Influence of vitamin D status on respiratory infection incidence and immune function during 4 months of winter training in endurance sport athletes

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Abstract

The purpose of this study was to examine the influence of vitamin D status on mucosal and systemic immunity and the incidence, severity and duration of upper respiratory tract illness (URTI) episodes in endurance athletes during a 16-week winter training period. Blood was collected from 225 subjects at the start of the study and plasma was analysed for total 25-hydroxy vitamin D (25(OH)D) and cathelicidin concentration. Blood was also collected at the end of the study and analysed for 25(OH)D and antigen-stimulated cytokine production. Unstimulated saliva samples were obtained at the start and at 4-week intervals during the study period. Saliva samples were analysed for salivary antimicrobial peptides and proteins (AMPs). Weekly training and daily illness logs were kept. At the start and end of the study 38% and 55%, respectively, of the athlete cohort had inadequate (plasma 25(OH)D 30-50 nmol/L) or deficient (plasma 25(OH)D < 30 nmol/L) vitamin D status. There was a significantly higher proportion of subjects who presented with symptoms of URTI in the vitamin D deficient status group (initial plasma 25(OH)D < 30 nmol/L) during the study period than in the optimal vitamin D group (> 120 nmol/L) and the total number of URTI symptom days and the median symptom-severity score in the vitamin D deficient group was signifi-

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cantly higher than in the other groups. The plasma cathelicidin concentration positively correlated with the plasma 25(OH)D concentration and the saliva secretory immunoglobulin A (SIgA) secretion rate in the optimal vitamin D status group was significantly higher than in the other groups. Low vitamin D status was associated with lower pro-inflammatory cytokine production by monocytes and lymphocytes. Low vitamin D status could be an important determinant of URTI risk in endurance athletes and mucosal as well as systemic immunity may be modified via vitamin D-dependent mechanisms.

Key words: exercise training, cholecalciferol, ergocalciferol, saliva antimicrobial proteins, common cold

Introduction

It has only recently been recognised that vitamin D plays an important role in up-regulating immunity [16]. Vitamin D is a key link between Toll-like receptor (TLR) activation and antimicrobial responses in innate immunity. Vitamin D has a vital role in up-regulating the expression of antimicrobial peptides and proteins (AMPs), such as cathelicidin and β-defensin [23]. These AMPs have a broad range of activities against microorganisms and may be involved in the direct inactivation of viruses [16]. They are produced by epithelial cells and macrophages and in the lungs are secreted into the biofilm covering the inner surface of the airways, thereby creating a barrier that is chemically lethal to microbes. The biologically active form of vitamin D (1,25 dihydroxy cholecalciferol) activates the genes for cathelicidin synthesis and enhances the effectiveness of monocytes and macrophages in killing microbes by enhancing the oxidative burst potential of these phagocytic cells [28]. Furthermore, vitamin D has been shown to be essential in activating and controlling the T-cell antigen receptor and thus enhancing the recognition of antigens by T lymphocytes [30]. Vitamin D may also influence cytokine production during periods of infection [3].

Several recent studies have found a negative association between vitamin D status and respiratory illness incidence in young and elderly adults [1,9,20]. The incidence of respiratory illnesses is generally higher in athletes [10] and low vitamin D status could be a contributing factor as vitamin D insufficiency has been reported to be common in athletes [21] especially if exposure to natural sunlight is limited (e.g. when training in the winter months or when training mostly indoors).

During a 4-month winter training period we conducted a study on a large cohort of endurance athletes who completed daily URTI symptom diaries and reported weekly training loads using validated questionnaires. We also collected blood samples from these athletes at the start and end of the study and saliva at the start and at 4-weekly intervals. Our aims were to determine the influence of vitamin D status on mucosal immunity, antigen-stimulated cytokine production and the incidence, severity and duration of upper respiratory tract illness (URTI) episodes in endurance athletes during a winter training period.

