Efficacy of High Dose Vitamin D Supplements for Elite Athletes

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Abstract

Purpose: Supplementation with dietary forms of vitamin D is commonplace in clinical medicine, elite athletic cohorts and the general population, yet the response of all major vitamin D metabolites to high doses of vitamin D is poorly characterized. We aimed to identify the responses of all major vitamin D metabolites to moderate and high dose supplemental vitamin D_3.

Methods: A repeated measures design was implemented in which 46 elite professional European athletes were block randomized based on their basal 25[OH]D concentration into two treatment groups. Athletes received either 35,000 or 70,000 IU.week^{-1} vitamin D_3 for 12 weeks and 42 athletes completed the trial. Blood samples were collected over 18 weeks to monitor the response to supplementation and withdrawal from supplementation.

Results: Both doses led to significant increases in serum 25[OH]D and 1,25$[OH]_2D_3$. 70,000 IU.week^{-1} also resulted in a significant increase of the metabolite 24,25$[OH]_2$D at weeks 6 and 12 that persisted following supplementation withdrawal at week 18, despite a marked decrease in 1,25$[OH]_2D_3$. Intact PTH was decreased in both groups by week 6 and remained suppressed throughout the trial.

Conclusions: High dose vitamin D_3 supplementation (70,000 IU.week^{-1}) may be detrimental for its intended purposes due to increased 24,25$[OH]_2$D production. Rapid withdrawal from high dose supplementation may inhibit the bioactivity of 1,25$[OH]_2D_3$ as a consequence of sustained increases in 24,25$[OH]_2$D that persist as 25[OH]D and 1,25$[OH]_2$D concentrations decrease. These data imply that lower doses of vitamin D_3 ingested frequently may be most appropriate and gradual withdrawal from supplementation as opposed to rapid withdrawal may be favorable.

Key Terms: 25-hydroxyvitamin D, 24,25-dihydroxyvitamin D, 1,25-dihydroxyvitamin D_3, parathyroid hormone, vitamin D

Text

Introduction

Vitamin D and its metabolites can be described as a group of seco-steroid hormones derived primarily from dermal synthesis following ultraviolet B (UVB) radiation exposure (sunlight) and also from the diet. Cholecalciferol (vitamin D_3), resulting from both skin exposure to UVB and in limited amounts from dairy products, oily fish and meat, is considered to be the major contributor to vitamin D concentration (13); whereas ergocalciferol (vitamin D_2) is exclusively derived from the diet of irradiated plants and mushrooms, and appears to have less biological significance. Following the photosynthetic conversion of 7-dehydrocholesterol to pre-vitamin D_3 and subsequently vitamin D_3 (or cholecalciferol, 13) or following dietary intake, vitamin D is transported in the circulation to the liver bound to the vitamin D binding protein (DBP), where it is hydroxylated at C-25 by the cytochrome P450 enzyme CYP27A1 (25-hydroxylase) to form 25-hydroxyvitamin D (25[OH]D or calcidiol). This metabolite is then carried, again by DBP, to the kidney where at the proximal renal tubule it is hydroxylated by CYP27B1 (1α-hydroxylase) at C-1α to form the biologically active metabolite, 1,25-dihydroxyvitamin D_3 (1,25$[OH]_2$D or calcitriol)(1). The active compound, 1,25$[OH]_2$D has long been known as a potent modulator of mineral homeostasis via transactivation of genes related to the maintenance of calcium and phosphate homeostasis. This biological activity is achieved through interaction of 1,25$[OH]_2D_3$ with its receptor, the vitamin D Receptor (VDR), which heterodimerizes with retinoid X receptor to form a transcriptional complex that can bind to vitamin D response elements in the promoter of vitamin D regulated genes (11).

