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 $24,25[OH]_2D$ 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 $24,25[OH]_2D$ production. Rapid withdrawal from high dose supplementation may inhibit the bioactivity of $1,25[OH]_2D_3$ as a consequence of sustained increases in $24,25[OH]_2D$ that persist as 25[OH]D and $1,25[OH]_2D$ 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

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 D_3), 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 (14); whereas ergocalciferol (vitamin D_2) 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 D_3 and subsequently vitamin D_3 (or
10 cholecalciferol, 14) 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 (1α-hydroxylase) at C-1α to form the biologically active metabolite, 1,25-
15 dihydroxyvitamin D_3 (1,25$[OH]_2$D or calcitriol)(1). The active compound, 1,25$[OH]_2$D 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$[OH]_2D_3$ 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 (12).

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 (22) 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 (21), innate and acquired
26 immunity (13), muscle development and repair (26, 27) 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 (34) as well as

29 athletic populations (4, 24). 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 (15, 19). 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 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 IU (1,250 µg) 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 $IU.day^{-1}$ (9), in line with advice portrayed by the US
41 Institute of Medicine (IoM) guidelines for vitamin D intake (33). Notably the US IoM also state a no
42 adverse effect limit (NOAEL) of 10,000 $IU.day^{-1}$. 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[OH]D and 1,25[OH]$_2$D$_3$ (16) 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[OH]D, 1,25[OH]$_2$D$_3$, 24,25[OH]$_2$D and iPTH to high dose vitamin D supplementation
52 (35,000 and 70,000 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[OH]D and the
54 active metabolite 1,25[OH]$_2$D$_3$ in a concomitant manner but would also increase the production of
55 24,25[OH]$_2$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 D_3
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 K_2EDTA 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 ^{o}C.
97 Serum/plasma was extracted and stored at -80 ^{o}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]D_3, 25[OH]D_2,
102 24,25[OH]$_2D_3$ and 24,25[OH]$_2D_2$ was performed using a Micromass Quattro Ultima Pt mass
103 spectrometer (Waters Corp., Milford, MA, USA). NIST SRM972a traceable 25[OH]D_3 and 25[OH]D_2
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]$_2D_3$ and
106 24,25[OH]$_2D_2$ 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]D_3-[$^2H_6$] and 24R,25[OH]$_2D_3$-[$^2H_6$] 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 μL 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 μL of water was added to stop the reaction. 20 μL
116 of the derivatised extracts were injected into LC-MS/MS. Separation of vitamin D metabolites were
117 achieved using core-shell C18 2.7μm 50 × 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. $24,25[OH]_2D_3/D_2$ and $25[OH]D_3/D_2$
121 peaks were eluted at 1.39, 1.42, 1.68 and 1.73 minutes respectively (Supplementary **Figure 1**).
122 Optimisation of MS/MS conditions were carried out by direct infusion of derivisated standards into
123 the ion source via a T-connector. The precursor to product ion transitions were based on the molecular
124 weight of the methylamine adduct of PTAD derived products (Supplementary **Table 1**).
125
126 Measurements of total $1,25[OH]_2D$ were carried out using a commercially available enzyme
127 immunoassay kit (IDS, Boldon, UK). Duplicate samples underwent immunoextraction with a
128 $1,25[OH]_2D$ specific solid phase monoclonal antibody and incubated overnight with sheep anti-
129 $1,25[OH]_2D$. $1,25[OH]_2D$ linked biotin was added the next day, followed by horseradish peroxidase
130 labelled avidin to selectively bind to biotin complex. After a wash step, colour was developed using a
131 chromogenic substrate (TMB). The absorbance of the stopped reaction mixtures were read in a
132 microtiter plate spectrophotometer (Multiskan Go, Thermo Scientific, Finland) at wavelength of 450
133 nm.
134
135 Intact parathyroid hormone in K_2EDTA plasma samples were determined by
136 electrochemiluminescence immunoassay (ECLIA) performed using Roche e601 analyser (Mannheim,
137 Germany). Two labelled monoclonal antibodies were employed to react with the N-terminal fragment
138 (1-37) and C-terminal fragment (38-84) of PTH; forming a sandwich complex. The antibody complex

