyroid hormone levels, were unchanged. IM injection of a large bolus of vitamin D effectively increases serum 25(OH)D levels without evidence of metabolic abnormality.

Guest edited as a special report on "The Importance of Vitamin D Assay Standardization and the Vitamin D Standardization Program" by Stephen P.J. Brooks and Christopher T. Sempos.

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Vitamin D deficiency may be widespread globally, even in sunny countries (1). In the latest Australian Health Survey, in which an assay standardized to the Vitamin D Standardization Program reference measurement protocol was used, the overall prevalence of vitamin D deficiency/ inadequacy (<50 nM; 2) in Australians aged 12 years and older was 23%, with less than 7% of the population estimated to be moderately or severely deficient (<30 nM; 3). The predominant source of vitamin D for most Australians is endogenous synthesis in the skin initiated by exposure to solar UV (UVB) radiation (4). The absorption of UVB photons by 7-dehydrocholesterol in epidermal cells results in conversion first to previtamin D₃ and then to vitamin D₃. Vitamin D (D₂ or D₃) can also be obtained through dietary sources such as oily fish and mushrooms and, increasingly, through oral supplementation. After oral ingestion, vitamin D is absorbed through the gut and then

transported in chylomicrons to the liver (5). Both ingested and endogenously synthesized vitamin D are hydroxylated in the liver to form 25-hydroxyvitamin D [25(OH)D]. In renal proximal tubule epithelial cells and other cells (reviewed in ref. 6), 25(OH)D is converted into the active vitamin D metabolite, 1,25-dihydroxyvitamin D [1,25(OH)₂D]. Serum 25(OH)D can also be catabolized to 24,25-dihydroxyvitamin D [24,25(OH)₂D], catalyzed by a 24-hydroxylase enzyme (encoded by the *CYP24A1* gene). There are a range of minor metabolites of 1,25(OH)₂D [such as 25(OH)D₃-26, 23-lactone], but these contribute <6% of the total concentration of vitamin D metabolites in blood (7). Vitamin D status is determined by measurement of serum/plasma levels of 25(OH)D because this metabolite has a longer half-life than other vitamin D metabolites and is detected at nanomolar concentrations [versus picomolar for 1,25(OH)₂D] in blood. In its active form, vitamin D has an essential role in maintaining plasma calcium and phosphate homeostasis, and, in turn, musculoskeletal health.

Vitamin D supplementation may be required to maintain vitamin D sufficiency when exposure to UVB radiation is limited, e.g., at high latitudes and/or during winter, and in people who wear clothing that covers most of the skin when outdoors. Vitamin D supplementation can be provided via oral or intramuscular (IM) routes and using daily dosing or a large bolus of vitamin D at less frequent intervals. Oral supplementation is most commonly used. Adherence to daily ingestion of vitamin D may be difficult, especially in elderly patients (8). Optimal levels are typically achieved after approximately 2 months of daily oral supplementation (assuming an appropriate dose), and serum 25(OH)D concentration is then maintained at a relatively constant level (reviewed in ref. 9). Daily oral dosing at <3000 IU/day has not been associated with any adverse effects on bone health (10). In contrast, a single oral treatment with a large bolus of 100 000–600 000 IU of vitamin D rapidly increased serum 25(OH)D concentrations for 1 to 2 months posttreatment, although these levels were not sustained (11, 12). In addition, with very high doses of oral supplementation (≥400 000 IU vitamin D), side effects have been reported, including gastrointestinal tract complaints, hypercalcemia, and hypercalciuria (11, 13). In one study, in the first 3 months after oral supplementation with a large bolus of vitamin D₃ (500 000 IU) in elderly women, there was an increased rate of falls and fractures (14).

An alternative means of administering high-dose vitamin D is via IM injection. The dynamics of changes in serum 25(OH)D level after IM injection differ to those after oral supplementation. Serum 25(OH)D levels increase at a slower rate (in response to IM treatment with 600 000 IU vitamin D₂ or D₃) as compared to oral supplementation (15) and can persist at levels greater than baseline for up to 12 months posttreatment (16). However, evidence on the effect of an IM bolus of vitamin D on blood biochemistry is limited. Studies have shown no change in plasma ionized calcium or phosphate levels in the 30–120 days (15) or 4–12 months (16) after a single IM injection of high-dose vitamin D₃ (600 000 IU). The earlier effects (<30 days) on blood biochemistry after a large IM bolus of vitamin D₃ have not been well described.