In the past decade, understanding of the biological effects of vitamin D has grown exponentially due to the development of the vitamin D knockout mouse (21) and high throughput gene microarray techniques (2). Vitamin D is now understood to be not only an important regulator of mineral homeostasis but may be influential in cell proliferation and differentiation (20), innate and acquired immunity (12), muscle development and repair (25, 26) and in the prevention of psychological diseases such as Alzheimer's. This is particularly pertinent given the growing understanding that low vitamin D concentrations (< 30 $nmol.L^{-1}$) are highly prevalent worldwide in general (33) as well as

athletic populations (3, 23). Despite a rich research base characterizing the biological importance of vitamin D, the regulation of the vitamin D endocrine system is less well understood. Practically this is an important consideration given that some authors advocate high doses of vitamin D, be administered to individuals with osteomalacia/rickets caused by severe vitamin D deficiency (defined < 12.5 nmol/L), to ameliorate symptoms (14, 18). In the context of professional sport it is commonplace to supplement entire teams with a blanket approach to vitamin D supplementation, often without basal concentrations being assessed, and with a target concentration of >100 nmol/L the aim. Perhaps even more concerning is that the sports teams have access to vitamin D in single capsule form at doses up to 50,000 IU (1,250 µg) making single dose weekly supplementation with mega doses practically very simple and without definitive guidelines for supplementation this could result in more harm than benefit. Such practice is in discord with recommendations set by the European Food Safety Authority, whom advise a safe daily upper limit of 4,000 $IU.day^{-1}$ (8), in line with advice portrayed by the US Institute of Medicine (IoM) guidelines for vitamin D intake (32). Notably the US IoM also state a no adverse effect limit (NOAEL) of 10,000 $IU.day^{-1}$. Evidence does not exist to appropriately define the effect that high dose blanket supplementation protocols, commonly employed in elite sport, have upon the negative regulators of the vitamin D endocrine system, notably 24,25-hydroxylase (CYP24A1 or 24-hydroxylase), which functions to inactivate both 25[OH]D and $1,25[OH]_2D_3$ (15) by hydroxylation at C-24. It is important to characterize the response high dose blanket approaches in order to avoid potentially detrimental effects of too much supplemental vitamin D and contribute toward the establishment of the most safe and effective vitamin D supplementation schemes for elite athletes.

The current study therefore aimed to characterize the serum responses of the major vitamin D metabolites, 25[OH]D, $1,25[OH]_2D_3$, $24,25[OH]_2D$ and iPTH to high dose vitamin D supplementation (35,000 and 70,000 IU vitamin D_3 weekly) in an elite professional team sport cohort. It was hypothesized that supplementation would dose dependently increase total serum 25[OH]D and the active metabolite $1,25[OH]_2D_3$ in a concomitant manner but would also increase the production of $24,25[OH]_2D$.

Methods

Participants

Forty-six elite male elite professional team sport athletes volunteered to participate in the current trial (Age = 26 ± 3 years, height = 1.86 ± 0.6 m, weight 101.5 ± 11 kg, fat mass 11.4 ± 3 %). Participants underwent a medical screening and provided full informed consent prior to inclusion into the study. Participants were excluded if they were currently taking vitamin D supplements, using sun beds or injured at the time of the study. Ethical approval was granted by the ethics committee of Liverpool John Moores University (Ethics code 12/SPS/047). The recruitment for the study began in November 2012 and testing commenced in the same month. The study was concluded in April 2013. The study was conducted at latitude 52°N during the winter months in order to limit sunlight exposure.

Supplementation

Participants were randomly allocated to either 35,000 or 70,000 IU.week^{-1} supplemental vitamin D_3 (Maxi Nutrition, UK), herein referred to as moderate and high, respectively. Randomisation was achieved with blocking based on baseline serum 25[OH]D and the use of a random number table to allocate participants into balanced groups. The random allocation sequence was allocated by a member of the research team and known by the rest of the research team at the point of supplement administration. The supplemental doses were chosen based on the fact that they represent widely reported supplement strategies (PubMed literature based search), applied experience of the authors in both clinical and elite sporting settings and also the NOAEL set by the US IoM. Supplements were taken orally as a bolus in capsule form on a weekly basis to increase compliance with the protocol, which was 100% as club staff were present during the weekly distribution of supplementation and monitored the ingestion of capsules in order to track compliance. Supplementation continued for 12 weeks at which point supplementation was ceased to monitor the response of vitamin D metabolites to withdrawal. Participants were blinded to the supplement they were receiving. Forty two players completed the trial whilst four dropped out or were excluded for the following reasons; one player did not tolerate venipuncture, one player would not comply with the supplementation protocol, two players used sun beds during the trial. The vitamin D supplements were batch screened by

