VITAMIN D AND OMEGA-3 POLYUNSATURATED FATTY ACID SUPPLEMENTATION IN ATHLETES WITH EXERCISE-INDUCED BRONCHOCONSTRICTION: A PILOT STUDY

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ABSTRACT

Objective: The aim of this pilot study was to determine the combined effect of vitamin D and omega-3 PUFA supplementation on airway function and inflammation in recreational athletes with exercise-induced bronchoconstriction (EIB). **Methods:** Ten recreational athletes with EIB participated in a single blind, placebo-controlled trial over six consecutive weeks. All subjects attended the laboratory on three occasions. Each visit was separated by a period of 3 weeks; visit 1 (usual diet), visit 2 (placebo) and visit 3 (SMARTFISH® NutriFriend 2000; 30µg vitamin D3 - 3000mg EPA, 3000mg DHA) consumed once daily for a period of 3-weeks. Venous blood was collected at the beginning of each trial to determine vitamin D status. Spirometry was performed pre and post eucapnic voluntary hyperpnea (EVH). **Results:** The ΔFEV_1max post EVH was not different between visits (usual diet: -15.9 ± 3.6%; placebo: -16.1 ± 6.1%; vitamin D + omega-3 PUFA: -17.8 ± 7.2%). Serum vitamin D remained unchanged between visits. **Conclusion:** Vitamin D and omega-3 PUFA supplementation does not attenuate the reduction in lung function post EVH. These findings should be viewed as preliminary until the results of randomised controlled trials are made available.

Key words: Airway dysfunction, Exercise-induced bronchoconstriction, Inflammation, Omega-3 polyunsaturated fatty acids, Vitamin D.

1

INTRODUCTION

Exercise-induced bronchoconstriction (EIB) describes the phenomenon of acute, transient airway narrowing in association with physical activity [1] and is highly prevalent in both recreational and elite level athletes [2,3]. Although the precise pathogenesis of EIB is not completely understood, it is generally acknowledged that exercise hyperpnea initiates bronchoconstriction by inducing osmotic changes at the distal airway surface [4]. This precipitates the release of pro-inflammatory mediators including histamine, neuropeptides, cytokines, cysteinyl leukotrienes and prostaglandins, ultimately resulting in airway smooth muscle contraction [5]. In the chronic setting, repeated, prolonged periods of exercise hyperpnea have been associated with injury-repair cycling of the airway epithelium resulting in smooth muscle remodelling [6,7] and the development of EIB in athletes [2].

The mainstay of treatment for EIB consists of pharmacological medication (e.g. short acting inhaled beta-2 agonists (SABA)) [1]. However, there is accumulating evidence that non-pharmacological interventions, such as dietary modification, may have utility in the treatment of EIB in athletes [8]. This is pertinent given the possible side effects of chronic beta-2 agonist therapy (e.g. development of tachyphlaxis and degenerative changes in lung function) [9]. One of the most promising dietary interventions is fish oil supplementation. Specifically, omega-3 polyunsaturated fatty acids (PUFA) (eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)) have previously been shown to attenuate airway inflammation and the bronchoconstrictor response to exercise hyperpnea [10,11]. The purported therapeutic effect of omega-3 PUFA for the treatment of EIB in athletes is biologically plausible; however the findings to date remain equivocal [10-16]. The proposed mechanism of omega-3 PUFA protecting against EIB consists of EPA and DHA competitively inhibiting arachidonic acid metabolism and therefore reducing the generation of pro-inflammatory leukotrienes, prostaglandins and cytokine production from inflammatory cells [17].