139 was magnetically captured and washed to remove unbound substances. A voltage was applied to
140 induce chemiluminiescent emission which was measured by a photomultiplier.
141
142 *Assay validation*
143 Summary of assay characteristics are described in **Supplementary Table 2**. Linearity of the methods
144 were evaluated by analysing stock standards made up from reference calibration solutions spiked into
145 human sera. Standard curve was constructed by plotting the analyte response against the concentration
146 of their respective standards. A calibration curves were accepted as linear if the weighted linear
147 regression produced a correlation coefficient (r^2) value of >0.999. Intra and inter-assay imprecision of
148 the methods were assessed by running quality control (QC) materials 10 times within a single run and
149 separately over a three-month period. Assays were deemed acceptable when the QC results fall within
150 ±2SD from the mean value. Lower limit of quantification (LLoQ) was determined by the lowest
151 concentration quantifiable with a precision CV of 20% over 12 replicates and minimum peak signal-
152 to-noise ratio of 10:1 (17). Assay recovery was determined by analysing samples containing a fixed
153 amount of the analyte and calculate the percentage of the measured value against the sum of
154 endogenous value plus spiking concentration.
155
156 *Statistical Analyses*
157 Comparisons of basal total serum 25[OH]D concentration for the two dose groups were made using an
158 independent t-test. The effects of vitamin D dose and time on all repeated measures variables was
159 determined using linear mixed modeling. Time (basal, weeks 6, 12 and 18) and dose (moderate and
160 high) were modeled as fixed effects and participants as a random effect, with time being modeled as a
161 continuous variable where linear or quadratic responses were observed. The covariance structure that
162 minimized the Hurvich and Tsai's criterion (AICC) value was used for the final fitted model for each
163 metabolite. Where significant main or interaction effects were observed, post hoc pairwise
164 comparisons were made with Sidak adjusted p-values. All statistical procedures were conducted using
165 SPSS v22 for Windows (IBM, Armonk, NY, USA), and two-tailed statistical significance was
166 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

195 concentrations at week 12 and were comparable with basal by this time point (*P* = 0.332).

196

197 The inactivated metabolite, 24,25[OH]D, showed comparable values between groups at basal
198 (moderate = 8.3 ± 2.5 and high = 7.1 ± 1.7 nmol.L^{-1}). Both groups showed significant increases in this
199 metabolite by week 6 (moderate, *P* = 0.011 and high, *P* = 0.000) that continued to increase between
200 weeks 6 and 12. A significant interaction effect was also detected as the high treatment group
201 displayed markedly higher peak 24,25[OH]D concentrations (17.3 ± 4.5 nmol.L^{-1}) versus moderate
202 treatment (11.8 ± 1.9 nmol.L^{-1}). Interestingly, whereas 1α,25$[OH]_2D_3$ declined following
203 supplementation withdrawal, 24,25[OH]D remained significantly elevated at week 18 when compared
204 with basal values in both treatment groups (moderate, 11.4 ± 2.2 and high, 15.7 ± 4.6 nmol.L^{-1}; *P* =
205 0.000 for both groups).

206

207 Intact parathyroid hormone was significantly suppressed in both groups by week 6 (moderate = 2.3 ±
208 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})
209 and remained suppressed throughout the trial and following the withdrawal of supplementation
210 (moderate = 2.1 ± 0.8 and high 2 ± 0.6 pmol.L^{-1}).