Methods

Subjects

Two hundred and sixty seven subjects who were engaged in regular sports training (predominantly endurance-based activities such as running, cycling, swimming, triathlon, team games and racquet sports) volunteered to participate in the study. Subjects ranged from recreationally active to Olympic triathletes and their self-reported training loads averaged 10 h/week. Subjects were required to complete a comprehensive health-screening questionnaire prior to starting the study and had not taken any regular medication or antibiotics in the 3 months prior to the study. All subjects were fully informed about the rationale for the study and of all experimental procedures to be undertaken. Subjects provided written consent to participate in the study, which had earlier received the approval of Loughborough University ethical advisory committee. Subjects were enrolled after having fulfilled all inclusion criteria, and presenting none of the exclusion criteria (determined by both questionnaire and interview). Subjects could be included if they were currently healthy, had been involved in endurance training for at least 2 years, engaged in at least 3 sessions and at least 3 h of total moderate/high-intensity training time per week and were between 18-40 years of age. Subjects representing one or more of the following criteria were excluded from participation: smoking or use of any medication, suffering from or had a history of cardiac, hepatic, renal, pulmonary, neurological, gastrointestinal, haematological or psychiatric illness. A total of 267 healthy individuals (83 females and 184 males) were recruited as subjects from Loughborough University, UK (latitude 53°N) during November 2011 with the mean age of the study cohort at recruitment being 21 ± 3 years (mean ± SD). For the first visit to the laboratory, subjects arrived in the morning at 08:30-10:30 following an overnight fast of approximately 12 h and their body mass and height were recorded. Information about the study was given to them and they then signed an informed consent form. Subjects then sat quietly for 10 min and completed a health-screening questionnaire and inclusion/exclusion criteria questionnaire before providing an unstimulated saliva sample by passive dribble into a pre-weighed sterile collection tube for a timed period (usually 2 min; longer was allowed if the volume of saliva collected after 2 min was insufficient). After centrifugation for 2 min at 5000 *g* to remove cells and insoluble matter, saliva samples were stored frozen at -80ºC prior to analysis. Subsequently, a resting venous blood sample (12 ml) was obtained by venepuncture from an antecubital forearm vein into two vacutainer tubes (Becton Dickinson, Oxford, UK) containing K_3EDTA and lithium heparin. Haematological analysis was immediately carried out on the the EDTA sample (including haemoglobin, haematocrit and total and differential leukocyte counts) using an automated cell-counter (A^c.T™5diff haematology analyser, Beckman Coulter, High Wycombe, UK). Subjects had to have normal haematology to be included in the study. The remaining EDTA blood was centifuged for 10 min at 1500 *g* and 4°C and the plasma stored at -80°C prior to analysis. Heparinised blood was used immediately for the measurement of antigen-stimulated cytokine production.

Study protocol

During the 4-month study period subjects were requested to continue with their

normal training programs. Subjects completed a validated health (URTI symptoms) questionnaire [14] on a daily basis. Subjects were not required to abstain from medication when they were suffering from illness symptoms but they were required, on a weekly basis, to report any unprescribed medications taken, visits to the doctor or any prescribed medications.

The illness symptoms listed on the questionnaire were: sneezing, headache, malaise, nasal discharge, nasal obstruction, sore throat, cough, ear ache, hoarseness, fever, chilliness and joint aches and pains. The non-numerical severity ratings of mild, moderate and severe of severity of symptoms were scored as 1, 2 or 3, respectively to provide a quantitative means of data analysis and the total symptom score for every subject each day was calculated as a sum of multiplied numbers of symptoms experienced by the numerical severity ratings. A URTI was deemed present when (i) total symptom score was ≥15 on any two consecutive days and (ii) when a subject positively indicated suffering a common cold on ≥ 3 days according to Jackson et al. [14]. Subjects were also asked to rate the impact of illness symptoms on their ability to train (above normal, at the same level, below normal or training stopped). The total number of URTI symptom days was also determined as the number of days with a symptom score of ≥5 according to Predy et al. [26].

Subjects were also asked to fill in a standard short form of International Physical Activity Questionnaire (IPAQ; http://www.ipaq.ki.se/downloads.htm) at weekly intervals, thus providing a quantitative information on training loads in metabolic equivalents (MET)-h/week [6]. Subjects attended the laboratory every 4 weeks following an overnight fast. Subjects were required to abstain from any strenuous physical activity for 24 h before coming to the laboratory. During these visits body mass was recorded and an unstimulated saliva sample was collected. Venous blood samples were collected only at the start and end of the study period. A total of 225 subjects completed the study and provided sufficient blood for routine haematology and vitamin D status analysis at the start of the study and sufficient saliva for analysis of AMPs on all 5 occasions. Plasma samples from 181 subjects were also analysed for Vitamin D status at the end of the study. After vitamin D status analysis of baseline samples, we also analyzed the initial plasma cathelicidin concentration from a subset of 80 subjects with high-level (all subjects with initial plasma 25(OH)D >90 nmol/L, n=26), a random selection of mid-level (33-89 nmol/L, n=27) and low-level (all subjects with <33 nmol/L, n=27) 25(OH)D concentration. After vitamin D status analysis of samples collected at the end of the study, we also measured antigen-stimulated cytokine production in a subset of 48 subjects with high-level (initial plasma 25(OH)D >90 nmol/L, n=24) and low-level (<30 nmol/L, n=24) 25(OH)D concentration.