chromatography and mass spectrometry for contaminants and confirmation of vitamin D content stated on the label. Screening was performed in accordance with ISO standard 17025. Sunlight exposure was minimal during the trial due to the latitude at which the players were based. Participants travelled for a two-day match fixture to a foreign climate at latitude 43ºN, in December during a period of significant cloud cover. Thus, players were exposed to minimal amounts of sunlight during the study period although no direct measurement of UV exposure was taken.

Blood Sampling

Blood samples were drawn prior to supplementation (basal and then at 6, 12 and 18 (withdrawal) weeks from the start of supplementation. Blood was obtained from the antecubital vein into two serum separator tubes and two K_2EDTA tubes (Becton, Dickinson and Co. Oxford, UK). Samples were then separated to isolate serum/plasma via centrifugation at 1500 RCF for 15 minutes at 4 ºC. Serum/plasma was extracted and stored at -80 ºC until required for analysis. All samples were collected in the medical room of a professional sports club.

Analysis of Vitamin D Metabolites and Parathyroid Hormone

Liquid chromatograph tandem mass spectrometry (LC-MS/MS) analysis of 25[OH]D_3, 25[OH]D_2, 24,25$[OH]_2D_3$ and 24,25$[OH]_2D_2$ was performed using a Micromass Quattro Ultima Pt mass spectrometer (Waters Corp., Milford, MA, USA). NIST SRM972a traceable 25[OH]D_3 and 25[OH]D_2 calibration standards (Chromsystems, München, Germany) and quality controls (UTAK Laboratories, CA, USA) were purchased commercially, ranged from 0-200 nmol/L. 24,25$[OH]_2D_3$ and 24,25$[OH]_2D_2$ calibration standards were prepared from certified standards (IsoSciences, King of Prussia, PA, USA) spiked into human vitamin D depleted serum (BBI Solutions, Cardiff, UK), ranged from 0-14.8 nmol/L. To 100 µL of human serum samples, calibration standards and quality controls, 200 µL of pretreatment solution consist of deuterated 25[OH]D_3-[$^2H_6$] and 24R,25$[OH]_2D_3$-[$^2H_6$] in isopropanol:water 50:50 (v/v) was added to displace binding proteins. After mixing, the samples were loaded onto Supported Liquid Extraction (SLE+) plates (Biotage, Uppsala, Sweden), which were

eluted with 1.5 mL of n-heptane. The extraction procedure was performed by Extrahera positive pressure automation system (Biotage). Eluents were dried under nitrogen, followed by reconstitution with 50 µL 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) (Sigma-Aldrich, Dorset, UK). After a 30 minute incubation period at room temperature, 50 µL of water was added to stop the reaction. 20 µL of the derivatised extracts were injected into LC-MS/MS. Separation of vitamin D metabolites were achieved using core-shell C18 2.7µm 50 × 2.1mm (Restek, PA, USA) reversed-phase column. A gradient elution profile was set up using mobile phase (A) LCMS grade water and (B) methanol containing 0.2 mM methylamine in 0.1% formic acid. The gradient at the start was 50:50 (v/v) at column flow rate of 0.4 mL/min, gradually increased to 99% B. 24,25$[OH]_2D_3/D_2$ and 25$[OH]D_3/D_2$ peaks were eluted at 1.39, 1.42, 1.68 and 1.73 minutes respectively (See **Supplementary Digital Content 1,** A Chromatogram from an extracted sample containing 86 nmol/L of 25[OH]D3/D2 and 5.3 nmol/L of 24,25[OH]2D3/D2). Optimisation of MS/MS conditions were carried out by direct infusion of derivisated standards into the ion source via a T-connector. The precursor to product ion transitions were based on the molecular weight of the methylamine adduct of PTAD derived products (See **Supplementary Digital Content 2**, a table highlighting mass spectrometer parameter settings and multiple reaction monitoring (MRM) precursor to product ion transitions for 25$[OH]D_3/D_2$ and 24,25$[OH]_2D_3/D_2$.).