Indeed, other dietary interventions may also be important. Recently, epidemiological studies have highlighted a direct association between vitamin D deficiency and the incidence and severity of asthma [18]. Although the evidence is sparse, low serum vitamin D levels have previously been associated with reduced lung function and increased airways hyper-reactivity to exercise in asthmatic children with EIB [19]. Mechanisms by which vitamin D may prevent EIB are likely multifactorial. The vitamin D receptor is expressed in most tissues and it has been proposed that vitamin D deficiency may result in an increase in mast cells, histamine release and apoptosis [20,21]. Furthermore, a reduction in the expression of pro-inflammatory interleukins (i.e. interleukin (IL)-13) associated with bronchoconstriction has been observed [22]. Vitamin D receptors in respiratory epithelial cells and bronchial smooth muscle have also been reported to regulate the expression of genes implicated in the pathogenesis of asthma [23] and smooth muscle proliferation (i.e. airway remodelling) [24]. Consequently, as vitamin D deficiency may play a role in the pathogenesis of lung disease, supplementation may present a novel preventative and/or therapeutic strategy for athletic individuals with EIB.

The principal aim of this pilot study was to evaluate the combined effect of a commercially available vitamin D and omega-3 PUFA supplement (SMARTFISH® NutriFriend 2000), on airway function in recreational athletes with EIB. We hypothesised that lower levels of vitamin D would be associated with reduced lung function, and that vitamin D and omega-3 PUFA supplementation would attenuate airway inflammation and bronchoconstriction following an indirect bronchoprovocation challenge. Eucapnic voluntary hyperpnea (EVH) was selected as the bronchoprovocation challenge since it is the test currently favoured by the International Olympic Committee-Medical Commission (IOC-MC) for diagnosing EIB in elite athletes [25].

METHODS

Preliminary screening

One hundred and one endurance trained recreational athletes (mean ± SD: 6 ± 1 hours training/week) were recruited and subsequently tested for EIB via a EVH challenge (described below). Sixteen athletes (17%) were positive for EIB (i.e. ≥10% fall in FEV_1 post EVH) and thus considered eligible for participation.

Study population

Ten athletes (runners, cyclists and triathletes) (male: $n = 9$) with EIB (63%) agreed to take part in the study. All subjects were non-smokers, free from respiratory, cardiovascular, metabolic and psychiatric disease, and any other significant medical condition except mild asthma. Four subjects had a previous physician-based diagnosis of clinical asthma and were prescribed a SABA; two of the four were also prescribed maintenance-inhaled corticosteroid.

Experimental design

The study was conducted as a single blind placebo-controlled trial over six consecutive weeks (June – September, United Kingdom). A randomised double-blind crossover design was not practical due to the half-life (~15 days) of vitamin D [26] (i.e. approximately 6-month wash-out period) and the effect of seasonal variation on airway calibre in atopic individuals [27]. All subjects were required to attend the laboratory on three occasions. Each visit was separated by a period of 3 weeks; visit 1 (usual diet), visit 2 (placebo; matching the treatment beverage for appearance, taste, quantity and packaging) and visit 3 (treatment; vitamin D + omega-3 PUFA consisting of a 600 ml fruit and berry flavoured beverage - SMARTFISH® NutriFriend 2000; 30µg vitamin D3 i.e. cholecalciferol, 3000mg EPA, 3000mg DHA) consumed once daily for a period of 3-weeks. SMARTFISH® provided

documented evidence (i.e. quality assurance) of the content of both placebo and experimental beverages.

Subjects arrived at the laboratory 1 h postprandial at a similar (± 1 h) time of day following their usual diet. At visit 1 an assessment of respiratory health and evaluation of allergy status was determined via completion of the Allergy Questionnaire for Athletes (AQUA) and aeroallergen skin prick testing. For all visits, venous blood was collected at the beginning of each trial to determine serum vitamin D status. Spirometry was performed pre- and post-EVH provocation. Airway inflammation was determined via fractional exhaled nitric oxide (FE_{NO}) (indirect marker for up-regulation of airway inflammation) pre- and 30 min post-EVH. Urine samples were obtained pre- and 60 min post-EVH for cysteinyl leukotriene (LTE_4) and prostaglandin (9α, 11β- prostaglandin F_2) quantification (markers of airway inflammation and mast cell activation, respectively). With the exception of AQUA and aeroallergen skin prick testing, all visits were replicated precisely on subsequent visits (Figure 1).