Saliva analysis

The saliva volume collected was estimated by weighing and the saliva flow rate was calculated. Saliva samples were analysed for secretory immunoglobulin A (SIgA) using an ELISA kit (Salimetrics, Philadelphia, USA) and α-amylase activity was measured as previously described [22]. Salivary lactoferrin and lysozyme were analysed using commercially available ELISA kits (Calbiochem, USA and

Biomedical Technologies, USA, respectively). Secretion rates for each of the salivary AMPs were calculated as the multiple of the saliva flow rate and the AMP concentration. Values obtained from the 5 visits at 4-week intervals were averaged for each subject. All saliva assays were carried out in duplicate. Coefficients of variation (CVs) for the assays were <5% for all salivary AMPs.

Plasma analysis

Vitamin D occurs in two forms: cholecalciferol (D3) which is formed by the action of UV light on the skin and ergocalciferol (D2) from plant food sources. The best measure of vitamin D status is considered to be the sum of the 25-hydroxy metabolites of D2 and D3 ($25(OH)D_3$ and $25(OH)D_2$) and the best way of measuring these is considered to be the high pressure liquid chromatography - tandem mass spectrometer method [31]. Our EDTA plasma samples were analysed for $25(OH)D_3$ and $25(OH)D_2$ with a high pressure liquid chromatography tandem mass spectrometer (Waters Acuity, Manchester, UK) after a maximum of 10 months in storage with no previous freeze-thaw cycles as described previously [29]. Briefly, $25(OH)D_2$, $25(OH)D_3$ and deuterated internal standard were extracted from plasma samples, following protein precipitation, using Isolute C18 solid phase extraction cartridges. Potential interfering compounds were removed by initial elution with 50% methanol followed by elution of the vitamins using 10% tetrahydrofuran in acetonitrile. Dried extracts were reconstituted prior to injection into a high performance liquid chromatography tandem mass spectrometer in the multiple reaction mode (MRM). The MRM transitions (*m/z*) used were 413.2 > 395.3, 401.1 > 383.3 and 407.5 > 107.2 for $25(OH)D_2$, $25(OH)D_3$ and hexa-deuterated$(OH)D_3$ (internal standard), respectively. Intra-assay CVs were <10% across a working range of 2.5–624 nmol/L for both $25(OH)D_3$ and $25(OH)D_2$. Measurements were performed in a laboratory meeting the performance target set by the Vitamin D External Quality Assessment Scheme (DEQAS) Advisory Panel for 25(OH)D assays.

EDTA plasma was assayed for cathelicidin concentration using a commercially available ELISA kit (Hycult Biotech, Uden, The Netherlands) according to the manufacturers' instructions. The intra-assay CV was 3%.

Antigen-stimulated cytokine production

Stimulated whole blood culture production of cytokines (interferon (IFN)-γ, tumour necrosis factor (TNF)-α, interleukin (IL)-1β, IL-2, IL-4, IL-6 and IL-10) was determined as described previously [11]. The stimulant was a commercially available multi-antigen vaccine (Pediacel Vaccine, Sanofi Pasteur, UK) containing diphtheria, tetanus, acellular pertussis, poliomyelitis and haemophilus influenzae type b antigens. Briefly, 0.25 mL of heparinized whole blood was added to 0.75 mL of RPMI 1640 medium (Sigma Chemicals, Poole, UK) with an added 40 μL of Pediacel vaccine cocktail (Sanofi Pasteur msd Limited, Maidenhead, UK) at a dilution of 1:100, before being incubated at 37°C and 5% CO_2 for 24 h. The stimulant dilution of 1:100 used in this study was based on a separate experiment (unpublished data), which established the dose–response curve for the measured cytokines over the dilution range of 1:100–1:20 000. Samples were then centrifuged at 15000 rpm for 4 min at 4°C, following which the supernatant

fluid was harvested and stored at -80°C prior to analysis of cytokine concentrations using an Evidence Investigator System using the high sensitivity cytokine biochip array EV3513 (Randox, County Antrim, UK). The intra-assay CV for all measured cytokines was less than 5.0%. The measured cytokine concentrations for the monocyte-derived cytokines (TNF-α, IL-1β and IL-6) and lymphocyte-derived cytokines (IL-2, IL-4 and IFN-γ) were divided by the monocyte and lymphocyte counts, respectively to give cytokine production per 10^6 cells.