In this study, we examined the effects of a single IM injection of high-dose vitamin D₃ (600 000 IU) on several vitamin D

metabolites and serum/plasma markers of bone metabolism, including calcium, phosphate, parathyroid hormone (PTH), and alkaline phosphatase, in 10 adult participants at baseline and at 1, 2, 3, 4, and 24 weeks postinjection. Because of the potential for high-dose therapy to substantially increase serum 25(OH)D levels, we hypothesized that this treatment would increase the concentration of minor vitamin D metabolites in blood, allowing them to be quantified.

Materials and Methods

Participants

Ten adult participants who were receiving an annual bolus of IM vitamin D₃ as part of routine clinical care for the treatment of previously diagnosed vitamin D deficiency were recruited from a general practitioner's clinical suite (by M. Zafir, The Surgery, Albany, Western Australia). The mean age of participants was 64 years, 60% were male, and the patients had been diagnosed with a range of medical conditions (Table 1).

Bolus Vitamin D₃ Supplementation

As part of their standard clinical care, participants were given an IM injection of 600 000 IU of vitamin D₃ (D3 Forte; Biological Therapies, Melbourne, Australia) into the deltoid muscle of the upper arm. Participants were treated in November 2015 in the clinic, when the average UV Index (maximum for the day) for Albany was 9.

Sample Collection

Blood was drawn from participants immediately before injection (baseline), as well as at 1, 2, 3, 4, and 24 weeks postinjection. Blood samples were transported on ice to Perth

Table 1. General characteristics of participants

Participant No.	Age, years ^a	Sex ^b	Past and current medical conditions
1	91	Male	Hemochromatosis, hypertension, type 1 diabetes, and scoliosis
2	71	Female	Lumpectomy (breast cancer)
3	82	Male	Asthma, gout, and glaucoma
4	82	Female	Hypertension and polymyalgia rheumatica
5	47	Female	Multiple sclerosis, depression, and hemochromatosis
6	35	Female	Scoliosis and Ross River virus infection
7	40	Male	Chronic fatigue, fibromyalgia, and scoliosis
8	64	Male	Vertigo, cholelithiasis, scoliosis, and eczema
9	56	Male	Asthma
10	63	Male	Gout and osteoporosis

^a Age at the beginning of the study; mean age ± SD was 63.9 ± 18.1 (*n* = 10).

^b 60% of the participants were male.

(Western Australia), and serum and plasma were extracted at PathWest Laboratory Medicine (Sir Charles Gairdner Hospital, Perth, Western Australia). Samples were assayed fresh or stored at -80°C until analyzed.

Measurement of Serum 25(OH)D

Serum 25(OH)D levels were measured using two different methods:

(a) *Standardized reference assay.*—An LC with tandem MS (MS/MS) method (17) was performed at a laboratory at Queen's University, Kingston, ON, Canada (by G. Jones), which was accredited by the Vitamin D External Quality Assessment Scheme for the period 2012–2016, for serum obtained at baseline and at 1, 2, 3, and 4 weeks postinjection only. The assay separately quantifies 25(OH)D₃ and 25(OH)D₂ [henceforth referred to individually or as their sum, total 25(OH)D].

(b) *Clinical assay as used for the general clinical management of patients.*—The Abbot ARCHITECT i2000 system (Abbott Core Laboratory, Abbott Park, IL) was performed at PathWest Laboratory Medicine. The assay is a chemiluminescent immunoassay (18) that does not discriminate between 25(OH)D₃ and 25(OH)D₂ [henceforth referred to as 25(OH)D], with results available for all time points.

Measurement of Other Vitamin D Metabolites

A slightly modified LC-MS/MS method using the derivatizing agent DMEQ-TAD (17), and adapted to include assay of vitamin D₃ (internal standard: vitamin D₃-[d₃]; IsoSciences), was used to separately quantify vitamin D₃, total 25(OH)D, 3-epi-25(OH)D₃, and 24,25(OH)₂D₃ in the sera at baseline and at 1, 2, 3 and 4 weeks after vitamin D IM bolus.