Subjects were excluded from follow-up assessment if changes in training and/or health status, respiratory tract infection, allergen or sunlight exposure were reported between visits. Subjects were asked to abstain from dietary supplements (e.g. vitamins and anti-oxidants) throughout the duration of the study and SABA and inhaled corticosteroid medication for 24 and 72 h, respectively, prior to each visit. Northumbria University ethics committee approved all tests and procedures, and all subjects provided written informed consent for experimentation with human subjects.

Atopic Status

Sensitivity to seven common airborne allergens (early blossom tree, mid blossom tree, grass, weed, mould, cat and dust mite) were assessed via skin prick testing [28]. A subject was classified as atopic if, in the skin prick test, at least 1 allergen caused a wheal of at least 3 mm

in diameter, in the presence of a negative saline control and positive histamine. Subjects also completed AQUA to assess allergic symptoms [29]. An athlete was considered to be allergic if they presented with a positive skin prick test and a positive AQUA score ≥5.

Pulmonary function

Spirometry

Lung function was assessed by forced flow-volume spirometry (MicroLoop ML3535; Cardinal Health, UK) [30].

Eucapnic voluntary hyperpnea

Bronchoprovocation challenge testing with EVH was performed as described previously [31,32]. In brief, subjects were required to inhale a mixture of dry compressed gas (21% O_2, 5% CO_2, balance N_2) at a ventilation rate equivalent to approximately 85% maximal voluntary ventilation (MVV)–calculated as $30^{*}FEV_1$ for a period of 6 min. Subjects viewed their ventilatory volume in real-time in order to ensure they maintained the target level. A positive diagnosis for EIB was defined by a post-EVH reduction in FEV_1 of ≥10% compared to resting spirometry.

Airway inflammation

Fraction of exhaled nitric oxide (FE_{NO}) was the first test performed during each visit and measured using a hand-held measuring device (NIOX MINO®) (Aerocrine AB, Stockholm, Sweden). FE_{NO} levels were obtained in accordance with international guidelines [33].

Vitamin D status

The Elecsys Total 25-hydroxyvitamin D assay (Roche Diagnostics GmbH, Germany) was used for the quantative determination of total serum 25-hydroxyvitamin D (25(OH)D) (nmol/L) [34]. Intra-assay coefficient of variation was <10%. Vitamin D status was classified

according to previous recommendations as sufficient: 75 – 100 nmol/L; insufficient: 50-75 nmol/L; deficient: < 50 nmol/L [19,35].

Urinary inflammatory markers

Enzyme immunoassays of LTE_4 and 9α, 11β- prostaglandin F_2 were performed in serially diluted urine (Cayman Chemical Company, Ann Arbor, MI) as previously described [36,37]. Inter- and intra-assay coefficient of variation was <10%. All data were normalised and presented as nanograms of excreted mediator per millimole of creatinine. Creatinine analyses were performed using a modification of Jaffe's creatinine protocol [38].

Nutrient intake and compliance

Subjects were instructed to maintain their usual diet (maximum of one fish meal per week) and physical activity levels throughout the duration of the study. Adherence to treatment regimens was monitored by athletes documenting the time and date of consumption and returning any supplements that were not consumed. In accordance with comparable research a compliance of ≥90% was considered acceptable [36].