Statistical analysis

The Shapiro-Wilk test was used to determine if data sets were normally distributed. The Kruskal-Wallis test (nonparametric equivalent of one-way ANOVA) with post-hoc Mann-Whitney test was used to examine differences in the salivary variables, blood leukocyte counts and the total number of URTI episodes and symptom days among groups classified by vitamin D status using the following ranges for plasma total 25(OH)D: 12-30 nmol/L (deficient); 30-50 nmol/L (inadequate), 50-120 nmol/L (adequate) and >120 nmol/L (optimal) [4]. For subjects with URTI symptoms, the symptom-severity score and the duration of URTI episodes among the 4 groups were also assessed using the Kruskal-Wallis test with post-hoc Mann-Whitney test. The plasma total 25(OH)D concentrations were compared between male and female subjects, and indoor and outdoor training locations using the Mann-Whitney test. The total plasma 25(OH)D concentrations at the start and the end of the study were compared by Wilcoxon signed-rank test. The difference in proportion of subjects who presented with symptoms of URTI during the trial between the vitamin D optimal and deficient groups was assessed by the chi-squared test. Correlation between the number of URTI episodes and the plasma 25(OH)D concentration as well as the plasma cathelicidin and 25(OH)D concentration was done using Spearman's rank correlation coefficient. One-way ANOVA with post-hoc Bonferroni test was used to examine differences in the plasma cathelicidin among high, middle and low level vitamin D status groups. Differences in antigen-stimulated cytokine production between high and low vitamin D status groups were compared with the Mann-Whitney test. We also evaluated the impact of URTI episodes on training volume by comparing physical activity levels (MET-h/week) on weeks when an URTI episode was present with the average MET-h/week when the subjects were healthy. Data are presented as mean (±SD) for data sets that were normally distributed; for data sets that were not normally distributed, the median and interquartile range (IQR) are shown. The accepted level of significance was $P<0.05$.

Results

Adherence to the study

Of the 267 subjects, 239 subjects (169 males, 70 females) completed the full 16 weeks of the study. Reasons for dropout included overseas travel, injury or persistent non-respiratory illness (preventing them from performing training) or due to undisclosed reasons. Saliva samples were obtained on all 5 visits from 236 subjects. Plasma samples were analysed for total 25(OH)D from 225 subjects

because there were 11 subjects having insufficient plasma volume for this analysis. At the end of the study blood samples with sufficient volume for analysis of plasma total 25(OH)D were obtained from 181 subjects.

Baseline characteristics and physical activity levels

Baseline characteristics of the 225 subjects who completed the study and for whom vitamin D status was established were (mean ± SD) age: 21 ± 3 years, body mass: 73.4 ± 11.4 kg, height: 176.2 ± 9.1 cm, body mass index: 23.5 ± 2.3 kg/m^2 and self-reported weekly training duration: 9.6 ± 5.2 h/week. Analysis of the IPAQ questionnaires indicated that the training loads were fairly consistent over the 16 weeks of the study. Mean training loads were 67.5 ± 31.2 MET-h/week which is equivalent to about 11 ± 5 hours of moderate-vigorous activity per week.

Vitamin D status

Plasma 25(OH)D_2 was below the detection limit (2.5 nmol/L) in 57% of subjects and the average plasma 25(OH)D_2 concentration was only 4.4 nmol/L (median, 0; IQR, 0-9 nmol/L) . The median (IQR) plasma 25(OH)D_3 concentration at the start of the study was 53 (40-66) nmol/L and the median total 25(OH)D concentration was 57 (44-71) nmol/L. The number (and percentage) of subjects classed as optimal, adequate, inadequate and deficient was 11 (5%), 128 (57%), 68 (30%) and 18 (8%). Plasma 25(OH)D concentration was not significantly different (p=0.478) between males (N=157; median, 56; IQR, 43-69 nmol/l) and females (N=68; median, 58; IQR, 45-72 nmol/L). Plasma 25(OH)D concentration was not significantly different (P = 0.120) between indoor (N=50; median, 64; IQR, 46-73 nmol/L) and outdoor sports (N=175; median, 55; IQR, 43-69 nmol/L). The total plasma 25(OH)D concentration at the end of the study (N=181; median, 47; IQR, 35-68 nmol/L) was significantly lower (P = 0.003) than that at the start of the study. At the start of the 4-month study period 38% of athletes had insufficient or deficient plasma 25(OH)D values (< 50 nmol/L) and by the end of the study 55% of athletes had plasma 25(OH)D values of less than 50 nmol/L.

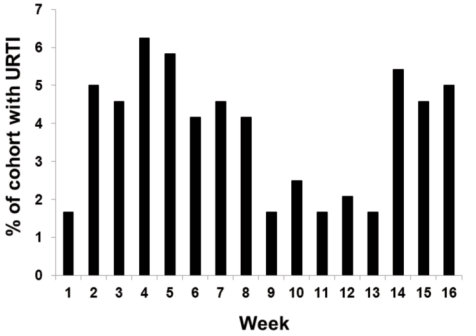


Figure 1. Percentage of the cohort reporting a URTI episode for each week of the study period.

URTI symptom incidence and its impact on training loads

Analysis of the URTI symptom questionnaires indicated that 4.0 ± 1.6% of the cohort experienced a URTI episode each week (Figure 1). One hundred and thirty six subjects did not experience a single URTI episode during the study period whereas 103 subjects experienced at least one URTI episode during the study period.

Table 1. Infection symptom incidence among different vitamin D status groups. Also shown are the severity score and duration of URTI episodes.

