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Efficacy of High Dose Vitamin D Supplements for Elite Athletes

#Daniel J. Owens¹, #Jonathan C.Y. Tang², Warren J. Bradley¹, Andy S. Sparks³, William D. Fraser², James P. Morton¹ & Graeme L. Close¹

#Authors contributed equally to the manuscript

Affiliations: ¹Research Institute for Sport and Exercise Science, Liverpool John Moores University, Liverpool, UK. ²Faculty of Medicine and Health Science, Norwich Medical School, University of East Anglia, Norwich, United Kingdom. ³Department of Sport and Physical Activity, Edge Hill University, Ormskirk, United Kingdom.

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Corresponding Author: Prof. Graeme L. Close

Research Institute for Sport and Exercise Science, Liverpool John Moores University

Tom Reilly Building, Byrom Street, Liverpool, L3 5AF.

g.l.close@ljmu.ac.uk

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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 D₃.

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 D₃ 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]₂D₃. 70,000 IU.week⁻¹ also resulted in a significant increase of the metabolite 24,25[OH]₂D at weeks 6 and 12 that persisted following supplementation withdrawal at week 18, despite a marked decrease in 1,25[OH]₂D₃. Intact PTH was decreased in both groups by week 6 and remained suppressed throughout the trial.

Conclusions: High dose vitamin D₃ supplementation (70,000 IU.week⁻¹) may be detrimental for its intended purposes due to increased 24,25[OH]₂D production. Rapid withdrawal from high dose supplementation may inhibit the bioactivity of 1,25[OH]₂D₃ as a consequence of sustained increases in 24,25[OH]₂D that persist as 25[OH]D and 1,25[OH]₂D concentrations decrease. These data imply that lower doses of vitamin D₃ 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 D₃, parathyroid hormone, vitamin D

1 Text

2 Introduction

3 Vitamin D and its metabolites can be described as a group of seco-steroid hormones derived primarily
4 from dermal synthesis following ultraviolet B (UVB) radiation exposure (sunlight) and also from the
5 diet. Cholecalciferol (vitamin D₃), resulting from both skin exposure to UVB and in limited amounts
6 from dairy products, oily fish and meat, is considered to be the major contributor to vitamin D
7 concentration (13); whereas ergocalciferol (vitamin D₂) is exclusively derived from the diet of
8 irradiated plants and mushrooms, and appears to have less biological significance. Following the
9 photosynthetic conversion of 7-dehydrocholesterol to pre-vitamin D₃ and subsequently vitamin D₃ (or
10 cholecalciferol, 13) or following dietary intake, vitamin D is transported in the circulation to the liver
11 bound to the vitamin D binding protein (DBP), where it is hydroxylated at C-25 by the cytochrome
12 P450 enzyme CYP27A1 (25-hydroxylase) to form 25-hydroxyvitamin D (25[OH]D or calcidiol). This
13 metabolite is then carried, again by DBP, to the kidney where at the proximal renal tubule it is
14 hydroxylated by CYP27B1 (1 α -hydroxylase) at C-1 α to form the biologically active metabolite, 1,25-
15 dihydroxyvitamin D₃ (1,25[OH]₂D or calcitriol)(1). The active compound, 1,25[OH]₂D has long been
16 known as a potent modulator of mineral homeostasis via transactivation of genes related to the
17 maintenance of calcium and phosphate homeostasis. This biological activity is achieved through
18 interaction of 1,25[OH]₂D₃ with its receptor, the vitamin D Receptor (VDR), which heterodimerizes
19 with retinoid X receptor to form a transcriptional complex that can bind to vitamin D response
20 elements in the promoter of vitamin D regulated genes (11).