Statistical analysis

Normality of data was assessed using a Kolmogorov-Smirnov test and Levene's test to check for homogeneity of variance between groups. A two-way repeated measures analysis of variance (ANOVA) was used to analyse within subject effects. Mauchly's test was conducted to determine if sphericity was violated. If sphericity was violated, the repeated measures ANOVA was corrected using a Greenhouse-Geisser adjustment factor. A Bonferroni *post hoc* analysis was employed for multiple comparisons (P<0.05). A one way repeated measures ANOVA was employed where relevant and relationships between variables were determined via liner regression analysis (Pearson correlation coefficients). $AUC_{0\text{-}20min}$ was calculated by the trapezoidal method and expressed as percentage fall in FEV_1. Data was analysed using

PASW Statistics 21 statistical software package (SPSS Inc., Version 21, Chicago, IL) and GraphPad Prism Version 5.0 (GraphPad Software, San Diego, California, USA). Data are expressed as mean (± SD) and significance was set at $P < 0.05$.

RESULTS

Baseline characteristics, allergy and pre-challenge lung function

Ten recreational athletes (male: n = 9) completed the study. Subjects' characteristics are presented in Table 1. Eight athletes were atopic to skin prick testing and eight had a positive (≥5) AQUA questionnaire. Seven athletes with a positive AQUA questionnaire were also atopic and therefore considered allergic. Five subjects reported respiratory symptoms (e.g. cough, wheeze, dyspnea etc.) in association with exercise. All pulmonary function measures were within normal predicted limits with no evidence of airflow obstruction. In addition, no difference in resting lung function was observed between visits (P>0.05) (Table 2).

Compliance to treatment regimens

Excellent adherence to treatment regimens was reported for placebo and vitamin D + omega-3 PUFA (99.5 ± 1.1% and 98.5 ± 3.4%) diets, respectively (P>0.05).

Airway response to eucapnic voluntary hyperpnea

Similar ventilation rates were achieved between all visits (usual diet: 105 ± 25 $L \cdot min^{-1}$; placebo: 101 ± 17 $L \cdot min^{-1}$; vitamin D + omega-3 PUFA: 100 ± 15 $L \cdot min^{-1}$) (P = 0.854). All athletes maintained >60% MVV throughout EVH thus achieving test validation [39]. The ΔFEV_1max post-EVH was no different between visits (usual diet: -15.9 ± 3.6%; placebo:-16.1 ± 6.1%; vitamin D + omega-3 PUFA: -17.8 ± 7.2%) (P = 0.719). No difference was observed in the reduction in FEV_1 between conditions at any time point (P>0.05) (Figure 2) (Table 3). Furthermore, no difference was observed for $AUC_{0\text{-}20\ min}$ % fall in FEV_1 between visits (usual diet: 198.0 ± 75.9%; placebo: 239.7 ± 99.4%; vitamin D + omega-3 PUFA: 256.9 ± 135.5%) (P = 0.455).

Vitamin D status

At visit one (usual diet), three athletes (30%) had sufficient levels of vitamin D, five were insufficient, and two were deficient. At visit two (placebo), two athletes were sufficient, six were insufficient and two were deficient. At visit three (vitamin D + omega-3 PUFA), three were sufficient, six were insufficient and one was deficient. No difference in serum vitamin D was observed between visits (usual diet: 64.2 ± 17.4 $nmol.L^{-1}$; placebo: 65.1 ± 16.5 $nmol.L^{-1}$; vitamin D + omega-3 PUFA: 69.0 ± 16.9 $nmol.L^{-1}$ (P = 0.798). In addition, change in serum vitamin D status between visits did not correlate with ΔFEV_1max (r = 0.11; P = 0.559).

Airway inflammation

No difference in FE_{NO} was observed pre-EVH between visits (usual diet: 28 ± 16ppb; placebo: 31 ± 23ppb; vitamin D + omega-3 PUFA: 37 ± 27ppb) (P = 0.182) or post-EVH between visits (usual diet: 27 ± 19ppb; placebo: 25 ± 19ppb; vitamin D + omega-3 PUFA: 28 ± 18ppb) (P = 0.834). However, a reduction in FE_{NO} post-EVH was observed within condition for placebo (-20.1%) and vitamin D + omega-3 PUFA (-28.9%), respectively (P<0.05) (Figure 3).

