

## **Oral spray wintertime vitamin D<sub>3</sub> supplementation has no impact on inflammation in Gaelic footballers**

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**Running head:** Vitamin D and inflammation in Gaelic footballers

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**Abstract**

Vitamin D inadequacy [total 25(OH)D <50nmol/L] is widespread in athletes. The biologically active metabolite, 1,25-dihydroxyvitamin D, may be involved in regulating inflammation although *in vitro* findings have not been consistently replicated in human intervention trials. This study, conducted at a latitude of 55°N, aimed to assess inflammatory biomarkers in Gaelic footballers before and after a wintertime vitamin D<sub>3</sub> intervention. Samples from a 12-week double-blind, randomised, placebo-controlled trial, in which 42 Gaelic footballers received 3000IU (75µg) vitamin D<sub>3</sub> daily or placebo via oral spray solutions, were analysed for a range of inflammatory biomarkers. Cytokines (interleukin-8 and tumour necrosis factor-α), cathelicidin and high sensitivity C-reactive protein were quantified by multiplex assay, ELISA and clinical biochemistry respectively. White blood cell, lymphocyte and neutrophil concentrations were determined by full blood profile. Data on total 25-hydroxyvitamin D, measured by LC-MS/MS, were available from the previous study. Vitamin D<sub>3</sub> supplementation significantly increased mean total 25-hydroxyvitamin D concentrations from 47 to 84nmol/L ( $P=0.006$ ); yet this had no effect on white blood cell count ( $P=0.699$ ), lymphocyte ( $P=0.694$ ), neutrophil ( $P=0.594$ ), interleukin-8 ( $P=0.334$ ), tumour necrosis factor-α ( $P=0.587$ ), cathelicidin ( $P=0.745$ ) or high sensitivity C-reactive protein concentration ( $P=0.621$ ) compared to placebo. 12-weeks vitamin D<sub>3</sub> supplementation did not impact the immune profile of Gaelic footballers. This is likely because biomarkers were within their respective normal range or at a concentration similar to that of the general population at baseline. Future studies are encouraged to use inflammation as their primary outcome measure and recruit athletes at risk of compromised immunity.

## Introduction

Vitamin D insufficiency and deficiency, defined as a total 25-hydroxyvitamin D concentration below 50 and 30nmol/L respectively, is widespread across different sport disciplines and geographical locations (Farrokhyar et al., 2015). Aside from the well-established role of vitamin D in facilitating calcium absorption, a growing number of *in vitro* and human studies indicate that this steroid pro-hormone is implicated in regulating systemic inflammation through mediating the balance of pro-inflammatory T-helper 1 (Th1) and anti-inflammatory T-helper 2 (Th2) derived cytokines and enhancing antimicrobial activity (Baeke et al., 2007;Guo et al., 2014;Schleithoff et al., 2006). The biologically active vitamin D metabolite, 1,25-dihydroxyvitamin D [1,25(OH)<sub>2</sub>D] and its interaction with the nuclear vitamin D receptor (VDR), which is expressed throughout the immune system, forms a retinoid-x-receptor complex that has been shown to trigger immunomodulatory actions *in vitro* through regulation of specific gene transcription factors. Immune cells, including macrophages and neutrophils, are also capable of intrinsically synthesising 1,25(OH)<sub>2</sub>D as they express the cytochrome P450 enzyme, 1- $\alpha$  hydroxylase, thereby enabling such cells to directly regulate inflammatory processes in their microenvironment (Christakos et al., 2013). Furthermore, vitamin D-mediated transcriptional regulation may facilitate production of the human cathelicidin (LL-37), an antimicrobial peptide that has an important role in innate immunity by negating viral, bacterial and fungal pathogens (Gombart, 2009). It is important to recognise that although 1,25(OH)<sub>2</sub>D can be quantified using a variety of assay methods, it is not considered an appropriate measure of vitamin D status or the *in vivo* response to supplementation. This is because 1,25(OH)<sub>2</sub>D has a short half-life, its concentration is tightly regulated and it is not possible to differentiate between 1,25(OH)<sub>2</sub>D synthesised as a result of a supplemental dose and that synthesised locally (Jones, 2008). Observational research in the general population has demonstrated that those with severe vitamin D deficiency (<25nmol/L) have a higher concentration of circulating Th1 (pro-