INCIDENCE	Optimal N = 11	Adequate N = 128	Inadequate N = 68	Deficient N = 18	P
Number of episodes	0 (0-1) *	0 (0-1)	0 (0-1)	0 (0-1)	0.062
Number of symptom days	1 (0-6)*	4 (0-8)*	4 (1-8)*	9 (3-17)	0.040
WHEN URTI PRESENT	Optimal N = 3	Adequate N = 56	Inadequate N = 27	Deficient N = 12	P
Symptom severity score	43 (38-52)*	47 (40-69)*	62 (46-74)*	102 (67-199)	0.013
Duration (days)	5 (5-7)*	8 (6-9)*	8 (5-14)*	13 (10-17)	0.059

Data are median and interquartile range (IQR). P value from Kruskal-Wallis test is shown in right hand column.

* Significantly different from Deficient (Mann-Whitney U test).

The proportion of subjects whose training was negatively affected when URTI was present was 70%. When mean MET-h/week physical activity during healthy (no URTI symptoms) weeks was compared with mean physical activity during weeks when an URTI episode occurred, there was a significant decrease from 68.3 ± 32.3 to 51.9 ± 40.4 MET-h/week (P = 0.001). Thus, when URTI was present subjects reduced their training load by an average of 24%.

Vitamin D status and URTI incidence, severity and duration

The proportion of subjects in the optimal vitamin D status group who experienced one or more URTI episodes during the trial was significantly lower than for the vitamin D deficient group (optimal 27%, deficient 67%; P = 0.039). There was a significant difference for URTI symptom days among the four vitamin D status groups and the total number of URTI symptom days in the deficient group was significantly higher than the other groups (Table 1). Vitamin D status tended to influence prevalence of URTI episodes but this fell just short of statistical significance (P = 0.061) and there was a tendency for the deficient group to have more episodes (Table 1). For subjects who experienced one or more URTI episodes, there was a significant difference in the median symptom-severity score per URTI episode among the four groups but no significant difference in the median duration of episodes (Table 1) although there was a tendency for episodes to be longer with low vitamin D status. The median symptom-severity score in the deficient group was significantly higher than the other groups.

Plasma cathelicidin concentration

The plasma cathelicidin concentrations were 32.2 ± 11.9, 27.7±10.6 and 24.5±7.5 ng/ml in the high, mid and low level vitamin D status groups, respectively. There was a significant influence of vitamin D status on the plasma cathelicidin concentration (P = 0.027). The plasma cathelicidin concentration in the high-level vitamin D status group was significantly higher than in the low-level group (P = 0.023). In addition, there was a positive correlation between the plasma 25(OH)D and cathelicidin concentrations (r = 0.234, P = 0.036).

Table 2. Salivary concentrations and secretion rates of antimicrobial peptides (AMPs) among different vitamin D status groups.

	Optimal N = 11	Adequate N = 128	Inadequate N = 68	Deficient N = 18	P
Lactoferrin concentration (ng/mL)	2006 (1413-3184)	2187 (1546-3184)	2491 (1606-3633)	2419 (1624-3001)	0.948
Lactoferrin SR (ng/min)	801 (587-1229)	756 (527-1151)	771 (367-1143)	872 (554-1645)	0.568
Lysozyme concentration (μg/mL)	1524 (641-2595)	1524 (926-2808)	1701 (1102-2693)	1361 (883-2437)	0.687
Lysozyme SR (μg/min)	435 (314-906)	517 (315-872)	504 (315-972)	609 (331-837)	0.970
SIgA concentration (μg/mL)	91 (82-130)	66 (49-100)	76 (46-111)	59 (46-78)*	0.103
SIgA SR (μg/min)	38.7 (30.3-48.6)	22.9 (14.2-36.6)*	19.5 (12.7-32.3)*	23.6 (14.8-32.9)*	0.018
Amylase activity (U/L)	131 (60-213)	142 (73-233)	131 (74-202)	121 (64-222)	0.839

Data are median and interquartile range (IQR). P value from Kruskal-Wallis test is shown in right hand column. SR = secretion rate.

* Significantly different from Optimal (Mann-Whitney U test).

Salivary variables

There was a significant difference in SIgA secretion rate among the four vitamin D groups (Figure 2) but no significant difference for the other salivary AMPs (Table 2). The SIgA secretion rate in the optimal vitamin D status group was significantly higher than in the other groups. The SIgA concentration tended to be lowest in the deficient group.