Measurements of total 1,25$[OH]_2$D were carried out using a commercially available enzyme immunoassay kit (IDS, Boldon, UK). Duplicate samples underwent immunoextraction with a 1,25$[OH]_2$D specific solid phase monoclonal antibody and incubated overnight with sheep anti-1,25$[OH]_2$D. 1,25$[OH]_2$D linked biotin was added the next day, followed by horseradish peroxidase labelled avidin to selectively bind to biotin complex. After a wash step, colour was developed using a chromogenic substrate (3, 5', 5, 5'- Tetramethylbenzidine; TMB). The absorbance of the stopped reaction mixtures were read in a microtiter plate spectrophotometer (Multiskan Go, Thermo Scientific, Finland) at wavelength of 450 nm.

Intact parathyroid hormone in K_2EDTA plasma samples were determined by electrochemiluminescence immunoassay (ECLIA) performed using Roche e601 analyser (Mannheim, Germany). Two labelled monoclonal antibodies were employed to react with the N-terminal fragment (1-37) and C-terminal fragment (38-84) of PTH; forming a sandwich complex. The antibody complex was magnetically captured and washed to remove unbound substances. A voltage was applied to induce chemiluminiescent emission, which was measured by a photomultiplier.

Assay validation

Summary of assay characteristics are described in **Supplementary Digital Content 3** (A table highlighting the assay characteristics for each parameter measured**)**. Linearity of the methods were evaluated by analysing stock standards made up from reference calibration solutions spiked into human sera. Standard curve was constructed by plotting the analyte response against the concentration of their respective standards. A calibration curves were accepted as linear if the weighted linear regression produced a correlation coefficient (r^2) value of >0.999. Intra and inter-assay imprecision of the methods were assessed by running quality control (QC) materials 10 times within a single run and separately over a three-month period. Assays were deemed acceptable when the QC results fall within ±2SD from the mean value. Lower limit of quantification (LLoQ) was determined by the lowest concentration quantifiable with a precision CV of 20% over 12 replicates and minimum peak signal-to-noise ratio of 10:1 (16). Assay recovery was determined by analysing samples containing a fixed amount of the analyte and calculate the percentage of the measured value against the sum of endogenous value plus spiking concentration.

Statistical Analyses

Comparisons of basal total serum 25[OH]D concentration for the two dose groups were made using an independent t-test. The effects of vitamin D dose and time on all repeated measures variables was determined using linear mixed modeling. Time (basal, weeks 6, 12 and 18) and dose (moderate and high) were modeled as fixed effects and participants as a random effect, with time being modeled as a continuous variable where linear or quadratic responses were observed. The covariance structure that

minimized the Hurvich and Tsai's criterion (Corrected Akaike Information Criterion; AICC) value was used for the final fitted model for each metabolite. Where significant main or interaction effects were observed, post hoc pairwise comparisons were made with Sidak adjusted p-values. All statistical procedures were conducted using SPSS v22 for Windows (IBM, Armonk, NY, USA), and two-tailed statistical significance was accepted at the $p < 0.05$ level. Descriptive statistics are displayed as means ± standard deviation (SD). For the calculation of sample size, Minitab software was used. Pilot work from our laboratories during the winter months suggested that the standard deviation for test–retest serum 25[OH]D concentrations (taken 6 weeks apart) in young athletes is ~12 nmol.L^{-1}. To enable the detection of a meaningful 50 nmol.L^{-1} increase in total serum 25[OH]D concentration between pre-supplementation and post-supplementation with 80% power; n = 6 participants per group was required. Thus, the recruitment of an entire squad of 42 players provided a large enough sample size to make valid conclusions from the derived data.