inflammatory) cytokines; compared to those whose total 25(OH)D concentration exceeds 75nmol/L (Barker et al., 2013b;Laird et al., 2014). Such evidence has led to the hypothesis that a total 25(OH)D concentration above 75nmol/L may be necessary in order to yield the purported anti-inflammatory actions of vitamin D; a threshold in-line with current Endocrine Society recommendations (Hart, 2012;Holick et al., 2011). With regard to athletes, the majority of studies investigating vitamin D and associations with inflammation have focused on those competing in endurance-based disciplines, owing to their increased risk of exercise-induced immunosuppression (He et al., 2016;He et al., 2013;Pedersen and Hoffman-Goetz, 2000;Willis et al., 2012). In endurance runners, total 25(OH)D concentration has been inversely correlated with the concentration of tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ), one of several Th1 cytokines linked to the pathogenesis of overtraining syndrome when chronically elevated, although findings have not been consistent across all studies (He et al., 2013;Willis et al., 2012). Cross sectional research has also demonstrated that the concentration of C-reactive protein (CRP) (a quintessential biomarker of systemic inflammation), is associated with vitamin D status and may be elevated in those with a total 25(OH)D concentration below 53nmol/L (Amer and Qayyum, 2012). Nevertheless, this association may not translate into a causal effect of treatment (Chandler et al., 2014;Liefgaard et al., 2015). LL-37 concentrations have been reported to be significantly higher in endurance athletes with a total 25(OH)D concentration >90nmol/L compared to <33nmol/L, suggesting that those below the latter cut-off may be at greater risk of infection through impaired LL-37 synthesis and a compromised immune response to pathogen exposure (He et al., 2013). However observational studies are unable to establish causality between total 25(OH)D concentration and inflammation as a diminished total 25(OH)D concentration may be the result of a shift in the Th1:Th2 balance rather than the cause (Autier et al., 2014;Mangin et al., 2014;Reid et al., 2011).

To our knowledge only two RCTs have examined the effects of vitamin D supplementation on inflammation in athletes and little is known regarding the effect of vitamin D supplementation on inflammation in athletes that compete in interval sports such as Gaelic football (He et al., 2015; Lewis et al., 2013). Previously studied cohorts were vitamin D sufficient ( $>50\text{nmol/L}$ ) at baseline and reported mixed findings however the response to supplementation may be greater in those who present with vitamin D insufficiency/deficiency ( $<50\text{nmol/L}$ ) (Didriksen et al., 2013).

The aim of this study was to characterise inflammation in Gaelic footballers before and after a wintertime vitamin D<sub>3</sub> intervention by quantifying systemic inflammation, the concentration of Th1 and Th2 peripheral cytokines and LL-37.

## **Materials and methods**

### Study design

The analysis conducted in this study formed part of a double-blind, randomised, placebo-controlled trial which took place at the University of Ulster Coleraine between November 2014 and April 2015 (Todd et al., 2016). All procedures were approved by the University Research Ethics Committee (REC/14/0087), registered at [www.clinicaltrials.gov](http://www.clinicaltrials.gov) (NCT02278172) and conducted in accordance to the declaration of Helsinki. Gaelic footballers signed informed consent and attended appointments to obtain fasted blood samples. Physical measures were taken before and after a 12-week intervention, the detailed methods of which have been reported previously (Todd et al., 2016).

### Participants

Gaelic footballers completed a screening questionnaire to determine eligibility and were provided with a study information sheet. Those that were over the age of 18 years and

apparently healthy were considered suitable for inclusion. Exclusion criteria were as follows: not a member of a University/local sports team; vitamin D supplementation and/or iron supplementation in the 30 days prior to baseline measurements; health concern(s)/physical disabilities as identified by the screening questionnaire that would prevent successful completion of the study; consumption of medication(s) known to influence vitamin D metabolism; those following a vegan diet; sun-bed users; those planning a sun holiday during the study or had been on a sun holiday in the 30 days prior to baseline measurements. In total, 42 footballers were deemed eligible for participation and were contacted via e-mail and telephone to arrange a date and time for their initial appointment. Aside from vitamin D inadequacy, recruited footballers were healthy individuals. This was confirmed through completion of a health screening questionnaire as part of the original study.