Urinary inflammatory markers

Cysteinyl leukotriene LTE_4

LTE_4 was higher pre-EVH following vitamin D + omega-3 PUFA: 104.1 ± 26.7 ng/mmol creatinine compared to both usual diet: 72.6 ± 16.6 ng/mmol creatinine and placebo: 72.6 ± 22.9 ng/mmol creatinine (P<0.05). No difference was observed between usual diet and placebo (P>0.05). LTE_4 was higher post-EVH following vitamin D + omega-3 PUFA: 99.1 ± 29.2 ng/mmol creatinine compared to placebo: 61.0 ± 13.7 ng/mmol creatinine (P = 0.007). No difference was observed between usual diet and placebo or usual diet and vitamin D +

omega-3 PUFA respectively (P>0.05) (Figure 4). LTE_4 did not correlate with ΔFEV_1max (r = 0.30; P = 0.107).

9α, 11β- prostaglandin F_2

No difference in 9α, 11β- prostaglandin F_2 was observed pre-EVH between visits (usual diet: 88.9 ± 59.1 ng/mmol creatinine; placebo: 82.8 ± 37.6 ng/mmol creatinine; vitamin D + omega-3 PUFA: 79.2 ± 43.7 ng/mmol creatinine) or post-EVH between visits (usual diet: (usual diet: 104.0 ± 41.7 ng/mmol creatinine; placebo: 101.1 ± 56.8 ng/mmol creatinine; vitamin D + omega-3 PUFA: 90.3 ± 48.0 ng/mmol creatinine) (P>0.05) (Figure 4). A correlation was observed between 9α, 11β- prostaglandin F_2 post-EVH and ΔFEV_1max (r = 0.45; P = 0.017).

DISCUSSION

This study has shown, contrary to our hypothesis, that the combination of vitamin D and omega-3 PUFA supplementation over a 3-week period does not reduce markers of airway inflammation or attenuate the reduction in lung function post EVH in recreational athletes with EIB. Furthermore, serum vitamin D status does not appear to correspond directly to the severity of bronchoconstriction following indirect bronchoprovocation. The study design and intervention of the present study was based on the premise that dietary modification with a commercially available self-administrated supplement would be pragmatic and overall applicable to 'real-life'.

Vitamin D deficiency (serum 25-hydroxyvitamin D <50 nmol.L^{-1}) has previously been associated with a reduction in lung function and increased reactivity to exercise in asthmatic children with EIB [19]. However, the precise role of vitamin D in the pathogenesis of EIB has yet to be determined. In the current study 20% (2/10) of athletes presented with vitamin D deficiency following their usual diet. This is in contrast to previous findings where 51% (23/45) of asthmatic children with EIB were vitamin D deficient [19]. The dissociation between studies is somewhat surprising, however supports the notion that physical activity is directly related to the level of sun light exposure [40]. However, it is important to acknowledge that the comparison of prevalence estimates of vitamin D deficiency between studies may be confounded by the population studied (i.e. adults versus children). In addition, as the current study was conducted in the summer months (June – September, United Kingdom), this may, in part, explain the limited number of athletes presenting with vitamin D deficiency. However, it must be acknowledged that the long half-life of vitamin D [26] combined with controlling environmental factors (e.g. sunlight exposure and diet) limits the standardisation of vitamin D trials *in vivo* (i.e. human studies). Nevertheless, further work is

required to fully determine the extent of vitamin D deficiency and thus requirement of supplementation in athletic individuals.

In the present study adherence to the treatment regimens was high, however no difference was observed in serum vitamin D following supplementation. Previous epidemiological studies have highlighted a positive correlation between lung function and serum vitamin D levels [19,41], whereas others have shown no association [42]. However, observational studies do not confirm causality. Our findings show a poor relationship between vitamin D status and severity of bronchoconstriction, thus disputing a direct association. These findings are supported by a recent comparable study demonstrating no effect of vitamin D supplementation in children with mild asthma [43]. However, a general consensus regarding the optimal vitamin D dose has yet to be established (see recent review by Owens et al. [44]). It is therefore reasonable to speculate that the dose employed within the current study (30 µg/day) or indeed length of supplementation was not sufficient to elicit a therapeutic effect. Thus, the optimal level of vitamin D supplementation remains elusive and clinical trials are required before informed recommendations can be employed.