Results

Of the 42 participants that were enrolled onto the trial, 40 were tested for all primary outcome measures. This was due to player commitment to international duty. However, no participants presented with adverse side effects to supplementation during the trial and thus no participant was withdrawn. Basal (pre-treatment) total serum 25[OH]D concentrations were 86 ± 20 and 85 ± 10 nmol.L^{-1} for high and moderate treatment groups, respectively (**Figure 1**). These concentrations were not significantly different between groups ($t = 0.20$, $P = 0.84$). .

<**Figure 1**>

Total serum 25[OH]D, displayed a significant interaction effect ($F = 4.30$, $P = 0.008$) between dose and time. Exploration of this interaction identified that both groups showed significantly elevated 25[OH]D concentrations at weeks 6 (moderate = 108 ± 22 and high = 122 ± 25 nmol.L^{-1}) and 12 (moderate = 163 ± 47 and high = 188 ± 66 nmol.L^{-1}). However, upon supplementation withdrawal the moderate treatment group demonstrated a return to 25[OH]D concentrations comparable with basal by week 18 ($P = 0.178$) whereas the high treatment did not ($P = 0.007$; **Figure 2A**).

Concentrations of the bioactive metabolite, $1\alpha,25[OH]_2D_3$ demonstrated a significant main effect for time ($F = 6.13$, $P = 0.001$). In the moderate treatment group, peak concentrations occurred at week 6 (141 ± 58 pmol.L^{-1}). However, a delayed response was observed in the high group with peak $1\alpha,25[OH]_2D_3$ levels occurring at week 12 (112 ± 66 pmol.L^{-1}, **Figure 2B**). Following supplementation withdrawal, the concentration of $1\alpha,25[OH]_2D_3$ declined significantly in both groups at week 18 (moderate, 107 ± 32 and high = 104 ± 42 pmol.L^{-1}, $P = 0.042$) compared with concentrations at week 12 and were comparable with basal by this time point ($P = 0.332$).

The inactivated metabolite, 24,25[OH]D, showed comparable values between groups at basal (moderate = 8.3 ± 2.5 and high = 7.1 ± 1.7 nmol.L^{-1}). Both groups showed significant increases in this metabolite by week 6 (moderate, $P = 0.011$ and high, $P = 0.000$) that continued to increase between weeks 6 and 12. A significant interaction effect was also detected as the high treatment group displayed markedly higher peak 24,25[OH]D concentrations (17.3 ± 4.5 nmol.L^{-1}) versus moderate treatment (11.8 ± 1.9 nmol.L^{-1}). Interestingly, whereas $1\alpha,25[OH]_2D_3$ declined following supplementation withdrawal, 24,25[OH]D remained significantly elevated at week 18 when compared with basal values in both treatment groups (moderate, 11.4 ± 2.2 and high, 15.7 ± 4.6 nmol.L^{-1}; $P = 0.000$ for both groups).

Intact parathyroid hormone was significantly suppressed in both groups by week 6 (moderate = 2.3 ± 0.8 and high = 1.9 ± 0.4 pmol.L^{-1} vs basal values in moderate = 3.2 ± 2.3 and high 2.8 ± 1 pmol.L^{-1}) and remained suppressed throughout the trial and following the withdrawal of supplementation (moderate = 2.1 ± 0.8 and high 2 ± 0.6 pmol.L^{-1}).

<**Figure 2**>

Several studies have also examined the ratios of 25[OH]D and $1\alpha,25[OH]_2D_3$ to 24,25[OH]D and it is evident that additional information can be obtained that is not always obvious when measuring absolute concentrations (17, 19, 22). In addition, it has been suggested that the ratio of 25[OH]D to 24,25[OH]D is predictive of the 25[OH]D response to supplementation (34) giving important information that surpasses simply measuring the absolute values for these metabolites. Therefore we