#### Supplements and compliance

The clinical trials manager randomised footballers to either a vitamin D<sub>3</sub> treatment (VD) or placebo group (PL). Researchers and athletes remained blinded to the treatment allocation until the study and data analyses were complete. Those allocated to VD received an oral spray solution which contained 3000IU (75µg) vitamin D<sub>3</sub> per spray and those allocated to PL received an oral spray solution that was identical in appearance, smell and taste but contained no vitamin D. The vitamin D<sub>3</sub> content of the oral spray solution was independently verified using high performance liquid chromatography (Eurofins Product Testing, Cheshire, UK). Compliance was calculated using the weight of the bottle upon study completion (Todd et al., 2016). Gaelic footballers were instructed to self-administer a single spray daily for 12-weeks, specifically aiming at the buccal membrane to promote absorption.

#### Sample collection

Blood samples were collected following an overnight fast and footballers were encouraged to continue drinking water. A trained phlebotomist obtained blood samples using a 21-gauge butterfly needle and 8mL serum separator and 9mL EDTA plasma vacutainer tubes (Greiner Bio-One GmbH, Kremsmunster, Austria). Following inversion, plasma samples were placed in refrigeration until full blood profile analysis. Whole blood was separated by centrifugation at 2200rpm for 15 minutes at 4°C. Following centrifugation, serum and plasma samples were pipetted into 0.5mL aliquots and stored at -80°C until further analysis.

#### Blood analysis

Serum total 25(OH)D [25(OH)D<sub>2</sub> and 25(OH)D<sub>3</sub>] concentrations were determined using an API 4000 liquid chromatography-tandem mass spectrometer (AB Sciex UK) at the Biochemistry Department, St. James's Hospital Dublin. The laboratory is fully accredited and partakes in the vitamin D external quality assessment (DEQAS) scheme. Vitamin D was assayed using a commercially available kit (MassChrom® 25-OH-Vitamin D3; Chromsystems GmbH, Germany) with calibrators traceable to the National Institute of Standards and Technology (NIST) reference standards. Inter- and intra-assay coefficients of variation were 6.5% and 7.5% respectively.

Plasma parathyroid hormone (PTH) concentrations were analysed, in duplicate, using a commercially available enzyme-linked immunosorbent assay (ELISA). Intra- and inter-assay coefficients of variation were 6.8% and 6.2% respectively.

Full blood profiles were measured, as part of the original study, in whole blood within 2 hours of sample collection (Sysmex KX21-N, Sysmex Ltd, UK). EDTA tubes and the internal quality control tube were placed on a roller for 10 minutes at ambient temperature to ensure adequate mixing. Quality control tests were run daily, prior to analysis of EDTA samples.

Peripheral concentrations of Th1-derived cytokines (interferon- $\gamma$ , TNF- $\alpha$  interleukins (IL)-1 $\beta$ , IL-2, IL-6, IL-8, IL-12p70) and Th2-derived cytokines (IL-4, IL-10, and IL-13) were analysed in EDTA plasma using a multi-spot electrochemiluminescent assay according to the manufacturer's instructions (Meso Scale Discovery, Gaithersburg, MD). The performance of this assay has been reported elsewhere (Dabitao et al., 2011). This assay was chosen owing to the broad range of pro- and anti-inflammatory cytokines included. For all cytokines that fell within range of the assay and are reported in the current paper, inter-assay coefficients of variation were below 10% and averaged 8.4% overall.

High sensitivity C-reactive protein (hs-CRP), was quantified in serum using a clinical chemistry analyser in order to obtain a measure of systemic inflammation (ILab 650, Instrumentation Laboratory, Lexington, MA, USA). If a footballer's hs-CRP concentration exceeded 10mg/L at either time point, this data was excluded because hs-CRP concentrations in excess of 10mg/L are indicative of acute infection (Ridker, 2003).

Plasma LL-37 concentrations were analysed in duplicate using a commercially available enzyme-linked immunosorbent assay as this antimicrobial peptide is thought to be vitamin D-regulated (Hycult Biotech, Fronstraat, The Netherlands). The assay was performed as per the manufacturer's instructions with a 20x sample dilution. All samples fell within the range of the assay (0-100ng/mL) with intra- and inter-assay coefficients of variation of 13.9% and 5.4% respectively.