21

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

29 athletic populations (3, 23). Despite a rich research base characterizing the biological importance of
30 vitamin D, the regulation of the vitamin D endocrine system is less well understood. Practically this is
31 an important consideration given that some authors advocate high doses of vitamin D, be administered
32 to individuals with osteomalacia/rickets caused by severe vitamin D deficiency (defined < 12.5
33 nmol/L), to ameliorate symptoms (14, 18). In the context of professional sport it is commonplace to
34 supplement entire teams with a blanket approach to vitamin D supplementation, often without basal
35 concentrations being assessed, and with a target concentration of $>100 \text{ nmol/L}$ the aim. Perhaps even
36 more concerning is that the sports teams have access to vitamin D in single capsule form at doses up to
37 $50,000 \text{ IU}$ ($1,250 \mu\text{g}$) making single dose weekly supplementation with mega doses practically very
38 simple and without definitive guidelines for supplementation this could result in more harm than
39 benefit. Such practice is in discord with recommendations set by the European Food Safety Authority,
40 whom advise a safe daily upper limit of $4,000 \text{ IU}\cdot\text{day}^{-1}$ (8), in line with advice portrayed by the US
41 Institute of Medicine (IoM) guidelines for vitamin D intake (32). Notably the US IoM also state a no
42 adverse effect limit (NOAEL) of $10,000 \text{ IU}\cdot\text{day}^{-1}$. Evidence does not exist to appropriately define the
43 effect that high dose blanket supplementation protocols, commonly employed in elite sport, have upon
44 the negative regulators of the vitamin D endocrine system, notably 24,25-hydroxylase (CYP24A1 or
45 24-hydroxylase), which functions to inactivate both $25[\text{OH}]\text{D}$ and $1,25[\text{OH}]_2\text{D}_3$ (15) by hydroxylation
46 at C-24. It is important to characterize the response high dose blanket approaches in order to avoid
47 potentially detrimental effects of too much supplemental vitamin D and contribute toward the
48 establishment of the most safe and effective vitamin D supplementation schemes for elite athletes.

49
50 The current study therefore aimed to characterize the serum responses of the major vitamin D
51 metabolites, $25[\text{OH}]\text{D}$, $1,25[\text{OH}]_2\text{D}_3$, $24,25[\text{OH}]_2\text{D}$ and iPTH to high dose vitamin D supplementation
52 ($35,000$ and $70,000 \text{ IU}$ vitamin D_3 weekly) in an elite professional team sport cohort. It was
53 hypothesized that supplementation would dose dependently increase total serum $25[\text{OH}]\text{D}$ and the
54 active metabolite $1,25[\text{OH}]_2\text{D}_3$ in a concomitant manner but would also increase the production of
55 $24,25[\text{OH}]_2\text{D}$.

56

57 **Methods**

58 Participants

59 Forty-six elite male elite professional team sport athletes volunteered to participate in the current trial
60 (Age = 26 ± 3 years, height = 1.86 ± 0.6 m, weight 101.5 ± 11 kg, fat mass 11.4 ± 3 %). Participants
61 underwent a medical screening and provided full informed consent prior to inclusion into the study.
62 Participants were excluded if they were currently taking vitamin D supplements, using sun beds or
63 injured at the time of the study. Ethical approval was granted by the ethics committee of Liverpool
64 John Moores University (Ethics code 12/SPS/047). The recruitment for the study began in November
65 2012 and testing commenced in the same month. The study was concluded in April 2013. The study
66 was conducted at latitude 52°N during the winter months in order to limit sunlight exposure.

67

68 Supplementation

69 Participants were randomly allocated to either 35,000 or 70,000 IU.week⁻¹ supplemental vitamin D₃
70 (Maxi Nutrition, UK), herein referred to as moderate and high, respectively. Randomisation was
71 achieved with blocking based on baseline serum 25[OH]D and the use of a random number table to
72 allocate participants into balanced groups. The random allocation sequence was allocated by a
73 member of the research team and known by the rest of the research team at the point of supplement
74 administration. The supplemental doses were chosen based on the fact that they represent widely
75 reported supplement strategies (PubMed literature based search), applied experience of the authors in
76 both clinical and elite sporting settings and also the NOAEL set by the US IoM. Supplements were
77 taken orally as a bolus in capsule form on a weekly basis to increase compliance with the protocol,
78 which was 100% as club staff were present during the weekly distribution of supplementation and
79 monitored the ingestion of capsules in order to track compliance. Supplementation continued for 12
80 weeks at which point supplementation was ceased to monitor the response of vitamin D metabolites to
81 withdrawal. Participants were blinded to the supplement they were receiving. Forty two players
82 completed the trial whilst four dropped out or were excluded for the following reasons; one player did
83 not tolerate venipuncture, one player would not comply with the supplementation protocol, two
84 players used sun beds during the trial. The vitamin D supplements were batch screened by

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

91

92 Blood Sampling

93 Blood samples were drawn prior to supplementation (basal and then at 6, 12 and 18 (withdrawal)
94 weeks from the start of supplementation. Blood was obtained from the antecubital vein into two serum
95 separator tubes and two K₂EDTA tubes (Becton, Dickinson and Co. Oxford, UK). Samples were then
96 separated to isolate serum/plasma via centrifugation at 1500 RCF for 15 minutes at 4 °C.