also calculated ratio data for the relationships between 25[OH]D and 1α,25$[OH]_2D_3$ to 24,25[OH]D. The ratio between 25[OH]D and 1α,25$[OH]_2D_3$ showed a significant main effect for time (F = 3.39, *P* = 0.023) but no group main effect with ratio's for both supplemental treatments decreasing over the duration of the study, reaching significance by week 12 (*P* = 0.039) and increasing toward pre treatment values at week 18 (**Figure 3A**). The ratio of 25[OH]D to the inactivated 24,25[OH]D also showed a main effect for time (F = 14.94, *P* = 0.000) and the absence of a group main effect. Both groups demonstrated a significant lower ratio at week 18 compared to basal (*P* = 0.000; **Figure 3B**). Finally, the ratio of the bioactive 1α,25$[OH]_2D_3$ against the inactivated 24,25[OH]D was assessed. A significant interaction effect was observed as at week 6 the moderate treatment group showed an increased ratio whilst the high treatment group showed an inverse relationship. However, by week 12 both treatment groups showed a significantly lower ratio of 1α,25$[OH]_2D_3$:24,25[OH]D (*P* = 0.005) that was maintained at week 18 following supplementation withdrawal (*P* = 0.003; **Figure 3C**).

<**Figure 3**>

Discussion

The current investigation sought to define the serum responses of the major vitamin D metabolites in a professional athletic cohort to establish the efficacy of a blanket supplementation approach using two commonly employed and commercially available doses of vitamin D_3.Our main findings demonstrate that both 35,000 and 70,000 $IU.week^{-1}$ oral vitamin D_3 supplementation significantly elevated total serum 25[OH]D concentrations. The highest dose led to an initial rapid increase in 1.25$[OH]_2D$ but then a decrease in serum 1,25$[OH]_2D$ at week 12 when there was a significant increase of 24,25$[OH]_2D_3$ which had also been significantly increased at week 6. Resultantly, these responses led to a significantly lower ratio of 1,25$[OH]_2$ D to 24,25$[OH]_2D$ from week 6 with the higher, 70,000 IU treatment. Following the withdrawal of supplementation, the concentrations of 25[OH]D and 1α,25$[OH]_2D$ return to basal values within 6 weeks. These data imply that high doses of supplemental vitamin D_3 are sufficient to markedly induce the expression of 24-hydroxylase leading to the negative control of 1,25$[OH]_2D$ activity. Finally, we demonstrate that elevating serum 25[OH]D and 1α,25$[OH]_2D_3$ suppresses iPTH appearance in circulation. This finding is in agreement with previous

data published by our group (27) and is underpinned by the understanding that DNA binding sequences exist in the PTH gene (6), permitting suppression of the gene when adequate ligand ($1,25[OH]_2D$) is available to induce transcriptional suppression by the VDR (30).

The fact that the concentration of serum $24,25[OH]_2D_3$ did not show a decline along with $1,25[OH]_2D$ following the withdrawal of supplementation has practical implications. The finding suggests that the activity of 24-hydroxylase is sustained following large increases in $1,25[OH]_2D$ and may persist and decrease both the concentration and subsequent biological activity of $1,25[OH]_2D$. Evidence is now emerging that the $24,25[OH]_2D$ metabolite may act at the VDR as a "blocking molecule" binding to the VDR decreasing 1,25 $[OH]_2D$ activity (5). Since $24,25[OH]_2D$ is present in the circulation in nmol/L concentration compared to pmol/L for $1,25[OH]_2D$ the significantly higher prevailing $24,25[OH]_2D$ concentrations are liable to contribute to a significant decrease in the activity of the biologically active $1,25[OH]_2D$. Thus a dual regulation would appear to be present in subjects receiving high dose vitamin D supplementation preventing possible toxic effects, namely, *1*) the positive stimulation of 24-hydroxylase and *2*) the negative control of the vitamin D receptor activity. This notion is supported by previous mechanistic evidence that has determined the function of $1\alpha,25[OH]_2D$ in regulating 24-hydroxylase activity *in vitro*. Identification of two VDREs in the 5' region of the CYP24A1 promoter demonstrated that $1\alpha,25[OH]_2D_3$ could potently trans-activate the CYP24A1 gene, inducing a 10 to 100 fold increase in CYP24A1 mRNA to limit the transcription of $1\alpha,25[OH]_2D_3$ responsive genes (24). Moreover, *in vitro* studies on primary human myoblasts indicate that the induction of 24-hydroxylase is dose dependent (10), which is in agreement with the serum response of 24,25[OH]D seen *in vivo* in the current study.