## Questionnaires

All questionnaires were completed in the presence of a researcher. Physical activity (hours/day) was estimated using the validated Recent Physical Activity Questionnaire (Besson et al., 2010). Footballers completed this pre and post-intervention. Dietary vitamin D intake was estimated

using a validated food frequency questionnaire (Weir et al., 2016). Gaelic footballers answered questions relating to their intake of foods that contain vitamin D. Typical portion sizes of such foods were estimated using a photographic food atlas.

#### Body composition

The height and weight of Gaelic footballers was ascertained using a stadiometer and calibrated scales. Fat mass (FM) and fat-free mass (FFM) were also measured by whole body densitometry pre and post-intervention as part of the original study (BOD POD, Life Measurement Inc, Concord, CA). FM and FFM (kg) were adjusted for athlete's height ( $m^2$ ) and presented as fat mass index (FMI) and fat-free mass index (FFMI) ( $kg/m^2$ ).

#### Statistical analysis

The Statistical Package for the Social Sciences (SPSS) was used to perform statistical analyses (IBM SPSS Statistics for Windows, version 21.0, IBM Corp, Armonk, NY). Statistical significance was set at  $P < 0.05$  throughout. Analyses were performed according to the intention-to-treat principle including all participants randomised to intervention ( $n=42$ ) (Moher et al., 2010). The Shapiro-Wilk test was used to determine if data followed a normal distribution and skewed variables were transformed, using the logarithmic function, to attain a normal distribution prior to multiple imputation and further analysis. Transformations were applied to total 25(OH)D and all inflammatory biomarker concentrations. Descriptive statistics were used to present participant characteristics at baseline. Using an independent  $t$ -test or Chi-Square test, significant differences between treatment groups at baseline were identified. Analysis of covariance (ANCOVA) tested the effect of intervention on immune marker concentrations compared to the placebo group and adjusted for *a priori* covariates. In observational analysis, data from pre and post-intervention were pooled and an ANCOVA used to determine whether inflammatory biomarker concentrations varied according to the 75nmol/L proposed threshold for immune

function after adjusting for sex and FMI (Hart, 2012). Potential differences in the distribution of males and females between vitamin D status groups was tested using a Chi-square test. *Post-hoc* power calculations were performed using the effect sizes generated from each ANCOVA model (GPower version 3.1).

## Results

The participant flow is detailed in (Todd et al., 2016). A total of seven footballers were lost to follow up ( $n=5$  VD and  $n=2$  PL) due to sun holidays or injury/illness that was unrelated to the study. Physical and biochemical characteristics pre and post-intervention for VD and PL groups are provided in Table 1. At baseline, only 3 footballers presented with a total 25(OH)D concentration above 75nmol/L. Overall, 21 footballers exhibited vitamin D insufficiency (31-49nmol/L) and 9 footballers exhibited clinical vitamin D deficiency (<30nmol/L) at baseline (Todd et al., 2016). Vitamin D<sub>3</sub> supplementation increased total 25(OH)D by 79%; a significant increase compared to footballers allocated to PL ( $P=0.006$ ), Figure 1. On average, footballers consumed  $5.87\pm 4.25\mu\text{g}$  vitamin D per day through dietary sources. There was no significant difference in the change over time in physical activity between VD and PL groups and therefore this variable was not included as a covariate in ANCOVA models. Concentrations of IL-1 $\beta$  and IL-4 were undetectable in all serum samples analysed and IL-2, IL-4, IL-6, IL-10, and IL-12p70 concentrations fell below the lower limit of detection (LLOD) of the assay and were therefore excluded from statistical analyses. Using ANCOVA, it was determined that there was no significant effect of vitamin D<sub>3</sub> supplementation on change over time in WBC, lymphocyte, neutrophil, hs-CRP, IL-8, TNF- $\alpha$  or LL-37 concentrations when compared to the PL group (all  $P > 0.05$ ). In observational analysis, there were 11 males and 11 females with 25(OH)D concentrations >75nmol/L, and 25 males and 37 females with 25(OH)D <75nmol/L. There was no significant difference in the distribution of males and females across these groups ( $P=0.294$ ). Athletes with a vitamin D status above 75nmol/L exhibited significantly lower neutrophil and

WBC concentrations and significantly higher TNF- $\alpha$  and hs-CRP concentrations compared to those below 75nmol/L, Table 2.