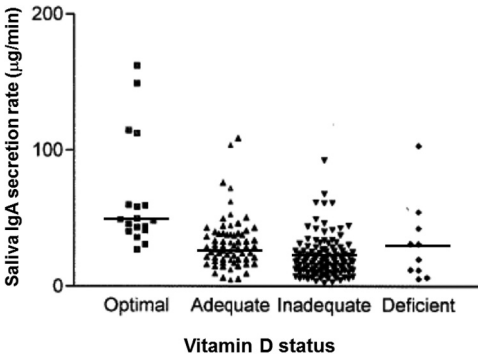


Figure 2. Influence of vitamin D status on the saliva IgA secretion rate. Median values for each group are indicated by the horizontal lines. Kruskal-Wallis test indicated a significant influence of vitamin D status on IgA secretion rate (P = 0.018). Saliva IgA section rate was significantly higher in the Optimal group than in the other groups (P < 0.05).

Blood leukocyte counts

Based on analysis of blood samples collected at the start of the study there was no influence of vitamin D status on circulating numbers of total leukocytes, neutrophils, lymphocytes or monocytes (Table 3).

Antigen-stimulated cytokine production

Both blood monocyte and lymphocyte counts were not significantly different in the subset of subjects for whom antigen-stimulated cytokine production was determined. However, production of all the monocyte-derived cytokines (TNF-α, IL-1β and IL-6) were significantly lower in the vitamin D deficient subjects compared with those with high vitamin D status (all P < 0.005; Figure 3). Production of the lymphocyte-derived pro-inflammatory cytokine IFN-γ was significantly lower in the vitamin D deficient subjects compared with those with high vitamin D status (P < 0.01; Figure 4) and

Table 3. Blood total and differential leukocyte counts among different vitamin D status groups.

	Optimal N = 11	Adequate N = 128	Inadequate N = 68	Deficient N = 18	P
Leukocytes (x10^9 cells/L)	5.9 (5.3-6.6)	5.9 (5.2-6.9)	5.9 (5.2-6.9)	6.5 (5.8-7.8)	0.495
Neutrophils (x10^9 cells/L)	2.8 (2.3-3.7)	2.9 (2.4-3.9)	3.0 (2.3-3.5)	3.4 (2.9-4.2)	0.352
Lymphocytes (x10^9 cells/L)	1.9 (1.7-2.3)	2.1 (1.7-2.3)	2.1 (1.8-2.4)	1.9 (1.8-2.0)	0.555
Monocytes (x10^9 cells/L)	0.5 (0.4-0.6)	0.6 (0.5-0.7)	0.6 (0.5-0.7)	0.6 (0.6-0.8)	0.136

Data are median and interquartile range (IQR). P value from Kruskal-Wallis test is shown in right hand column.

IL-4 also tended to be lower (P = 0.062). Production of IL-2 was not significantly influenced by vitamin D status. The anti-inflammatory cytokine IL-10 is produced by both monocytes and lymphocytes so it is not appropriate to normalise its production by cell counts. The antigen-stimulated production of IL-10 was not significantly different between the high and deficient vitamin D status groups (high: median 4.0; IQR 2.4-8.8 pg/ml *versus* deficient: median 2.8; IQR 2.0-6.0 pg/ml; P = 0.164).

Discussion

This research is of direct relevance to on-going study of the factors that determine illness susceptibility in athletes. The main findings of the present study were as follows: (1) there was a higher proportion of subjects who experienced one or more URTI episodes in the vitamin D deficient status group during the 4-month study period than in the optimal vitamin D group; (2) the total number of URTI symptom days and the median symptom-severity score in the vitamin D deficient group were significantly higher than in the other groups; (3) the plasma cathelicidin concentration was positively associated with vitamin D status; (4) the SIgA secretion rate in the optimal vitamin D status group was significantly higher than in the other groups; (5) pro-inflammatory cytokine production in response to multi- antigen stimulation was substantially lower in vitamin D deficient subjects.

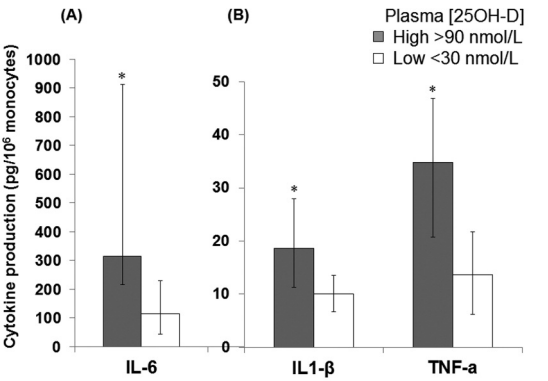
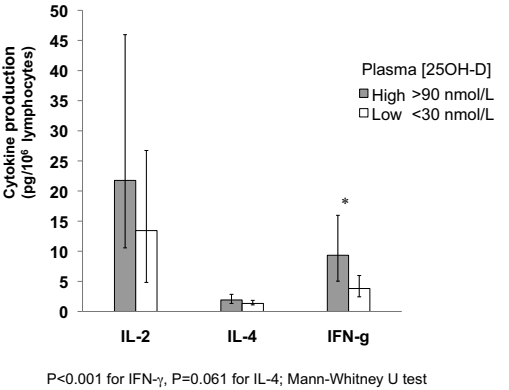


Figure 3. Influence of high or deficient vitamin D status on the antigen-stimulated production of (A) IL-6 and (B) IL-1β and TNF-α by monocytes. Production of all cytokines was significantly higher in the high vitamin D status group compared with the deficient group (all P < 0.005 as indicated by the asterisks).