Measurement of Other Metabolites

Serum and plasma were extracted from blood samples, and, with urine, were tested as part of a routine pathology “fasting bone metabolic study” or “fasting calcium metabolism panel” at PathWest Laboratory Medicine between November 2015 and May 2016. This panel is used for investigation of calcium disorders, osteoporosis, or other metabolic bone disease, as described previously (19, 20). Plasma and urine biochemistry, including total calcium, phosphate, alkaline phosphatase, and plasma creatinine, were measured on the Abbott c16000 Clinical Chemistry Analyzer. Serum ionized calcium was measured using an ion-selective electrode on the ABL 800 FLEX analyzer (Radiometer, Copenhagen, Denmark). Plasma intact PTH was measured by electrochemiluminescence on the E170 (Roche Diagnostics GmbH, Mannheim, Germany).

Statistical Analyses

Data were compared using one-way analysis of variance (with Tukey's post hoc analysis), linear correlation (Pearson test), or a Bland-Altman analysis using the Prism 6.0 for Mac OS X statistical analysis program. Differences were considered significant with a P -value <0.05 .

Ethics Approval

This study was performed according to the ethical guidelines of the Australian National Health and Medical Research Council and with approval from the Australian National University Human Research Ethics Committee (No. 2015/643).

Results

Large IM Bolus of Vitamin D₃ Increased Serum Total 25(OH)D Levels 4 Weeks After IM Injection

As measured by LC-MS/MS, all participants had total 25(OH)D levels >50 nM at baseline (Figure 1A). Total 25(OH)D levels significantly increased from baseline to 4 weeks postinjection (Figure 1A).

Large IM Bolus of Vitamin D₃ Modified Serum Calcium, but not Alkaline Phosphatase, PTH, or Phosphate

Serum ionized calcium levels were increased from baseline at 1, 3, and 4 weeks postinjection (Figure 1B) but were largely maintained within the normal clinical range (1.12–1.32 mM; Figure 2A). By 24 weeks, the serum calcium levels were not significantly different to baseline (data not shown). At baseline, ionized serum calcium levels were below the normal clinical range for one individual (participant 5), but these were normalized by the IM vitamin D₃ by 1 week postinjection.

PTH levels were not significantly changed after vitamin D₃ treatment (Figure 1C), including at the 24-week time point (data not shown), and were maintained within the clinically normal range (1.6–6.9 pM; Figure 2B). As has been previously reported (21), there was a significant nonlinear relationship (second-order polynomial, quadratic $r^2 = 0.156$, $P < 0.05$) between plasma PTH and serum total 25(OH)D levels. This weak correlation is consistent with the variable findings around this relationship as reviewed and reported by the U.S. Institute of Medicine (IOM; 2). Other biochemical parameters were unchanged by the high-dose injection, including plasma levels of alkaline phosphatase (Figure 1D), phosphate (Figure 1E), albumin, and creatinine, as well as urinary markers including creatinine, calcium, phosphate, and the calcium/creatinine excretion index (data not shown).

IM Injection of High-Dose Vitamin D Had Modest Effects on Other Serum Vitamin D Metabolites

The results from the LC-MS/MS assay showed that there was an increase in total 25(OH)D (Figure 1A) and 25(OH)D₃ (Figure 3A) at 4 weeks postinjection, with a nonsignificant decrease in 25(OH)D₂ (Figure 3B). Serum vitamin D₃ (Figure 3C) and 3-epi-25(OH)D₃ (Figure 3D) appeared to increase from baseline, but this increase was not statistically significant. Levels of 24,25(OH)₂D₃ were significantly increased from baseline at 3 and 4 weeks postinjection (Figure 3E). The 25(OH)D₃-to-24,25(OH)₂D₃ ratio was significantly reduced at 4 weeks postinjection (Figure 3F). Because 25(OH)D₃ levels increased only moderately, i.e., none above 150 nM, we were unable to measure levels of minor vitamin D metabolites such as 25(OH)D₃-26,23-lactone in serum.

Modest Correlation Between 25(OH)D Levels as Measured by Immunoassay and Total 25(OH)D as Measured with the Standardized LC-MS/MS Assay

Data for 25(OH)D levels for the 24-week time point were available only for the immunoassay. This immunoassay is typically used by doctors in their clinical management of patients; therefore, understanding the change in 25(OH)D levels that would be seen in clinical practice is of value. We assessed the accuracy of the immunoassay as compared to the standardized reference assay. There was a significant (but weak) correlation between 25(OH)D levels measured using the immunoassay and total 25(OH)D levels measured using LC-MS/MS (Figure 4A). There was a positive bias of 13.3 nM, with higher 25(OH)D levels measured using the immunoassay compared to the LC-MS/MS assay (Figure 4B).