## Discussion

Whilst total 25(OH)D cut-offs for bone health are well-established (Ross et al., 2011), no consensus has been met over what constitutes an 'optimal' total 25(OH)D concentration for extra-skeletal outcomes such as inflammation. Although it has been suggested that a total 25(OH)D concentration in excess of 75nmol/L may be necessary in order to achieve immune-related benefits (He et al., 2016; Laird et al., 2014), these thresholds are currently speculative owing to inconsistent results from randomised controlled trials (Barnes et al., 2011; Das et al., 2014; He et al., 2015). Furthermore, it is not uncommon for cytokines to fall below the LLOD of a multiplex assay when samples are derived from healthy adults (Kleiner et al., 2013). A wealth of *in vitro* data lends support to an immunoregulatory role of 1,25(OH)<sub>2</sub>D (Cantorna et al., 2004); although few have investigated the impact of vitamin D supplementation on inflammation in athletes *in vivo* (He et al., 2015; Lewis et al., 2013). Despite 93% of the cohort in the present study presenting with total 25(OH)D concentrations below 75nmol/L at baseline and an endpoint mean total 25(OH)D concentration of 84nmol/L, supplementation with vitamin D<sub>3</sub> did not impact measures of inflammation including peripheral cytokine or antimicrobial peptide concentrations. This finding is comparable to that of Lewis *et al.* whose 6-month intervention (4000IU/100 $\mu$ g vitamin D<sub>3</sub> daily) in collegiate swimmers and divers did not detect an effect of supplementation on TNF- $\alpha$ , IL-6 and IL-1 $\beta$  concentrations when compared to a placebo group (Lewis et al., 2013). It is likely that vitamin D<sub>3</sub> supplementation did not impact immune parameters in the current study because biomarkers were within their respective normal range, or at a concentration comparable to that of the general population at baseline (Black et al., 2004; Kim et al., 2011). It is plausible that vitamin D<sub>3</sub> supplementation may only deliver an immunological benefit in those with a compromised immune system or underlying inflammation at baseline; a

concept supported by a recent review of randomised controlled trials (Cannell et al., 2014). For that reason, future placebo-controlled trials testing the immunological effects of vitamin D supplementation in healthy athletes may wish to consider obtaining blood samples immediately following high-intensity exercise. Emerging evidence suggests that this type of study design may be necessary in order to identify if vitamin D supplementation bolsters the compensatory anti-inflammatory cytokine response to high intensity exercise (Barker et al., 2013a; Pedersen, 2000).

Circulating LL-37 concentrations were similar to those reported in a large observational study of endurance athletes (He et al., 2013); yet universal reference ranges for this antimicrobial peptide have yet to be established and the clinical implications of LL-37 require further investigation. Vitamin D supplementation did not have an effect on LL-37 concentrations which contrasts with a recent vitamin D intervention trial conducted in male athletes (He et al., 2015). Over 14-weeks, He and colleagues reported a greater percentage increase in the LL-37 concentration of athletes supplemented with 5000IU (125µg) vitamin D<sub>3</sub> compared to athletes assigned to a placebo. However, when looking at absolute change, LL-37 concentrations increased significantly in both vitamin D-supplemented and placebo groups suggesting that use of percentage change, which does not account for variation between groups at baseline, may be masking a null result (Vickers, 2001). Furthermore, secondary analysis from a randomised controlled trial conducted in healthy females determined that vitamin D<sub>3</sub> supplementation (60,000 IU weekly for 2 months followed by 60,000 IU fortnightly for six months) did not have an impact on the mRNA expression of LL-37 (Das et al., 2014). Such findings question whether theory driven by promising *in vitro* studies can be applied to intervention trials in a healthy athlete population.

Observational findings have also been inconsistent with regard to the proposed link between vitamin D and inflammation in athletes and healthy adults (Dixon et al., 2012; Willis et al., 2012).