211 <**Figure 2**>

212 Several studies have also examined the ratios of 25[OH]D and 1α,25$[OH]_2D_3$ to 24,25[OH]D and it is
213 evident that additional information can be obtained that is not always obvious when measuring
214 absolute concentrations (18, 20, 23). In addition, it has been suggested that the ratio of 25[OH]D to
215 24,25[OH]D is predictive of the 25[OH]D response to supplementation (35) giving important
216 information that surpasses simply measuring the absolute values for these metabolites. Therefore we
217 also calculated ratio data for the relationships between 25[OH]D and 1α,25$[OH]_2D_3$ to 24,25[OH]D.
218 The ratio between 25[OH]D and 1α,25$[OH]_2D_3$ showed a significant main effect for time (F = 3.39, *P*
219 = 0.023) but no group main effect with ratio's for both supplemental treatments decreasing over the
220 duration of the study, reaching significance by week 12 (*P* = 0.039) and increasing toward pre
221 treatment values at week 18 (**Figure 3A**). The ratio of 25[OH]D to the inactivated 24,25[OH]D also
222 showed a main effect for time (F = 14.94, *P* = 0.000) and the absence of a group main effect. Both

223 groups demonstrated a significant lower ratio at week 18 compared to basal ($P = 0.000$; **Figure 3B**).
224 Finally, the ratio of the bioactive 1α,25[OH]$_2$D$_3$ against the inactivated 24,25[OH]D was assessed. A
225 significant interaction effect was observed as at week 6 the moderate treatment group showed an
226 increased ratio whilst the high treatment group showed an inverse relationship. However, by week 12
227 both treatment groups showed a significantly lower ratio of 1α,25[OH]$_2$D$_3$:24,25[OH]D ($P = 0.005$)
228 that was maintained at week 18 following supplementation withdrawal ($P = 0.003$; **Figure 3C**).
229 <**Figure 3**>
230
231 ***Discussion***
232 The current investigation sought to define the serum responses of the major vitamin D metabolites in a
233 professional athletic cohort to establish the efficacy of a blanket supplementation approach using two
234 commonly employed and commercially available doses of vitamin D$_3$.Our main findings demonstrate
235 that both 35,000 and 70,000 IU.week^{-1} oral vitamin D$_3$ supplementation significantly elevated total
236 serum 25[OH]D concentrations. The highest dose led to an initial rapid increase in 1.25[OH]$_2$D but
237 then a decrease in serum 1,25[OH]$_2$D at week 12 when there was a significant increase of
238 24,25[OH]$_2$D$_3$ which had also been significantly increased at week 6. Resultantly, these responses led
239 to a significantly lower ratio of 1,25[OH]$_2$ D to 24,25[OH]$_2$D from week 6 with the higher, 70,000 IU
240 treatment. Following the withdrawal of supplementation, the concentrations of 25[OH]D and
241 1α,25[OH]$_2$D return to basal values within 6 weeks. These data imply that high doses of supplemental
242 vitamin D$_3$ are sufficient to markedly induce the expression of 24-hydroxylase leading to the negative
243 control of 1,25[OH]$_2$D activity. Finally, we demonstrate that elevating serum 25[OH]D and
244 1α,25[OH]$_2$D$_3$ suppresses iPTH appearance in circulation. This finding is in agreement with previous
245 data published by our group (28) and is underpinned by the understanding that DNA binding
246 sequences exist in the PTH gene (7), permitting suppression of the gene when adequate ligand
247 (1,25[OH]$_2$D) is available to induce transcriptional suppression by the VDR (31).
248
249 The fact that the concentration of serum 24,25[OH]$_2$D$_3$ did not show a decline along with 1,25[OH]$_2$D
250 following the withdrawal of supplementation has practical implications. The finding suggests that the