Serum 25(OH)D Peaked 4 Weeks After IM Injection, with Levels Remaining Higher than Baseline at 24 Weeks Posttreatment

At baseline, 25(OH)D levels measured in serum using the immunoassay were >50 nM, peaking at 4 weeks postinjection for most participants (Figure 4C). At 24 weeks postinjection, serum 25(OH)D levels were still significantly greater than baseline (Figure 4C).

Discussion

In this study, we undertook detailed monitoring of changes in vitamin D and its metabolites and in bone-related parameters measured in serum/plasma and urine immediately before and after a single large IM bolus of vitamin D₃

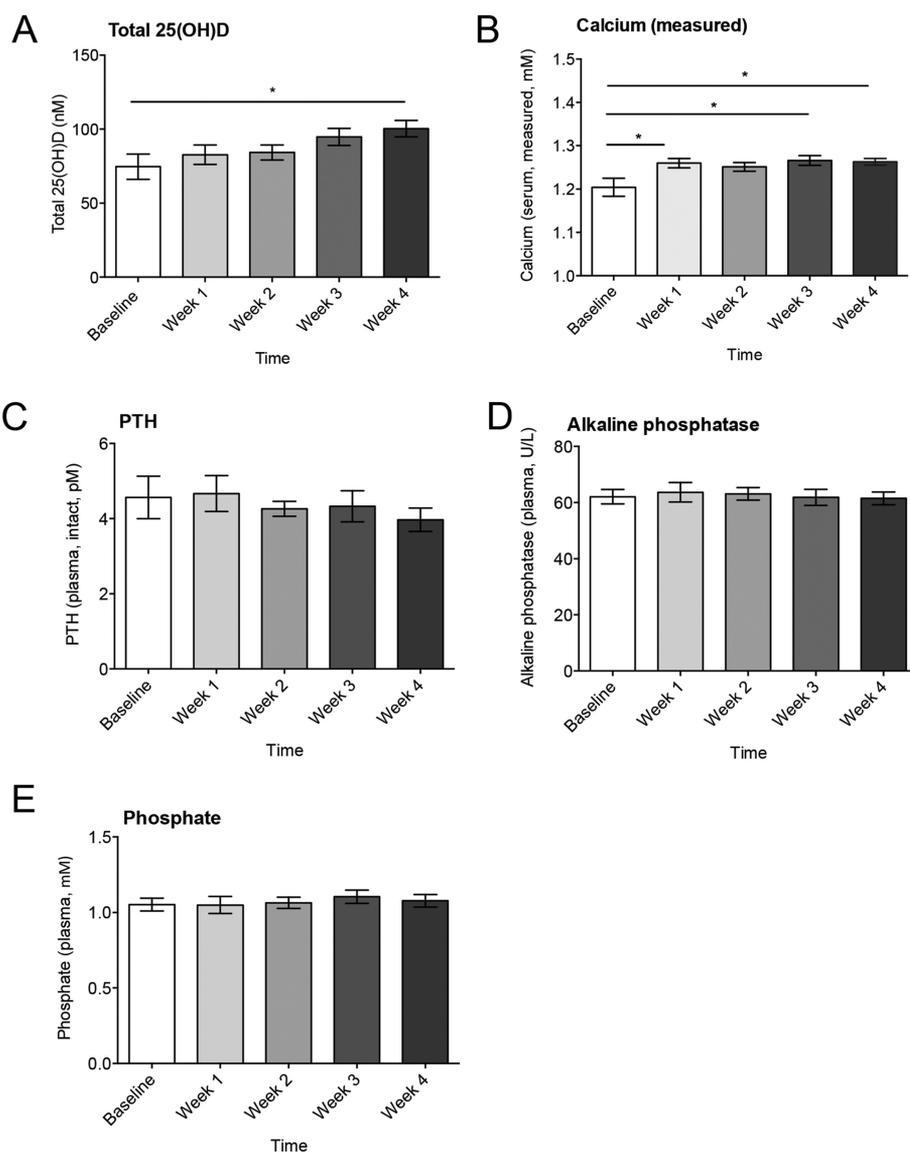


Figure 1. High-dose vitamin D increased circulating total 25(OH)D levels after IM injection. Blood levels (serum or plasma, as indicated) of (A) total 25(OH)D (measured via LC-MS/MS in serum), (B) calcium, (C) parathyroid hormone (PTH), (D) alkaline phosphatase, and (E) phosphate were tracked weekly for 4 weeks after the injection. Data are shown as mean \pm SEM (* $P < 0.05$).