This is likely owing to statistically underpowered studies as well as differences in assay methodology, the type of athletes recruited and the total 25(OH)D cut-off used to categorise participants. Although the current study identified significant differences in neutrophil, WBC, TNF- $\alpha$  and hs-CRP concentrations between those with a vitamin D status  $\pm 75$ nmol/L; the abovementioned immune markers were within their respective normal range and so differences are unlikely to be clinically relevant.

To our knowledge this is the first study to explore vitamin D and inflammation in Irish athletes. Despite being one of the largest vitamin D intervention trials in athletes to date, sample size was calculated to detect a significant change in  $VO_2$  max the primary outcome measure of the original study and not a significant change in inflammatory biomarkers. The current study was powered as follows to detect a significant change in WBC, 28%; lymphocytes, 50%; neutrophils, 78%; LL-37, 13%; IL-8, 100%, TNF- $\alpha$ , 98% and hs-CRP, 65%. Nonetheless, few have investigated the effects of vitamin D supplementation on inflammation in athletes and results of this study provide useful preliminary data to inform future adequately powered studies. A limitation of the current study is absence of an infection symptoms questionnaire and functional measures of immune function, such as response to an antigen challenge. Strengths of the study include the randomised controlled trial design, gender balance of athletes and the wide range of inflammatory biomarkers assessed. In conclusion, supplementation for 12-weeks with 3000IU (75 $\mu$ g) vitamin D<sub>3</sub> per day did not have any effect on biomarkers of inflammation in this cohort of collegiate Gaelic footballers despite optimising vitamin D concentrations.

### **Perspectives**

Based on the results of this study, it is clear that further research is necessary before vitamin D<sub>3</sub> supplementation can be deemed an important mediator of inflammation in healthy athletes. The current study demonstrated that restoring vitamin D status, from below 50nmol/L to above 75nmol/L, does not influence circulating inflammatory biomarker concentrations; a conclusion

supported by a previous randomised controlled trial conducted in adolescent swimmers and divers (Lewis et al., 2013). Future vitamin D intervention studies in this area should focus on athlete cohorts at risk of a compromised immune system, those exhibiting underlying chronic inflammation, or sample athletes following vigorous exercise or rehabilitation from injury in order to establish any beneficial effects on inflammation.

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## Tables

**Table 1.** Physical and biochemical characteristics of Gaelic footballers at baseline and week 12 presented as mean  $\pm$  SD

Treatment group	Vitamin D ( <i>n</i> =22)		Placebo ( <i>n</i> =20)			
Measure	Baseline	Week 12	Baseline	Week 12	<i>P</i>	<i>P</i>
<i>General characteristics</i>						
Age, y	20 $\pm$ 2	-	20 $\pm$ 2	-	0.819	-
Height, cm	171.39 $\pm$ 8.65	-	165.65 $\pm$ 10.18	-	0.048 <sup>a</sup>	-
Weight, Kg	70.52 $\pm$ 11.49	71.1 $\pm$ 11.91	61.92 $\pm$ 10.69	62.30 $\pm$ 10.56	0.009 <sup>a</sup>	0.493
BMI, kg/m <sup>2</sup>	23.89 $\pm$ 2.66	24.30 $\pm$ 2.82	22.31 $\pm$ 2.19	22.57 $\pm$ 2.35	0.031 <sup>a</sup>	0.605
Physical activity, hr/day	1.06 $\pm$ 1.04	0.70 $\pm$ 0.59	0.88 $\pm$ 0.89	0.51 $\pm$ 0.47	0.466	0.749
FMI, kg/m <sup>2</sup>	5.23 $\pm$ 2.71	5.70 $\pm$ 2.97	5.81 $\pm$ 1.94	5.76 $\pm$ 2.03	0.439	0.047 <sup>b</sup>
FFMI, kg/m <sup>2</sup>	18.95 $\pm$ 3.11	18.34 $\pm$ 2.51	16.46 $\pm$ 1.88	16.89 $\pm$ 2.12	0.004 <sup>a</sup>	0.082
Vitamin D intake, $\mu$ g/day	6.73 $\pm$ 5.33	-	4.93 $\pm$ 2.47	-	0.227	-
PTH, pg/mL	41.21 $\pm$ 15.98	42.24 $\pm$ 20.96	39.28 $\pm$ 19.12	43.93 $\pm$ 23.65	0.502	0.204
Adjusted calcium, mmol/L	2.29 $\pm$ 0.07	2.29 $\pm$ 0.09	2.29 $\pm$ 0.08	2.31 $\pm$ 0.08	0.961	0.427
<i>Inflammation</i>						
WBC count, x10 <sup>3</sup> / $\mu$ L	5.78 $\pm$ 1.63	5.77 $\pm$ 1.60	5.94 $\pm$ 1.13	5.83 $\pm$ 1.09	0.551	0.766
Lymphocytes, x10 <sup>3</sup> / $\mu$ L	1.96 $\pm$ 0.48	1.77 $\pm$ 0.56	1.95 $\pm$ 0.55	1.76 $\pm$ 0.50	0.885	0.727
Neutrophils, x10 <sup>3</sup> / $\mu$ L	3.36 $\pm$ 1.38	3.54 $\pm$ 1.42	3.47 $\pm$ 0.88	3.57 $\pm$ 1.07	0.426	0.756
IL-8, pg/mL	5.36 $\pm$ 2.80	4.59 $\pm$ 1.18	4.61 $\pm$ 1.31	4.90 $\pm$ 1.47	0.347	0.101
TNF- $\alpha$ , pg/mL	2.15 $\pm$ 0.42	5.61 $\pm$ 1.51	2.01 $\pm$ 0.60	5.95 $\pm$ 1.63	0.271	0.422
LL-37, ng/mL	36.21 $\pm$ 13.43	29.69 $\pm$ 21.50	36.94 $\pm$ 24.75	21.71 $\pm$ 27.86	0.033 <sup>a</sup>	0.347
hs-CRP, mg/L	1.01 $\pm$ 1.28	1.81 $\pm$ 2.99	1.90 $\pm$ 1.45	2.81 $\pm$ 4.85	0.979	0.474