These findings may explain reported observations of deterioration in skeletal muscle function, increased risk of falls and increased fracture risk in individuals supplemented with extreme dose vitamin D_3 to correct for severe vitamin D deficiency. As an example, in a large-scale trial (n = 2256), women $\geq$ 70 years old were randomized to either 500,000 IU of vitamin D_3 or placebo. The women randomized to the supplemental vitamin D_3 experienced significantly more falls than the placebo

group in the year following dosing of which the falls ratio was greatest in the first month following the one off 500,000 IU dose (29). Furthermore, in a retrospective observational cohort study, very low (< 10 nmol.L^{-1}) and high (above 140 nmol.$^{-1}$) concentrations of 25[OH]D showed an increased risk of all cause mortality indicating not only a lower limit but also an upper limit for serum 25[OH]D (7). This hypothesis also lends an explanation for the inconsistency in positive outcomes related to supplemental vitamin D reported by large-scale meta-analyses (4). It is reasonable to suggest that mega dose vitamin D supplements are detrimental to vitamin D target tissues by increasing the production of 24,25$[OH]_2$D, which may act to block the activity of the VDR. It will be necessary to now perform mechanistic studies that clarify the function of 24,25$[OH]_2$D and to determine whether high dose supplementation is detrimental to vitamin D signaling through the VDR.

The current trial also had limitations that are important to consider for the design of future work. Firstly, although one of our goals was to use a 'real world' blanket supplementation approach, we acknowledge that the same protocol used in other athletic cohorts with different body composition, genotype and lower basal serum 25[OH]D concentrations may yield different results. Determining the response of the vitamin D metabolites to a similar protocol as we have used here across wider athletic cohorts will allow more conclusive recommendations to be made on dosing concentration and frequency. Indeed, we have previously shown that basal 25[OH]D concentrations vary across athletes from different professional sports (3). This assumption is also true for female cohorts and as such we appreciate that our findings cannot be conclusively extended to the female athletic population. Regarding genotype, genotypic variation in the vitamin D binding protein influences the response to exogenous vitamin D (9) and little is known of the variation in genes encoding other vitamin D metabolizing enzymes such as CY24A1 and CYP24B1. We did not perform genotyping and in light of recent evidence, we fully support genotype-phenotype studies in the context of vitamin D in future. Combining a genotyping approach with vitamin D metabolite ratio data, the latter as we have performed in this study, will offer a great advancement in the understanding of how genotype and supplementation interact and how this can be managed. We also did not measure serum or urinary calcium concentrations, which are markers of vitamin D toxicity and also regulate the PTH response.

Measuring Ca^{2+} excretion would add another aspect to our findings, however we do maintain that the observed increases in 24,25[OH]D are indicative of too much exposure to exogenous vitamin D. Finally, future work should aim to monitor FGF-23, a bone derived hormone that can function to lower both 25[OH]D and 1α,25$[OH]_2D_3$ by inducing the CYP24 genes (28). FGF-23 may also be a player in regulating the metabolite response to high dose supplementation and at present its role in lowering 25[OH]D by promoting 24-hydroxylase expression is still disputed (31). Extending the current findings to a broader range of vitamin D concentrations, coupled with intracellular signaling cascades related to the vitamin D axis will yield the most inferential data, moving towards safer and more effective vitamin D supplementation practices in athletes.