(600 000 IU). After a large IM bolus, there was a modest and sustained increase in serum 25(OH)D₃ levels and elevated metabolism of 25(OH)D₃, as shown by the increase in levels of the breakdown product, 24,25(OH)₂D₃. There was an increase in serum levels of ionized calcium within the normal range. There were no significant changes in other markers of bone metabolism in blood or urine.

The active form of vitamin D, 1,25(OH)₂D plays a critical role in bone metabolism through regulation of calcium and phosphate homeostasis. Falling calcium levels stimulate the release of PTH, which, in turn, upregulates the conversion of 25(OH)D to 1,25(OH)₂D. The latter enhances the intestinal uptake and renal retention of calcium and the immobilization of phosphate within

bone (22). When this process is insufficient to return calcium levels to homeostasis (e.g., because there is insufficient dietary calcium), 1,25(OH)₂D induces the mobilization of skeletal calcium from the bone matrix (causing an elevation of bone-specific alkaline phosphatase; 23) to restore blood calcium levels (24). Thus, vitamin D is required for bone health; severe vitamin D deficiency results in loss of mineralization of bone, leading to rickets in children and osteomalacia in adults. Similarly, vitamin D toxicity manifests as hypercalcemia and hyperphosphatemia, and the suppression of PTH secretion has been explored as a marker of vitamin D adequacy (2).

In a previous study, high-dose oral vitamin D supplementation modified bone metabolism and increased the incidence of falls