<sup>a</sup> Significant difference between treatment groups at baseline using an independent *t* test or chi-squared test (*P*<0.05).

<sup>b</sup> Significant difference in change over time between treatment groups using an independent *t* test (*P*<0.05).

**Abbreviations:** 25-hydroxyvitamin D, 25(OH)D; body mass index, BMI; parathyroid hormone, PTH; white blood cell count, WBC; interleukin, IL; tumour necrosis factor, TNF; cathelicidin antimicrobial peptide, LL-37; high-sensitivity C-reactive protein, hs-CRP; fat mass index, FMI; fat-free mass index, FFMI

**Table 2.** Characteristics of footballers according to total 25(OH)D concentrations above and below 75nmol/L with values provided as mean  $\pm$  SD (pooled data)

Measure	Total 25(OH)D concentration		P
	<75nmol/L (n=62)	>75nmol/L (n=22)	
<i>General characteristics</i> <sup>a</sup>			
Age, y	20 $\pm$ 2	20 $\pm$ 2	0.846
BMI, kg/m <sup>2</sup>	23.17 $\pm$ 2.56	23.60 $\pm$ 2.94	0.544
Physical activity, hr/day	0.75 $\pm$ 0.72	0.79 $\pm$ 0.97	0.749
FMI, kg/m <sup>2</sup>	5.68 $\pm$ 2.31	5.55 $\pm$ 2.79	0.597
FFMI, kg/m <sup>2</sup>	17.61 $\pm$ 2.68	18.03 $\pm$ 2.40	0.467
Total 25(OH)D, nmol/L	43.34 $\pm$ 13.22	92.92 $\pm$ 19.07	0.001 <sup>d</sup>
PTH, pg/mL	41.63 $\pm$ 18.46	41.76 $\pm$ 14.08	0.823
Adjusted calcium, mmol/L	2.30 $\pm$ 0.07	2.27 $\pm$ 0.53	0.122
<i>Inflammation</i> <sup>b</sup>			
WBC count, x10 <sup>3</sup> / $\mu$ L	6.00 $\pm$ 1.28	5.33 $\pm$ 1.39	0.034 <sup>c</sup>
Lymphocytes, x10 <sup>3</sup> / $\mu$ L	1.87 $\pm$ 0.49	1.81 $\pm$ 0.50	0.594
Neutrophils, x10 <sup>3</sup> / $\mu$ L	3.65 $\pm$ 1.18	3.03 $\pm$ 1.01	0.028 <sup>c</sup>
LL-37, ng/mL	31.02 $\pm$ 21.68	31.80 $\pm$ 18.91	0.522
IL-8, pg/mL	4.88 $\pm$ 1.92	4.82 $\pm$ 1.41	0.919
TNF- $\alpha$ , pg/mL	3.38 $\pm$ 2.08	5.28 $\pm$ 1.57	0.001 <sup>d</sup>
hs-CRP, mg/L	1.57 $\pm$ 2.83	2.03 $\pm$ 2.07	0.025 <sup>c</sup>