97 Serum/plasma was extracted and stored at -80 °C until required for analysis. All samples were
98 collected in the medical room of a professional sports club.

99

100 Analysis of Vitamin D Metabolites and Parathyroid Hormone

101 Liquid chromatograph tandem mass spectrometry (LC-MS/MS) analysis of 25[OH]D₃, 25[OH]D₂,
102 24,25[OH]₂D₃ and 24,25[OH]₂D₂ was performed using a Micromass Quattro Ultima Pt mass
103 spectrometer (Waters Corp., Milford, MA, USA). NIST SRM972a traceable 25[OH]D₃ and 25[OH]D₂
104 calibration standards (Chromsystems, München, Germany) and quality controls (UTAK Laboratories,
105 CA, USA) were purchased commercially, ranged from 0-200 nmol/L. 24,25[OH]₂D₃ and
106 24,25[OH]₂D₂ calibration standards were prepared from certified standards (IsoSciences, King of
107 Prussia, PA, USA) spiked into human vitamin D depleted serum (BBI Solutions, Cardiff, UK), ranged
108 from 0-14.8 nmol/L. To 100 µL of human serum samples, calibration standards and quality controls,
109 200 µL of pretreatment solution consist of deuterated 25[OH]D₃-[²H₆] and 24R,25[OH]₂D₃-[²H₆] in
110 isopropanol:water 50:50 (v/v) was added to displace binding proteins. After mixing, the samples were
111 loaded onto Supported Liquid Extraction (SLE+) plates (Biotage, Uppsala, Sweden), which were

112 eluted with 1.5 mL of n-heptane. The extraction procedure was performed by Extrahera positive
113 pressure automation system (Biotage). Eluents were dried under nitrogen, followed by reconstitution
114 with 50 μ L 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) (Sigma-Aldrich, Dorset, UK). After a 30
115 minute incubation period at room temperature, 50 μ L of water was added to stop the reaction. 20 μ L
116 of the derivatised extracts were injected into LC-MS/MS. Separation of vitamin D metabolites were
117 achieved using core-shell C18 2.7 μ m 50 \times 2.1mm (Restek, PA, USA) reversed-phase column. A
118 gradient elution profile was set up using mobile phase (A) LCMS grade water and (B) methanol
119 containing 0.2 mM methylamine in 0.1% formic acid. The gradient at the start was 50:50 (v/v) at
120 column flow rate of 0.4 mL/min, gradually increased to 99% B. 24,25[OH]₂D₃/D₂ and 25[OH]D₃/D₂
121 peaks were eluted at 1.39, 1.42, 1.68 and 1.73 minutes respectively (See **Supplementary Digital**
122 **Content 1**, A Chromatogram from an extracted sample containing 86 nmol/L of 25[OH]D₃/D₂ and
123 5.3 nmol/L of 24,25[OH]₂D₃/D₂). Optimisation of MS/MS conditions were carried out by direct
124 infusion of derivatised standards into the ion source via a T-connector. The precursor to product ion
125 transitions were based on the molecular weight of the methylamine adduct of PTAD derived products
126 (See **Supplementary Digital Content 2**, a table highlighting mass spectrometer parameter settings
127 and multiple reaction monitoring (MRM) precursor to product ion transitions for 25[OH]D₃/D₂ and
128 24,25[OH]₂D₃/D₂).

129
130 Measurements of total 1,25[OH]₂D were carried out using a commercially available enzyme
131 immunoassay kit (IDS, Boldon, UK). Duplicate samples underwent immunoextraction with a
132 1,25[OH]₂D specific solid phase monoclonal antibody and incubated overnight with sheep anti-
133 1,25[OH]₂D. 1,25[OH]₂D linked biotin was added the next day, followed by horseradish peroxidase
134 labelled avidin to selectively bind to biotin complex. After a wash step, colour was developed using a
135 chromogenic substrate (3, 5', 5, 5'- Tetramethylbenzidine; TMB). The absorbance of the stopped
136 reaction mixtures were read in a microtiter plate spectrophotometer (Multiskan Go, Thermo Scientific,
137 Finland) at wavelength of 450 nm.