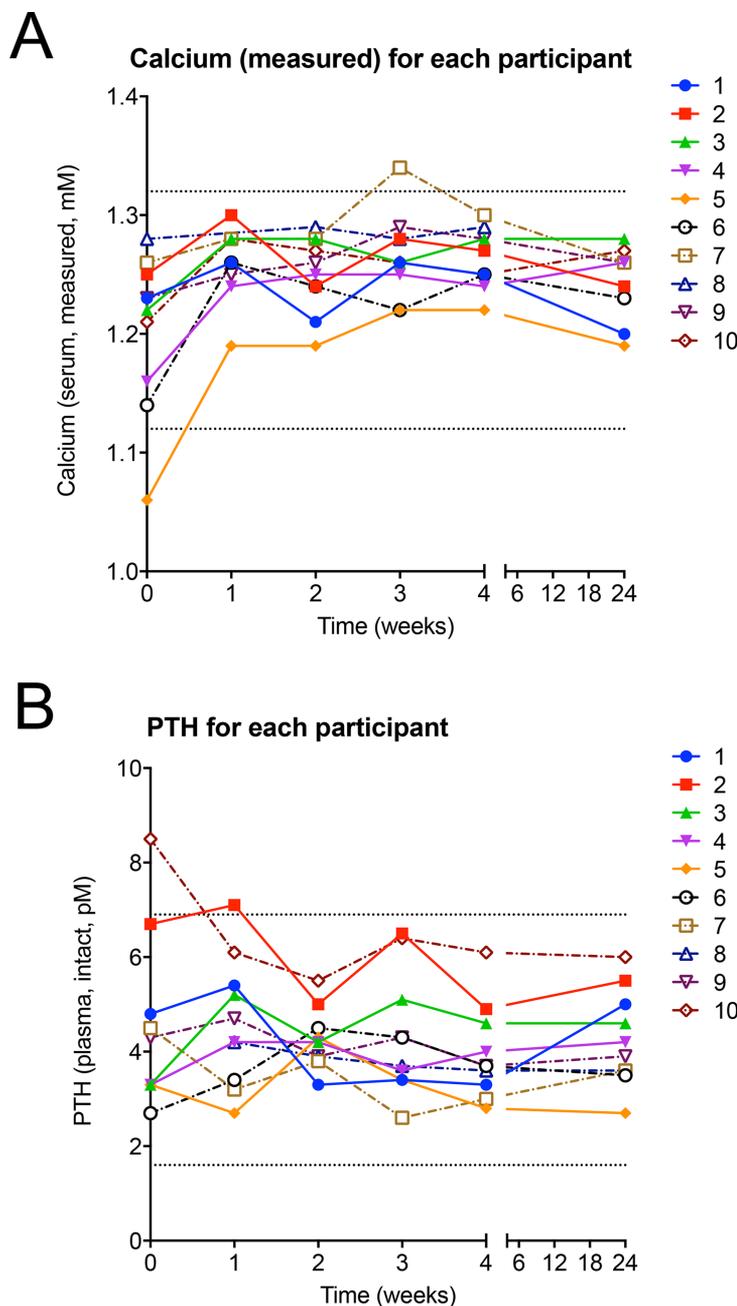


Figure 2. There was little change in markers of bone metabolism up to 24 weeks after IM injection of a large bolus of vitamin D. Blood levels of (A) calcium and (B) parathyroid hormone (PTH) are shown for each individual, measured weekly for 4 weeks and then 24 weeks after the injection. Broken lines in (A) and (B) represent the clinically normal ranges for calcium and PTH, respectively.

and fractures in the first few months post-treatment (14). There is similar evidence suggesting that IM injection of vitamin D may perturb bone health in elderly people, although this effect may be sex specific. Although an annual IM injection of high-dose vitamin D₂ (150 000–300 000 IU) reduced the proportion of fractures in elderly male participants in comparison to that observed in a control group (25), increased rates of hip fracture were observed in women (but not men) receiving an IM injection of 300 000 IU vitamin D₂ (compared to those receiving placebo; 26). Notably, we did not observe any early effects on alkaline phosphatase or PTH, and calcium levels remained within the normal range, although serum calcium levels increased during the first month after supplementation. In a study in which the incidence of increased falls was observed in elderly people administered higher monthly oral doses of vitamin D₃ (60 000 IU or 24 000 IU plus 300 µg calcifediol; 25(OH)D) compared to a control group (24 000 IU vitamin D₃), a transient increase in calcium excretion (in urine)

was observed in the treatment groups (with no change in serum calcium; 27). Further studies examining the effects of high-dose vitamin D supplementation on biochemical markers of bone health in the months immediately after supplementation are needed.

One motivation for doing this study was to quantify the levels of minor vitamin D metabolites such as 25(OH)D₃-26, 23-lactone, anticipating that we would observe a very high spike in 25(OH)D₃ levels such as can be seen after high-dose oral vitamin D supplementation (reviewed in ref. 13). However, the IM dosing strategy did not cause extreme changes in total 25(OH)D concentrations and we were not able to quantify these metabolites. We did note an increase in 24,25(OH)₂D₃ levels, along with a reduction in the 25(OH)D₃-to-24,25(OH)₂D₃ ratio, probably as a result of upregulation of the vitamin D catabolism pathway. There was a nonsignificant reduction in 25(OH)D₂ as 25(OH)D₃