P<0.001 for IFN-γ, P=0.061 for IL-4; Mann-Whitney U test

Figure 4. Influence of high or deficient vitamin D status on the antigen-stimulated production of cytokines by lymphocytes. * Significant difference between groups (P < 0.05).

According to the findings from the present study, it seems likely that vitamin D status has an influence on URTI symptom incidence. We found that a higher proportion of subjects (67%) in the vitamin D deficient status group experienced one or more URTI episodes during the 4-month study period than in the optimal vitamin D group (27%). Recent studies have also shown an inverse association between vitamin D status and respiratory infection incidence in young and elderly adults [1,9]. In the Third National Health and Nutrition Examination Survey involving 18,883 participants [9], it was found that individuals with plasma 25(OH)D concentration less than 25 nmol/L had a significantly higher risk of respiratory infections (24%) than those with 25(OH)D levels higher than 75 nmol/L (17%). In addition, a population-based study on 6,789 British adults demonstrated that plasma 25(OH)D concentrations are inversely associated with recent URTI [1]. There was a 7% reduction in the risk of respiratory infections for each 10 nmol/L increase in plasma 25(OH)D.

Furthermore, we also found that the total number of URTI symptom days in the vitamin D deficient group was significantly higher than in the other groups. There was a similar result in a Finnish study in which young male Finnish soldiers with plasma 25(OH)D concentration less than 40 nmol/L had more days of absence from duty due to respiratory infections during the 6-month study period than soldiers with plasma 25(OH)D concentration more than 40 nmol/L [20]. In addition, an observational study has shown that adults with 25(OH)D status less than 95 nmol/L had a longer duration of illness compared with those whose 25(OH)D status was higher than 95 nmol/L [27]. In the present study, it was also shown that there was a tendency for episodes to be longer with low vitamin D status. Moreover, we found that subjects with plasma 25(OH)D higher than 30 nmol/L had less severe symptoms during URTI episodes compared with those having plasma 25(OH)D below 30 nmol/L. Thus, vitamin D might play a role in reducing both the severity and duration of URTI symptoms.

There is a plausible mechanism for the inverse association between plasma 25(OH)D status and risk/severity of URTI episodes: vitamin D is a key link between Toll-like receptor (TLR) activation and antimicrobial responses in innate immunity. Following activation of the TLR signalling cascade in the presence of microbes, vitamin D has a vital role in up-regulating the production of AMPs, such as cathelicidin and β-defensin [23]. These AMPs are produced by both

epithelial cells and macrophages and have a broad range of activities against microorganisms including the direct inactivation of viruses [16]. They are secreted into the biofilm covering the epithelial surface, thereby creating a barrier that is chemically lethal to microbes. One of these AMPs, cathelicidin, enhances the microbicidal capability of monocytes and macrophages by increasing the oxidative burst potential of these phagocytic cells [28] and has a defined vitamin D-dependent mechanism. Pathogenic antigens interact with TLRs on the epithelial cells and macrophages to upregulate the expression of the 1α-hydroxylase enzyme that converts 25(OH)D to the biologically active 1,25-dihydroxyvitamin D. This in turn activates a suite of genes which enhance the production of cathelicidin [19,23]. Our finding that the plasma cathelicidin concentration in the high-level vitamin D status group was significantly higher than in the low-level group and the significant correlation between plasma 25(OH)D and cathelicidin concentrations are in agreement with other studies [2,7,15]. Furthermore, vitamin D has been shown to be essential in activating and controlling the T-cell antigen receptor and thus enhancing the recognition of antigens by T lymphocytes [30].