138

139 Intact parathyroid hormone in K₂EDTA plasma samples were determined by
140 electrochemiluminescence immunoassay (ECLIA) performed using Roche e601 analyser (Mannheim,
141 Germany). Two labelled monoclonal antibodies were employed to react with the N-terminal fragment
142 (1-37) and C-terminal fragment (38-84) of PTH; forming a sandwich complex. The antibody complex
143 was magnetically captured and washed to remove unbound substances. A voltage was applied to
144 induce chemiluminescent emission, which was measured by a photomultiplier.

145

146 Assay validation

147 Summary of assay characteristics are described in **Supplementary Digital Content 3** (A table
148 highlighting the assay characteristics for each parameter measured). Linearity of the methods were
149 evaluated by analysing stock standards made up from reference calibration solutions spiked into
150 human sera. Standard curve was constructed by plotting the analyte response against the concentration
151 of their respective standards. A calibration curves were accepted as linear if the weighted linear
152 regression produced a correlation coefficient (r^2) value of >0.999 . Intra and inter-assay imprecision of
153 the methods were assessed by running quality control (QC) materials 10 times within a single run and
154 separately over a three-month period. Assays were deemed acceptable when the QC results fall within
155 $\pm 2SD$ from the mean value. Lower limit of quantification (LLoQ) was determined by the lowest
156 concentration quantifiable with a precision CV of 20% over 12 replicates and minimum peak signal-
157 to-noise ratio of 10:1 (16). Assay recovery was determined by analysing samples containing a fixed
158 amount of the analyte and calculate the percentage of the measured value against the sum of
159 endogenous value plus spiking concentration.

160

161 Statistical Analyses

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

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

179

180 **Results**

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

187 <Figure 1>

188 Total serum 25[OH]D, displayed a significant interaction effect ($F = 4.30$, $P = 0.008$) between dose
189 and time. Exploration of this interaction identified that both groups showed significantly elevated
190 25[OH]D concentrations at weeks 6 (moderate = 108 ± 22 and high = 122 ± 25 nmol.L⁻¹) and 12
191 (moderate = 163 ± 47 and high = 188 ± 66 nmol.L⁻¹). However, upon supplementation withdrawal the
192 moderate treatment group demonstrated a return to 25[OH]D concentrations comparable with basal by
193 week 18 ($P = 0.178$) whereas the high treatment did not ($P = 0.007$; **Figure 2A**).

194 Concentrations of the bioactive metabolite, $1\alpha,25[\text{OH}]_2\text{D}_3$ demonstrated a significant main effect for
195 time ($F = 6.13$, $P = 0.001$). In the moderate treatment group, peak concentrations occurred at week 6
196 ($141 \pm 58 \text{ pmol.L}^{-1}$). However, a delayed response was observed in the high group with peak
197 $1\alpha,25[\text{OH}]_2\text{D}_3$ levels occurring at week 12 ($112 \pm 66 \text{ pmol.L}^{-1}$, **Figure 2B**). Following
198 supplementation withdrawal, the concentration of $1\alpha,25[\text{OH}]_2\text{D}_3$ declined significantly in both groups
199 at week 18 (moderate, 107 ± 32 and high = $104 \pm 42 \text{ pmol.L}^{-1}$, $P = 0.042$) compared with
200 concentrations at week 12 and were comparable with basal by this time point ($P = 0.332$).

201
202 The inactivated metabolite, $24,25[\text{OH}]_2\text{D}$, showed comparable values between groups at basal
203 (moderate = 8.3 ± 2.5 and high = $7.1 \pm 1.7 \text{ nmol.L}^{-1}$). Both groups showed significant increases in this
204 metabolite by week 6 (moderate, $P = 0.011$ and high, $P = 0.000$) that continued to increase between
205 weeks 6 and 12. A significant interaction effect was also detected as the high treatment group
206 displayed markedly higher peak $24,25[\text{OH}]_2\text{D}$ concentrations ($17.3 \pm 4.5 \text{ nmol.L}^{-1}$) versus moderate
207 treatment ($11.8 \pm 1.9 \text{ nmol.L}^{-1}$). Interestingly, whereas $1\alpha,25[\text{OH}]_2\text{D}_3$ declined following
208 supplementation withdrawal, $24,25[\text{OH}]_2\text{D}$ remained significantly elevated at week 18 when compared
209 with basal values in both treatment groups (moderate, 11.4 ± 2.2 and high, $15.7 \pm 4.6 \text{ nmol.L}^{-1}$; $P =$
210 0.000 for both groups).