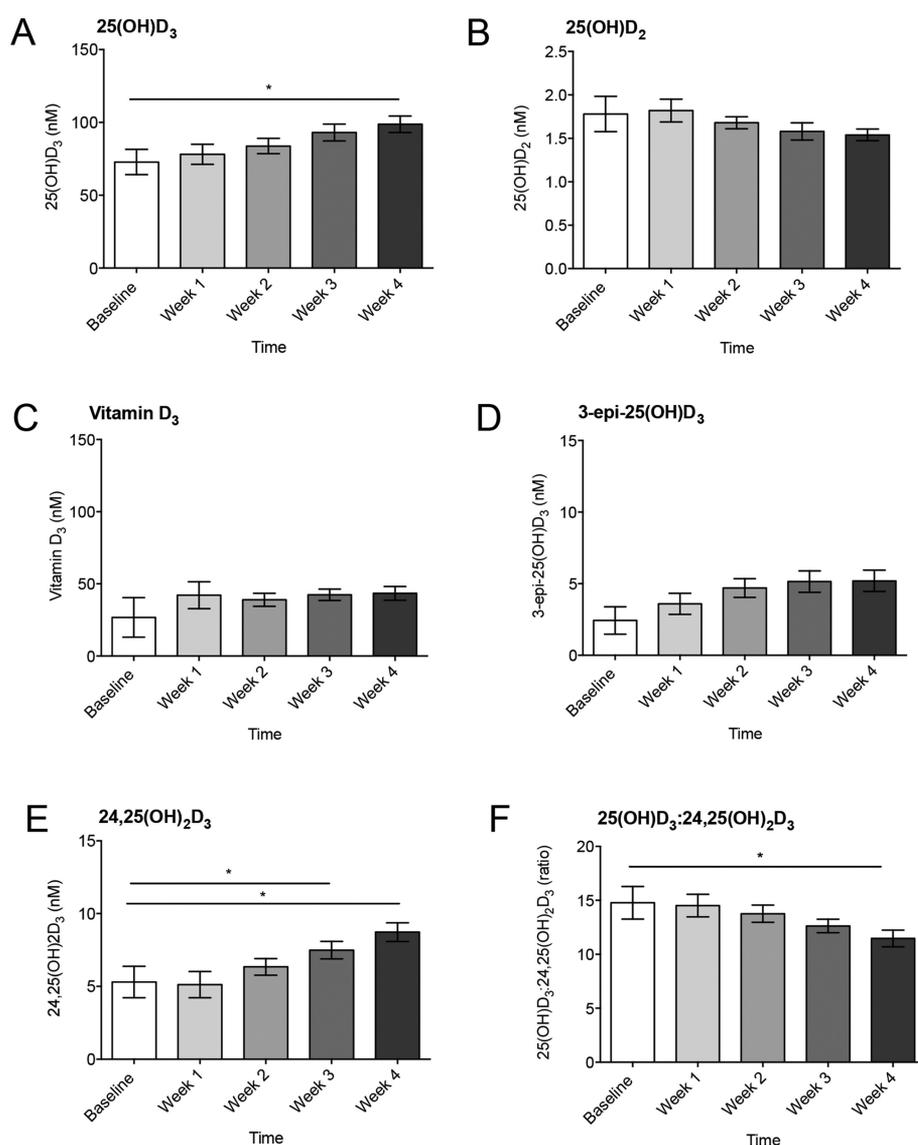


Figure 3. Serum levels of 25(OH)D₃ and 24,25(OH)₂D₃ increased 4 weeks after IM injection, as determined by LC-MS/MS. Ten individuals with a prior history of vitamin D insufficiency were injected IM with 600 000 IU of vitamin D₃. The serum levels of (A) 25(OH)D₃, (B) 25(OH)D₂, (C) vitamin D₃, (D) 3-epi-25(OH)D₃ [the C₃-epimer of 25(OH)D₃], (E) 24,25(OH)₂D₃, and (F) 25(OH)D₃-to-24,25(OH)₂D₃ ratio were tracked weekly for 4 weeks (using the LC-MS/MS assay). Data are shown as mean ± SEM (**P* < 0.05).