Summary and implications for practice

The data presented here are the first to characterize the response of two major metabolites of vitamin D in response to two high-dose supplementation protocols in healthy professional athletes. The results demonstrate that a blanket approach of high-dose supplementation with 70,000 $IU.week^{-1}$ leads not only to increased 25$[OH]D_3$ and 1,25$[OH]_2D_3$ concentrations but also stimulates elevated concentrations of the vitamin D metabolite 24,25$[OH]_2D_3$, which has been previously shown to limit the transcriptional activity of 1,25$[OH]_2D_3$. We demonstrated that this negative regulatory effect persists following cessation of vitamin D_3 supplementation even as 1,25$[OH]_2D_3$ concentrations decrease. There are a number of novel key implications for practice that arise from our current observations. Firstly, we speculate that 'high dose' bolus supplementation with vitamin D_3 is likely to be detrimental to the intended targeted downstream biological functions due to significant increases in the negative regulatory molecule, 24,25$[OH]_2D$. Weekly doses amounting to more than 5,000 $IU.day^{-1}$ may need to be reassessed in light of our data. Rapid withdrawal from high dose supplementation may result in adverse outcomes as the concentration of 24,25$[OH]_2D_3$ remains elevated for several weeks following withdrawal from supplementation despite declines in 1,25$[OH]_2D_3$. If moderate to high doses of vitamin D_3 have been administered, a gradual withdrawal from supplementation is advisable. At present the optimal approach has not been established. Lower doses administered often (daily) may offer the most potent beneficial biological effects and limit the transactivation of CYP24A1 and

subsequent production of the negative regulatory molecule, 24,25$[OH]_2D_3$. Future research must aim to establish the appropriate dose and frequency of administration to achieve a positive increase in both 25$[OH]D_3$ and 1,25$[OH]_2D_3$ whilst limiting the appearance of increased 24,25$[OH]_2D_3$ concentrations. The generation of 24,25 $[OH]_2D$ may be an aspect of the body's defense mechanism to prevent "toxicity" when administered high doses of vitamin D. We postulate that single "super" doses of vitamin D_3 administered on a weekly basis as is common practice in many professional sporting teams, may result in similar rapid transient increases in 1,25$[OH]_2D_3$ leading to significant increases in the negative regulatory metabolite, 24,25$[OH]_2D_3$. Further studies will be required to determine if the relationship we have observed is seen with higher and lower doses of vitamin D.

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Author Contributions

DJO designed the research, conducted the research, analyzed data and wrote the manuscript. **JCYT** designed the research, conducted the research, designed and optimized all analytical techniques and analyzed the data. **WJB** conducted the research and provided essential materials for research. **AS** designed the research and conducted the statistical analyses. **WDF** designed the research, provided essential reagents for the research, analyzed the data and wrote the paper. **JPM** designed the research, analyzed the data and wrote the paper. **GLC** designed the research, conducted the research, analyzed the data, performed statistical analysis, wrote the paper and had primary responsibility for the final content.

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Supplementary Digital Content

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Figure Captions

Figure 1. Distribution plot of basal serum total 25[OH]D concentrations in participants allocated to the 35,000 (moderate) and 70,000 IU.week^{-1} (high) supplemental treatment groups. No significant differences were detected between groups for basal total serum 25[OH]D. Data were normally distributed with no significant difference detected between groups.

Figure 2. Serum responses of the major vitamin D metabolites with treatment of either 35,000 IU.week^{-1} or 70,000 IU.week^{-1} vitamin D_3 **a**) 25-hydroxyvitamin D (25[OH]D) **b**) 1α,25-dihydroxyvitamin D_3 (1α,25$[OH]_2D_3$) **c**) 24,25-hydroxyvitamin D and **d**) intact parathyroid hormone (iPTH). Samples were collected prior to supplementation (basal) and then at weeks 6, 12 and 18 of supplementation. At week 12, supplementation was stopped in both groups. * denotes significance for both groups compared with basal and # denotes significance for the 70,000 IU.week^{-1} compared with basal.

Figure 3. Relationships between the major vitamin D metabolites, expressed as ratio at all test time points. **a**) ratio of 25-hydroxyvitamin D (25[OH]D) to the biologically active 1α,25-dihydroxyvitamin D_3 (1α,25$[OH]_2D_3$). **b**) Ratio of 25[OH]D to the inactive metabolite 24,25[OH]D and **c**) ratio of 1α,25$[OH]_2D_3$ to 24,25[OH]D. * denotes significance for both groups compared with basal.