211
212 Intact parathyroid hormone was significantly suppressed in both groups by week 6 (moderate = $2.3 \pm$
213 0.8 and high = $1.9 \pm 0.4 \text{ pmol.L}^{-1}$ vs basal values in moderate = 3.2 ± 2.3 and high $2.8 \pm 1 \text{ pmol.L}^{-1}$)
214 and remained suppressed throughout the trial and following the withdrawal of supplementation
215 (moderate = 2.1 ± 0.8 and high $2 \pm 0.6 \text{ pmol.L}^{-1}$).

216 <Figure 2>

217 Several studies have also examined the ratios of $25[\text{OH}]_2\text{D}$ and $1\alpha,25[\text{OH}]_2\text{D}_3$ to $24,25[\text{OH}]_2\text{D}$ and it is
218 evident that additional information can be obtained that is not always obvious when measuring
219 absolute concentrations (17, 19, 22). In addition, it has been suggested that the ratio of $25[\text{OH}]_2\text{D}$ to
220 $24,25[\text{OH}]_2\text{D}$ is predictive of the $25[\text{OH}]_2\text{D}$ response to supplementation (34) giving important
221 information that surpasses simply measuring the absolute values for these metabolites. Therefore we

222 also calculated ratio data for the relationships between 25[OH]D and $1\alpha,25[\text{OH}]_2\text{D}_3$ to 24,25[OH]D.
223 The ratio between 25[OH]D and $1\alpha,25[\text{OH}]_2\text{D}_3$ showed a significant main effect for time ($F = 3.39$, P
224 $= 0.023$) but no group main effect with ratio's for both supplemental treatments decreasing over the
225 duration of the study, reaching significance by week 12 ($P = 0.039$) and increasing toward pre
226 treatment values at week 18 (**Figure 3A**). The ratio of 25[OH]D to the inactivated 24,25[OH]D also
227 showed a main effect for time ($F = 14.94$, $P = 0.000$) and the absence of a group main effect. Both
228 groups demonstrated a significant lower ratio at week 18 compared to basal ($P = 0.000$; **Figure 3B**).
229 Finally, the ratio of the bioactive $1\alpha,25[\text{OH}]_2\text{D}_3$ against the inactivated 24,25[OH]D was assessed. A
230 significant interaction effect was observed as at week 6 the moderate treatment group showed an
231 increased ratio whilst the high treatment group showed an inverse relationship. However, by week 12
232 both treatment groups showed a significantly lower ratio of $1\alpha,25[\text{OH}]_2\text{D}_3:24,25[\text{OH}]_2\text{D}$ ($P = 0.005$)
233 that was maintained at week 18 following supplementation withdrawal ($P = 0.003$; **Figure 3C**).

234 <**Figure 3**>

235

236 **Discussion**

237 The current investigation sought to define the serum responses of the major vitamin D metabolites in a
238 professional athletic cohort to establish the efficacy of a blanket supplementation approach using two
239 commonly employed and commercially available doses of vitamin D₃. Our main findings demonstrate
240 that both 35,000 and 70,000 IU.week⁻¹ oral vitamin D₃ supplementation significantly elevated total
241 serum 25[OH]D concentrations. The highest dose led to an initial rapid increase in 1,25[OH]₂D but
242 then a decrease in serum 1,25[OH]₂D at week 12 when there was a significant increase of
243 24,25[OH]₂D₃ which had also been significantly increased at week 6. Resultantly, these responses led
244 to a significantly lower ratio of 1,25[OH]₂D to 24,25[OH]₂D from week 6 with the higher, 70,000 IU
245 treatment. Following the withdrawal of supplementation, the concentrations of 25[OH]D and
246 $1\alpha,25[\text{OH}]_2\text{D}$ return to basal values within 6 weeks. These data imply that high doses of supplemental
247 vitamin D₃ are sufficient to markedly induce the expression of 24-hydroxylase leading to the negative
248 control of 1,25[OH]₂D activity. Finally, we demonstrate that elevating serum 25[OH]D and
249 $1\alpha,25[\text{OH}]_2\text{D}_3$ suppresses iPTH appearance in circulation. This finding is in agreement with previous

250 data published by our group (27) and is underpinned by the understanding that DNA binding
251 sequences exist in the PTH gene (6), permitting suppression of the gene when adequate ligand
252 (1,25[OH]₂D) is available to induce transcriptional suppression by the VDR (30).