A novel and potentially important finding of the present study is that vitamin D deficiency was associated with a significant and substantially lower production of pro-inflammatory cytokines by both monocytes and lymphocytes in response to a multi-antigen challenge. The consequence of this could be an impaired immune response to an infectious pathogen, increasing the likelihood of an infection occurring. Our finding is in contrast to other studies that have indicated a reduced pro-inflammatory cytokine response when the biologically active form of vitamin D ($1,25(OH)_2D$) is added in concentrations of 10-100 nmol/L to stimulated cultures of peripheral blood mononuclear cells [17,18]. The reason for this discrepancy is unclear. The stimulant we used was a vaccine containing antigens from a virus and both gram-positive and gram-negative bacteria and we used a whole blood culture. The studies by Khoo and colleagues [17,18] used lipopolysaccharide or *Candida albicans* to stimulate isolated peripheral blood mononuclear cells (PBMC). Inhibition of pro-inflammatory cytokine production in these studies was only observed when PBMC were incubated with $1,25(OH)_2D$ in concentrations that were 50-500-fold above the normal healthy range for plasma $1,25(OH)_2D$ (50-250 pmol/L), so the physiological relevance is questionable. Moreover, the multiple antigen challenge used in our study provides valuable information on cytokine production since not all cytokines respond to the same antigen. The capacity of leukocytes to produce cytokines upon adequate challenge (e.g. with mitogen, antigen, endotoxin or pathogen exposure) has potentially far reaching consequences for the entire functional capacity of the immune system. It is highly likely to reflect the capacity of an individual to defend itself against intruding microorganisms and hence is a suitable measure to examine the impact of nutritional interventions (e.g., vitamin D supplementation) designed to boost immune function. We chose to examine whole blood culture rather than isolated PBMC as the former retains the normal cellular, hormonal and cytokine milieu that the leukocytes are normally exposed to in the circulation. This model probably comes closest to the natural environment avoiding artefacts from cell isolation and preparation and allowing natural interactions between immune components and antigens within the normal hormonal milieu. Essentially it is an *in vitro* method of

simulating responses to an infection. Our findings indicate that low vitamin D status is associated with an impaired ability to mount a pro-inflammatory cytokine response to a multi-antigen challenge, whereas the production of anti-inflammatory cytokines (IL-4 and IL-10) was not significantly influenced by vitamin D status. An impaired pro-inflammatory response to antigen challenge could increase the risk of succumbing to infection and increase severity and/or duration of symptoms of infection.

Another interesting finding in the present study is that the SIgA secretion rate in the optimal vitamin D status group was significantly higher than in the other groups. The mucosal immune system, especially SIgA, functions as the first line of defence against pathogen invasion by preventing antigens and microbes adhering to mucosal surfaces and interrupting replication of intracellular pathogens during transcytosis through epithelial cells [10]. Previous studies have shown an inverse relationship between SIgA values and URTI prevalence. For example, low SIgA values have been reported to be associated with increased incidence of URTI in athletes [8,13,24]. The finding in the present study that a significantly lower proportion of subjects in the vitamin D optimal status group experienced URTI episodes during the 4-month study period could be explained partially by the protective effect of their higher SIgA secretion rate. To our knowledge, this is the first study to report an association between SIgA values and plasma vitamin D status. It would be interesting to know if high dose Vitamin D supplementation could elevate SIgA in people with low SIgA secretion.

The inter-individual variation in vitamin D status within our athlete cohort is most likely due to differences in sunlight exposure rather than diet since the D3 form (derived primarily from synthesis in the skin) made up >90% of total plasma 25(OH)D. None of the subjects were taking vitamin supplements but we did not assess dietary vitamin D intake in this study. Vitamin D insufficiency has been reported to be common in athletes [21] especially if exposure to natural sunlight is limited (e.g. when training in the winter months or when training mostly indoors). On the basis of the present data, we found that 38% of athletes had insufficient plasma 25(OH)D values (< 50 nmol/L) at the start of the 4-month period and 55% of athletes had plasma 25(OH)D values of less than 50 nmol/L at the end of the study. This proportion is slightly lower than previously reported in a study on UK athletes [5] that reported 61% had serum 25(OH)D concentrations of less than 50 nmol/L during the winter months. Moreover, we also found that there was a significant drop in plasma 25(OH)D concentration from the start of the 4-month period to the end of the study. This is most likely due to insufficient UV radiation of appropriate wavelength between November and March in the UK (Loughborough latitude is 53°N) to produce vitamin D in the skin [32]. This probably also explains why there was no difference in plasma 25(OH)D concentration between indoor and outdoor athletes during the winter months in the present study, an observation also reported by other studies [5,13]. Given that the incidence of URTI is generally higher in athletes [10] and that low vitamin D status could be a contributing factor, it seems probable that vitamin D supplementation could be desirable for athletes during the winter months. Our results also provide confirmation that the presence of URTI episodes in athletes results in a significant reduction of their training load.

In conclusion, our study suggests that low vitamin D status could be an important determinant of URTI risk in endurance athletes. Athletes with low vitamin D status may have a higher risk of URTI and suffer more severe symptoms when URTI is present. This may be due to impaired mucosal and systemic immunity as SIgA secretion, cathelicidin levels and antigen-stimulated pro-inflammatory cytokine production appear to be increased by vitamin D-dependent mechanisms. Overall, with regard to URTI and vitamin D status the results indicate that it is not good to be deficient in vitamin D (plasma 25(OH)D < 30 nmol/L) and it is probably best to be optimal (plasma 25(OH)D > 120 nmol/L). Further studies are needed to establish if vitamin D supplementation can improve immunity and reduce URTI risk in athletes.

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