251 activity of 24-hydroxylase is sustained following large increases in 1,25[OH]$_2$D and may persist and
252 decrease both the concentration and subsequent biological activity of 1,25[OH]$_2$D. Evidence is now
253 emerging that the 24,25[OH]$_2$D metabolite may act at the VDR as a "blocking molecule" binding to
254 the VDR decreasing 1,25 [OH]$_2$D activity (6). Since 24,25[OH]$_2$D is present in the circulation in
255 nmol/L concentration compared to pmol/L for 1,25[OH]$_2$D the significantly higher prevailing
256 24,25[OH]$_2$D concentrations are liable to contribute to a significant decrease in the activity of the
257 biologically active 1,25[OH]$_2$D. Thus a dual regulation would appear to be present in subjects
258 receiving high dose vitamin D supplementation preventing possible toxic effects, namely, *1*) the
259 positive stimulation of 24-hydroxylase and *2*) the negative control of the vitamin D receptor activity.
260 This notion is supported by previous mechanistic evidence that has determined the function of
261 1α,25[OH]$_2$D in regulating 24-hydroxylase activity *in vitro*. Identification of two VDREs in the 5'
262 region of the CYP24A1 promoter demonstrated that 1α,25[OH]$_2$D$_3$ could potently trans-activate the
263 CYP24A1 gene, inducing a 10 to 100 fold increase in CYP24A1 mRNA to limit the transcription of
264 1α,25[OH]$_2$D$_3$ responsive genes (25). Moreover, *in vitro* studies on primary human myoblasts indicate
265 that the induction of 24-hydroxylase is dose dependent (11), which is in agreement with the serum
266 response of 24,25[OH]D seen *in vivo* in the current study.
267
268 These findings may explain reported observations of deterioration in skeletal muscle function,
269 increased risk of falls and increased fracture risk in individuals supplemented with extreme dose
270 vitamin D$_3$ to correct for severe vitamin D deficiency. As an example, in a large-scale trial (n = 2256),
271 women ≥ 70 years old were randomized to either 500,000 IU of vitamin D$_3$ or placebo. The women
272 randomized to the supplemental vitamin D$_3$ experienced significantly more falls than the placebo
273 group in the year following dosing of which the falls ratio was greatest in the first month following the
274 one off 500,000 IU dose (30). Furthermore, in a retrospective observational cohort study, very low (<
275 10 nmol.L^{-1}) and high (above 140 nmol.$^{-1}$) concentrations of 25[OH]D showed an increased risk of all
276 cause mortality indicating not only a lower limit but also an upper limit for serum 25[OH]D (8). This
277 hypothesis also lends an explanation for the inconsistency in positive outcomes related to
278 supplemental vitamin D reported by large-scale meta-analyses (5). It is reasonable to suggest that

279 mega dose vitamin D supplements are detrimental to vitamin D target tissues by increasing the
280 production of 24,25[OH]$_2$D, which may act to block the activity of the VDR. It will be necessary to
281 now perform mechanistic studies that clarify the function of 24,25[OH]$_2$D and to determine whether
282 high dose supplementation is detrimental to vitamin D signaling through the VDR.
283
284 The current trial also had limitations that are important to consider for the design of future work.
285 Firstly, although one of our goals was to use a 'real world' blanket supplementation approach, we
286 acknowledge that the same protocol used in other athletic cohorts with different body composition,
287 genotype and lower basal serum 25[OH]D concentrations may yield different results. Determining the
288 response of the vitamin D metabolites to a similar protocol as we have used here across wider athletic
289 cohorts will allow more conclusive recommendations to be made on dosing concentration and
290 frequency. Indeed, we have previously shown that basal 25[OH]D concentrations vary across athletes
291 from different professional sports (3). This assumption is also true for female cohorts and as such we
292 appreciate that our findings cannot be conclusively extended to the female athletic population.
293 Regarding genotype, genotypic variation in the vitamin D binding protein influences the response to
294 exogenous vitamin D (10) and little is known of the variation in genes encoding other vitamin D
295 metabolizing enzymes such as CY24A1 and CYP24B1. We did not perform genotyping and in light of
296 recent evidence, we fully support genotype-phenotype studies in the context of vitamin D in future.
297 Combining a genotyping approach with vitamin D metabolite ratio data, the latter as we have
298 performed in this study, will offer a great advancement in the understanding of how genotype and
299 supplementation interact and how this can be managed. We also did not measure serum or urinary
300 calcium concentrations, which are markers of vitamin D toxicity and also regulate the PTH response.
301 Measuring Ca^{2+} excretion would add another aspect to our findings, however we do maintain that the
302 observed increases in 24,25[OH]D are indicative of too much exposure to exogenous vitamin D.
303 Finally, future work should aim to monitor FGF-23, a bone derived hormone that can function to
304 lower both 25[OH]D and 1α,25[OH]$_2$D$_3$ by inducing the CYP24 genes (29). FGF-23 may also be a
305 player in regulating the metabolite response to high dose supplementation and at present its role in
306 lowering 25[OH]D by promoting 24-hydroxylase expression is still disputed (32). Extending the