253

254 The fact that the concentration of serum 24,25[OH]₂D₃ did not show a decline along with 1,25[OH]₂D
255 following the withdrawal of supplementation has practical implications. The finding suggests that the
256 activity of 24-hydroxylase is sustained following large increases in 1,25[OH]₂D and may persist and
257 decrease both the concentration and subsequent biological activity of 1,25[OH]₂D. Evidence is now
258 emerging that the 24,25[OH]₂D metabolite may act at the VDR as a “blocking molecule” binding to
259 the VDR decreasing 1,25 [OH]₂D activity (5). Since 24,25[OH]₂D is present in the circulation in
260 nmol/L concentration compared to pmol/L for 1,25[OH]₂D the significantly higher prevailing
261 24,25[OH]₂D concentrations are liable to contribute to a significant decrease in the activity of the
262 biologically active 1,25[OH]₂D. Thus a dual regulation would appear to be present in subjects
263 receiving high dose vitamin D supplementation preventing possible toxic effects, namely, 1) the
264 positive stimulation of 24-hydroxylase and 2) the negative control of the vitamin D receptor activity.
265 This notion is supported by previous mechanistic evidence that has determined the function of
266 1α,25[OH]₂D in regulating 24-hydroxylase activity in vitro. Identification of two VDREs in the 5’
267 region of the CYP24A1 promoter demonstrated that 1α,25[OH]₂D₃ could potently trans-activate the
268 CYP24A1 gene, inducing a 10 to 100 fold increase in CYP24A1 mRNA to limit the transcription of
269 1α,25[OH]₂D₃ responsive genes (24). Moreover, in vitro studies on primary human myoblasts indicate
270 that the induction of 24-hydroxylase is dose dependent (10), which is in agreement with the serum
271 response of 24,25[OH]₂D seen in vivo in the current study.

272

273 These findings may explain reported observations of deterioration in skeletal muscle function,
274 increased risk of falls and increased fracture risk in individuals supplemented with extreme dose
275 vitamin D₃ to correct for severe vitamin D deficiency. As an example, in a large-scale trial (n = 2256),
276 women ≥ 70 years old were randomized to either 500,000 IU of vitamin D₃ or placebo. The women
277 randomized to the supplemental vitamin D₃ experienced significantly more falls than the placebo

278 group in the year following dosing of which the falls ratio was greatest in the first month following the
279 one off 500,000 IU dose (29). Furthermore, in a retrospective observational cohort study, very low (<
280 10 nmol.L⁻¹) and high (above 140 nmol.L⁻¹) concentrations of 25[OH]D showed an increased risk of all
281 cause mortality indicating not only a lower limit but also an upper limit for serum 25[OH]D (7). This
282 hypothesis also lends an explanation for the inconsistency in positive outcomes related to
283 supplemental vitamin D reported by large-scale meta-analyses (4). It is reasonable to suggest that
284 mega dose vitamin D supplements are detrimental to vitamin D target tissues by increasing the
285 production of 24,25[OH]₂D, which may act to block the activity of the VDR. It will be necessary to
286 now perform mechanistic studies that clarify the function of 24,25[OH]₂D and to determine whether
287 high dose supplementation is detrimental to vitamin D signaling through the VDR.

288

289 The current trial also had limitations that are important to consider for the design of future work.
290 Firstly, although one of our goals was to use a ‘real world’ blanket supplementation approach, we
291 acknowledge that the same protocol used in other athletic cohorts with different body composition,
292 genotype and lower basal serum 25[OH]D concentrations may yield different results. Determining the
293 response of the vitamin D metabolites to a similar protocol as we have used here across wider athletic
294 cohorts will allow more conclusive recommendations to be made on dosing concentration and
295 frequency. Indeed, we have previously shown that basal 25[OH]D concentrations vary across athletes
296 from different professional sports (3). This assumption is also true for female cohorts and as such we
297 appreciate that our findings cannot be conclusively extended to the female athletic population.
298 Regarding genotype, genotypic variation in the vitamin D binding protein influences the response to
299 exogenous vitamin D (9) and little is known of the variation in genes encoding other vitamin D
300 metabolizing enzymes such as CYP24A1 and CYP24B1. We did not perform genotyping and in light of
301 recent evidence, we fully support genotype-phenotype studies in the context of vitamin D in future.
302 Combining a genotyping approach with vitamin D metabolite ratio data, the latter as we have
303 performed in this study, will offer a great advancement in the understanding of how genotype and
304 supplementation interact and how this can be managed. We also did not measure serum or urinary
305 calcium concentrations, which are markers of vitamin D toxicity and also regulate the PTH response.