<sup>a</sup> Differences between groups assessed using an independent *t* test or chi-squared test

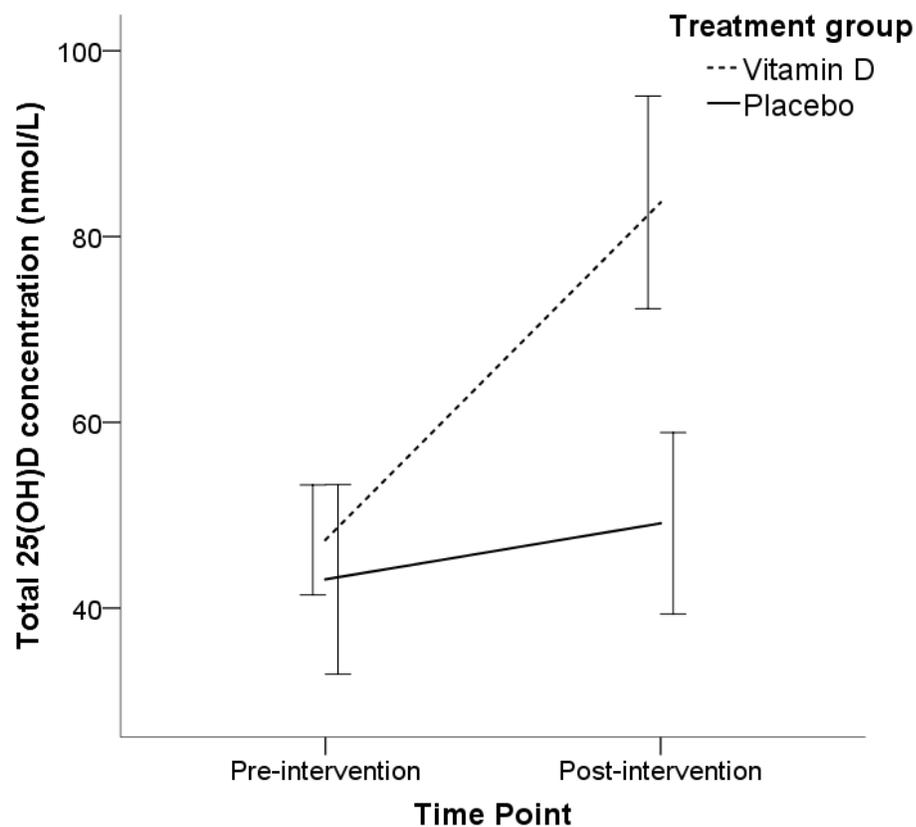
<sup>b</sup> Differences between groups assessed using an ANCOVA adjusting for FMI and sex

<sup>c</sup> Statistically significant difference between vitamin D status groups  $P < 0.05$

<sup>d</sup> Statistically significant difference between vitamin D status groups  $P < 0.001$

**Abbreviations:** 25-hydroxyvitamin D, 25(OH)D; body mass index, BMI; parathyroid hormone, PTH; fat mass index, FMI; white blood cell count, WBC; interleukin, IL; tumour necrosis factor, TNF; cathelicidin antimicrobial peptide, LL-37, high-sensitivity C-reactive protein, hs-CRP; fat mass index, FMI; fat-free mass index, FFMI.

## Figures



**Figure 1.** Total 25(OH)D response to intervention versus placebo presented as mean (95% CI). Daily supplementation for 12-weeks with 3000IU (75 $\mu$ g) vitamin D<sub>3</sub> increased mean total 25(OH)D concentration from 47 to 84nmol/L (+79%) compared with 43 to 49nmol/L (+14%) in those allocated to placebo (ANCOVA,  $P= 0.006$ ).

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