307 current findings to a broader range of vitamin D concentrations, coupled with intracellular signaling
308 cascades related to the vitamin D axis will yield the most inferential data, moving towards safer and
309 more effective vitamin D supplementation practices in athletes.
310

311 *Summary and implications for practice*

312 The data presented here are the first to characterize the response of two major metabolites of vitamin
313 D in response to two high-dose supplementation protocols in healthy professional athletes. The results
314 demonstrate that a blanket approach of high-dose supplementation with 70,000 $IU.week^{-1}$ leads not
315 only to increased $25[OH]D_3$ and $1,25[OH]_2D_3$ concentrations but also stimulates elevated
316 concentrations of the vitamin D metabolite $24,25[OH]_2D_3$, which has been previously shown to limit
317 the transcriptional activity of $1,25[OH]_2D_3$. We demonstrated that this negative regulatory effect
318 persists following cessation of vitamin D_3 supplementation even as $1,25[OH]_2D_3$ concentrations
319 decrease. There are a number of novel key implications for practice that arise from our current
320 observations. Firstly, we speculate that 'high dose' bolus supplementation with vitamin D_3 is likely to
321 be detrimental to the intended targeted downstream biological functions due to significant increases in
322 the negative regulatory molecule, $24,25[OH]_2D$. Weekly doses amounting to more than 5,000 $IU.day^{-1}$
323 may need to be reassessed in light of our data. Rapid withdrawal from high dose supplementation may
324 result in adverse outcomes as the concentration of $24,25[OH]_2D_3$ remains elevated for several weeks
325 following withdrawal from supplementation despite declines in $1,25[OH]_2D_3$. If moderate to high
326 doses of vitamin D_3 have been administered, a gradual withdrawal from supplementation is advisable.
327 At present the optimal approach has not been established. Lower doses administered often (daily) may
328 offer the most potent beneficial biological effects and limit the transactivation of CYP24A1 and
329 subsequent production of the negative regulatory molecule, $24,25[OH]_2D_3$. Future research must aim
330 to establish the appropriate dose and frequency of administration to achieve a positive increase in both
331 $25[OH]D_3$ and $1,25[OH]_2D_3$ whilst limiting the appearance of increased $24,25[OH]_2D_3$ concentrations.
332 The generation of $24,25\ [OH]_2D$ may be an aspect of the body's defense mechanism to prevent
333 "toxicity" when administered high doses of vitamin D. We postulate that single "super" doses of
334 vitamin D_3 administered on a weekly basis as is common practice in many professional sporting

335 teams, may result in similar rapid transient increases in 1,25[OH]$_2$D$_3$ leading to significant increases in
336 the negative regulatory metabolite, 24,25[OH]$_2$D$_3$. Further studies will be required to determine if the
337 relationship we have observed is seen with higher and lower doses of vitamin D.
338

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345

346 ***Author Contributions***
347 **DJO** designed the research, conducted the research, analyzed data and wrote the manuscript. **JCYT**
348 designed the research, conducted the research, designed and optimized all analytical techniques and
349 analyzed the data. **WJB** conducted the research and provided essential materials for research. **AS**
350 designed the research and conducted the statistical analyses. **WDF** designed the research, provided
351 essential reagents for the research, analyzed the data and wrote the paper. **JPM** designed the research,
352 analyzed the data and wrote the paper. **GLC** designed the research, conducted the research, analyzed
353 the data, performed statistical analysis, wrote the paper and had primary responsibility for the final
354 content.
355

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451

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.

Supplementary Figure 1. 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.