306 Measuring Ca^{2+} excretion would add another aspect to our findings, however we do maintain that the
307 observed increases in 24,25[OH]D are indicative of too much exposure to exogenous vitamin D.
308 Finally, future work should aim to monitor FGF-23, a bone derived hormone that can function to
309 lower both 25[OH]D and $1\alpha,25[\text{OH}]_2\text{D}_3$ by inducing the CYP24 genes (28). FGF-23 may also be a
310 player in regulating the metabolite response to high dose supplementation and at present its role in
311 lowering 25[OH]D by promoting 24-hydroxylase expression is still disputed (31). Extending the
312 current findings to a broader range of vitamin D concentrations, coupled with intracellular signaling
313 cascades related to the vitamin D axis will yield the most inferential data, moving towards safer and
314 more effective vitamin D supplementation practices in athletes.

315

316 Summary and implications for practice

317 The data presented here are the first to characterize the response of two major metabolites of vitamin
318 D in response to two high-dose supplementation protocols in healthy professional athletes. The results
319 demonstrate that a blanket approach of high-dose supplementation with $70,000 \text{ IU}\cdot\text{week}^{-1}$ leads not
320 only to increased 25[OH]D₃ and $1,25[\text{OH}]_2\text{D}_3$ concentrations but also stimulates elevated
321 concentrations of the vitamin D metabolite $24,25[\text{OH}]_2\text{D}_3$, which has been previously shown to limit
322 the transcriptional activity of $1,25[\text{OH}]_2\text{D}_3$. We demonstrated that this negative regulatory effect
323 persists following cessation of vitamin D₃ supplementation even as $1,25[\text{OH}]_2\text{D}_3$ concentrations
324 decrease. There are a number of novel key implications for practice that arise from our current
325 observations. Firstly, we speculate that ‘high dose’ bolus supplementation with vitamin D₃ is likely to
326 be detrimental to the intended targeted downstream biological functions due to significant increases in
327 the negative regulatory molecule, $24,25[\text{OH}]_2\text{D}$. Weekly doses amounting to more than $5,000 \text{ IU}\cdot\text{day}^{-1}$
328 may need to be reassessed in light of our data. Rapid withdrawal from high dose supplementation may
329 result in adverse outcomes as the concentration of $24,25[\text{OH}]_2\text{D}_3$ remains elevated for several weeks
330 following withdrawal from supplementation despite declines in $1,25[\text{OH}]_2\text{D}_3$. If moderate to high
331 doses of vitamin D₃ have been administered, a gradual withdrawal from supplementation is advisable.
332 At present the optimal approach has not been established. Lower doses administered often (daily) may
333 offer the most potent beneficial biological effects and limit the transactivation of CYP24A1 and

334 subsequent production of the negative regulatory molecule, 24,25[OH]₂D₃. Future research must aim
335 to establish the appropriate dose and frequency of administration to achieve a positive increase in both
336 25[OH]D₃ and 1,25[OH]₂D₃ whilst limiting the appearance of increased 24,25[OH]₂D₃ concentrations.
337 The generation of 24,25 [OH]₂D may be an aspect of the body's defense mechanism to prevent
338 "toxicity" when administered high doses of vitamin D. We postulate that single "super" doses of
339 vitamin D₃ administered on a weekly basis as is common practice in many professional sporting
340 teams, may result in similar rapid transient increases in 1,25[OH]₂D₃ leading to significant increases in
341 the negative regulatory metabolite, 24,25[OH]₂D₃. Further studies will be required to determine if the
342 relationship we have observed is seen with higher and lower doses of vitamin D.

343

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351

352 **Author Contributions**

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

361

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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 D₃ **a)** 25-hydroxyvitamin D (25[OH]D) **b)** 1 α ,25-dihydroxyvitamin D₃ (1 α ,25[OH]₂D₃) **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 1 α ,25-dihydroxyvitamin D₃ (1 α ,25[OH]₂D₃). **b)** Ratio of 25[OH]D to the inactive metabolite 24,25[OH]D and **c)** ratio of 1 α ,25[OH]₂D₃ to 24,25[OH]D. * denotes significance for both groups compared with basal.