Mickleborough et al. [10,11] previously reported that omega-3 PUFA (3.2g/day EPA and 2.2g/day DHA) derived from fish oil results in a reduction in markers of airway inflammation (e.g. LTE_4 and 9α, 11β- prostaglandin F_2) and an attenuated bronchoconstrictor response following exercise in EIB and asthmatic patients, respectively. More recently, similar findings have been reported by the same group following EVH bronchoprovocation [12,36]. Although Arms et al. [16] also observed a 50% inhibition of total leukotriene count in peripheral blood in mild asthmatics following 10 weeks of daily fish oil supplementation (3.2g EPA and 2.2g DHA), in agreement with our findings no change was observed in ΔFEV_1max post indirect bronchoprovocation. In further support of this concept, Brannan et al. [15] recently found that a 3-week period of omega-3 supplementation (4.0g/day EPA and

2.0g/day DHA) does not improve bronchial hyper-responsiveness to mannitol or inhibit urinary excretion of mast cell mediators in adults with mild-moderate asthma.

This observation is comparable with findings from the present study where no difference was observed in urinary 9α, 11β- prostaglandin F_2 between visits. Although urinary LTE_4 increased pre and post EVH following vitamin D + omega-3 PUFA, the majority of athletes within our cohort were atopic (80%) and allergic (70%), and thus any potential anti-inflammatory effect of vitamin D and omega-3 PUFA may have been counteracted by the variation in allergen exposure (e.g. pollen count, house dust mite etc.) between visits [27]. In keeping with our findings however, Moreira et al. [45] observed no difference in FE_{NO} following short-term dietary supplementation with omega-3 PUFA in woman with stable asthma.

Our finding of a correlation between ΔFEV_1max and urinary excretion of 9α, 11β-prostaglandin F_2 ($P<0.05$) further supports the role of mast cells in EIB [37]. Although the urine sampling time-points post challenge were not identical, similar to Kippelen et al. [37] no association existed between ΔFEV_1max and urinary excretion of LTE_4. This observation could suggest that 9α, 11β- prostaglandin F_2 is a more sensitive marker of EIB in atopic individuals than LTE_4, which warrants further investigation.

Although Mickleborough and Rundell [17] have highlighted statistical limitations to explain the inconsistency in results between studies [17], the majority of trials have consisted of a comparable sample size to the present study [10,11,16]. However, it should be acknowledged that the diagnostic methodology used to quantify the extent of bronchoconstriction often varies between studies [10-12,15]. Furthermore, it has previously been shown that a poor relationship exists between indirect bronchoprovocation challenges (i.e. exercise and EVH) [46,47]. It is therefore possible that the purported therapeutic effect of treatment varies according to the specific bronchoprovocation challenge employed.

Nonetheless, the disparities in findings are still somewhat surprising given the similarities in study design, population, sample size and similar dose of the respective interventions [10,11,16]. Whilst the form of vitamin D and omega-3 PUFA administration in the present study differed from previous research, there is currently no consensus in the literature to suggest that the absorption or indeed effect of supplementation significantly varies according to the form of consumption (i.e. encapsulated supplement versus commercially available nutritional beverage). However, it should be acknowledged that in contrast to previous work [6,10,14,19,40,41] equal quantities of EPA and DHA (3.0g/day) were employed in the current study. It is therefore possible that EPA may be more important than DHA in attenuating EIB. This theory is consistent with a previous pilot study by Head et al. [13] where supplementation with 4.0g/day of DHA did not attenuate bronchoconstriction or airway inflammation in asthmatic patients following EVH. Moreover, a recent mouse model of asthma observed pro-inflammatory effects following the consumption of DHA over a six week period [48].