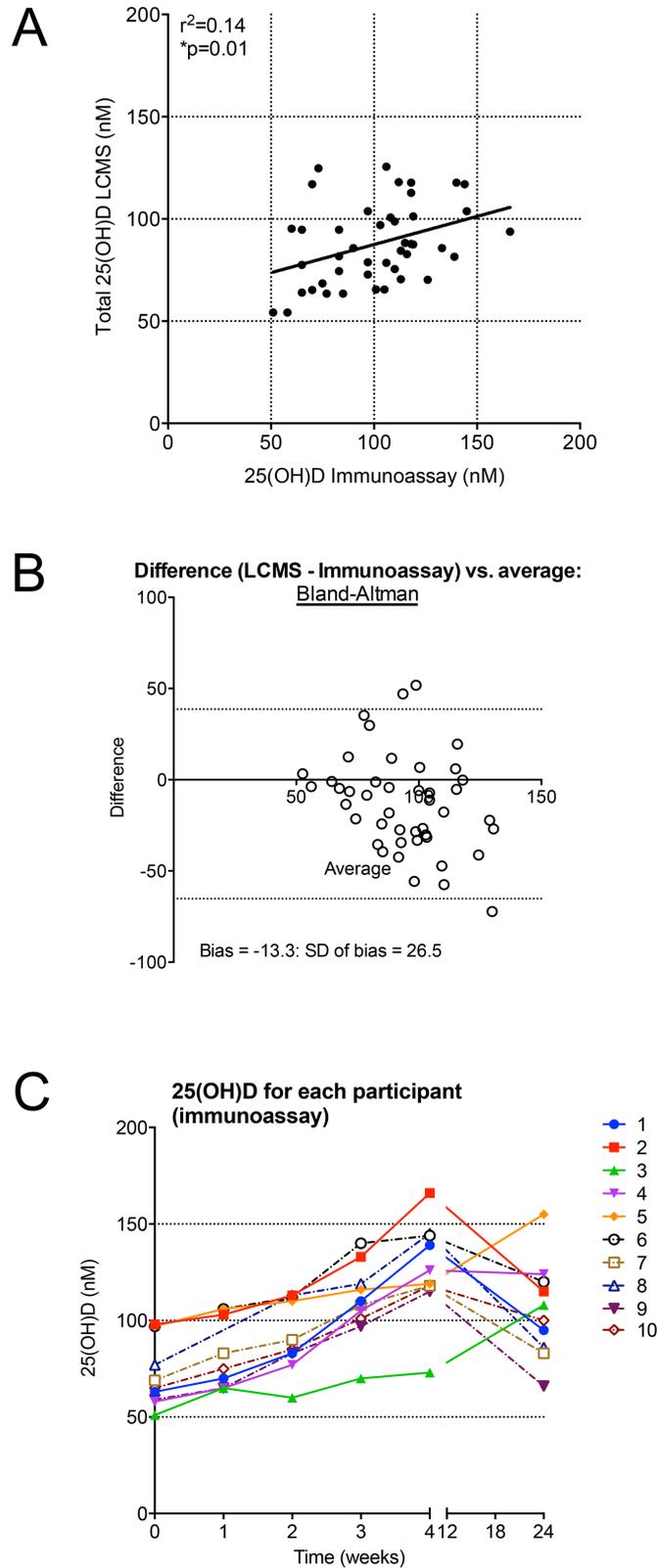


Figure 4. (A, B) Comparison of serum 25(OH)D levels measured by immunoassay and by LC-MS/MS and (C) serum 25(OH)D levels up to 24 weeks after IM injection bolus measured by immunoassay. (A) A significant linear correlation (Pearson test, $*P < 0.05$) was detected. (B) A Bland-Altman plot illustrates the agreement between the tests. There was a positive bias toward the immunoassay (mean \pm SD was 13.3 ± 26.5). The broken lines show the 95% confidence interval limits. (C) Serum 25(OH)D levels as measured by the immunoassay are shown for each individual, measured weekly for 4 weeks and then 24 weeks after the IM injection of a large bolus of vitamin D₃. The broken lines denote vitamin D deficiency/inadequacy [50 nM 25(OH)D] and two upper levels of vitamin D sufficiency [100 and 150 nM 25(OH)D].

concentration increased, also likely due to the induction of vitamin D catabolism through increased 24-hydroxylase enzyme activity.

Some limitations of the current study include the small sample size and the heterogeneous nature of the recruited population. Compared to daily supplementation, treatment with an IM bolus of vitamin D₃ is far less common as a treatment for vitamin D deficiency, limiting the size of the potential study population. Participants covered a wide age range (from 35 to 91 years) and were diagnosed with a range of conditions, some of which could affect aspects of vitamin D synthesis (e.g., underlying liver diseases like hemochromatosis). Therefore, it is not clear how applicable the findings here are to a healthy population, and further work is required to confirm that these findings are applicable more widely. A final caveat is that these participants were all vitamin D sufficient at baseline, and steeper changes in 25(OH)D level occur after supplementation when the baseline concentration is lower (11).

On the basis of the results from this small study, this dosing strategy of a large IM bolus of vitamin D₃ appears to be safe and effective in raising and sustaining serum 25(OH)D levels, at least for a 24-week period, such as for maintaining vitamin D sufficiency through winter. The IM dosing gave a slower-than-anticipated rise in total 25(OH)D levels, and all of the changes in biochemical parameters remained within the normal clinical ranges. None of the participants achieved total 25(OH)D levels (measured using the standardized reference assay) that exceeded the IOM guidelines for an upper limit (125 nM); however, the immunoassay had a positive bias of 13 nM, so clinicians testing follow-up vitamin D status may indeed record higher levels than in the IOM guidelines (2). This variability underlines the importance of assay standardization and informing clinicians of the potential errors in the values returned from nonstandardized assays.

Acknowledgments

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