Overall however, the results of the present study support the current recommendation by the American Thoracic Society that the evidence is not currently strong enough to confirm that omega-3 PUFA's are effective in the large majority of patients with EIB [1].

Pertinent to the present study and previous research [10-12,16,36], poor short-term test re-test clinical reproducibility of indirect bronchoprovocation (i.e. exercise and EVH) [49,50] has recently been observed in patients with mild EIB. Therefore, although the combination of vitamin D and omega-3 PUFA does not appear to attenuate the ΔFEV_1max post bronchoprovocation, the inherent variability of a test employed to determine changes in lung function should be considered when advocating the efficacy of a treatment intervention to avoid masking or overestimating the proposed therapeutic benefit. Likewise, the use of FE_{NO}

as a marker of airway inflammation may be confounded given the high ventilatory demand of EVH (i.e. exhaled nitric oxide often falls from baseline values even when EIB is confirmed).

Methodological considerations / future research

Although this study is the first interventional trial to address the impact of combining vitamin D and omega-3 PUFA supplementation in athletic individuals with EIB, there are a number of important considerations. Firstly, given the small sample size of the cohort, the results should be viewed with some caution. Whilst we are confident that false negative results (i.e. type II error) have not been reported, further work with a larger sample size is still required to provide a definitive answer. Secondly, the optimal level of vitamin D supplementation remains elusive and clinical trials are required before informed recommendations can be employed. Once established, randomised controlled trials are required to determine the individual and combined efficacy of vitamin D and omega-3 PUFA for the treatment of EIB in athletes. Whilst highly speculative, the possibility exists that the lipophilic properties of vitamin D may compete with omega-3 PUFA by an unknown mechanism. Thirdly, to understand the mechanism of action of specific interventions, future studies should assess nutritional deficiencies (i.e. vitamin D and omega-3 PUFA status) prior to study entry and recruit homogenous cohorts of athletes according to severity of disease and specific clinical phenotypes (e.g. asthma, EIB, airway hyper-responsiveness, atopy etc.) rather than 'pooling' heterogeneous cohorts. Finally, the longitudinal impact of vitamin D and/or omega-3 PUFA supplementation has yet to be established. Conducting randomised double-blind crossover design studies (acknowledging the limitations of vitamin D washout) may provide value in this setting.

Conclusion

In conclusion, this pilot study has shown that a 3-week period of vitamin D and omega-3 PUFA supplementation does not reduce markers of airway inflammation nor attenuate the reduction in lung function post EVH. In addition, vitamin D status does not appear to correspond directly to the severity of bronchoconstriction in recreational athletes with EIB. However, these findings should be viewed as preliminary until the results of randomised controlled trials are made available.

KEY ISSUES

- Vitamin D deficiency has previously been associated with the development and severity of asthma, with low serum vitamin D levels associated with reduced lung function and increased reactivity to exercise in children with EIB.

- Omega-3 PUFA supplementation has been shown to attenuate airway inflammation and bronchoconstriction following indirect bronchoprovocation.

- The aim of this pilot study was to determine the combined effect of acute vitamin D and omega-3 PUFA supplementation on airway function in recreational athletes with EIB.

- The combination of vitamin D and omega-3 PUFA supplementation does not reduce markers of airway inflammation nor attenuate the reduction in lung function following EVH.

- Serum vitamin D status does not appear to directly correspond to the severity of bronchoconstriction.

- The inherent variability of a test (i.e. indirect bronchoprovocation) employed to determine changes in lung function should be considered when advocating the efficacy of a treatment intervention to avoid masking or overestimating the proposed therapeutic benefit.

- Further work is required to determine the individual and combined effect of omega-3 PUFA and vitamin D as a non-pharmacological treatment for EIB. The findings of the present study should be viewed as preliminary until the results of randomised controlled trials are made available.

TABLE HEADINGS

Table 1: Subject clinical characteristics.

Definitions of abbreviations: BMI, body mass index.

Table 2: Baseline pulmonary function.

Definitions of abbreviations: **FEV_1,** forced expiratory volume in 1^{-s}; **FVC**, forced vital capacity; **PEF,** peak flow rate.

Table 3: Baseline lung function and response to eucapnic voluntary hyperpnea.

Definitions of abbreviations: **FEV_1,** forced expiratory volume in 1^{-s}

377 **Table 1.**

Subject	Sex (M:F)	Age (years)	Height (cm)	Weight (kg)	BMI ($kg \bullet m^{-2}$)	Training ($hrs \bullet wk^{-1}$)	Physician diagnosed asthma	Medication	Self-report symptoms	Allergy
1	M	42	177.7	90.3	28.6	6	No	Nil	Asymptomatic	No
2	M	27	185.6	87.4	25.4	6	No	Nil	Asymptomatic	No
3	M	36	178.5	72.5	22.8	6	No	Nil	Asymptomatic	Yes
4	M	28	181.3	79.4	24.2	6	No	Nil	Asymptomatic	Yes
5	M	48	173.7	75.6	25.1	6	No	Nil	Asymptomatic	Yes
6	M	28	177.0	78.8	25.2	6	Yes	SABA + ICS	Symptomatic	Yes
7	F	42	166.6	64.2	23.1	6	Yes	SABA	Symptomatic	No
8	M	39	177.9	88.7	28.0	6	Yes	SABA + ICS	Symptomatic	Yes
9	M	34	181.1	72.7	22.2	6	Yes	SABA + ICS	Symptomatic	Yes
10	M	24	183.3	84.5	25.1	4.5	No	Nil	Symptomatic	Yes
Total	9:1	35 ± 8	178.3 ± 5.5	79.4 ± 8.4	25.0 ± 2.1	6 ± 1	4/10	4/10	5/10	7/10

Table 2.

Baseline pulmonary function			
	Visit 1	**Visit 2**	**Visit 3**
	Usual diet	Placebo	Vitamin D + Omega-3
FEV_1 (L)	4.04 ± 0.85	4.12 ± 0.77	4.00 ± 0.80
FEV_1 (% predicted)	96.5 ± 15.4	98.4 ± 12.0	95.4 ± 12.2
FVC (L)	5.61 ± 0.81	5.69 ± 0.78	5.61 ± 0.86
FVC (% predicted)	111.6 ± 10.7	113.1 ± 9.5	111.2 ± 10.4
FEV_1/FVC (%)	71.4 ± 5.4	71.9 ± 4.2	71.0 ± 4.7
PEF (L/min)	552.4 ± 103.3	569.5 ± 85.6	556.1 ± 107.5
PEF (% predicted)	97.7 ± 13.7	100.6 ± 7.9	97.9 ± 11.5

Data presented as Mean ± SD. *n* =10.

Table 3.

	Baseline	**ΔFEV$_1$max**		
Subject	Visit 1: FEV$_1$ (% predicted)	Visit 1: Usual diet	Visit 2: Placebo	Visit 3: Vitamin D + Omega-3 PUFA
1	87.0	-19.6	-12.5	-17.5
2	104.9	-17.2	-20.8	-20.5
3	102.6	-11.5	-20.1	-16.5
4	95.2	-12.9	-13.2	-14.7
5	89.8	-12.1	-12.1	-7.5
6	130.0	-13.6	-9.0	-12.0
7	80.2	-14.4	-17.6	-14.7
8	95.8	-16.8	-9.4	-25.1
9	104.4	-18.2	-16.9	-16.1
10	75.4	-22.6	-28.9	-33.4
Mean ± SD	96.5 ± 15.4	-15.9 ± 3.6	-16.1 ± 6.1	-17.8 ± 7.2

FIGURE LEGENDS

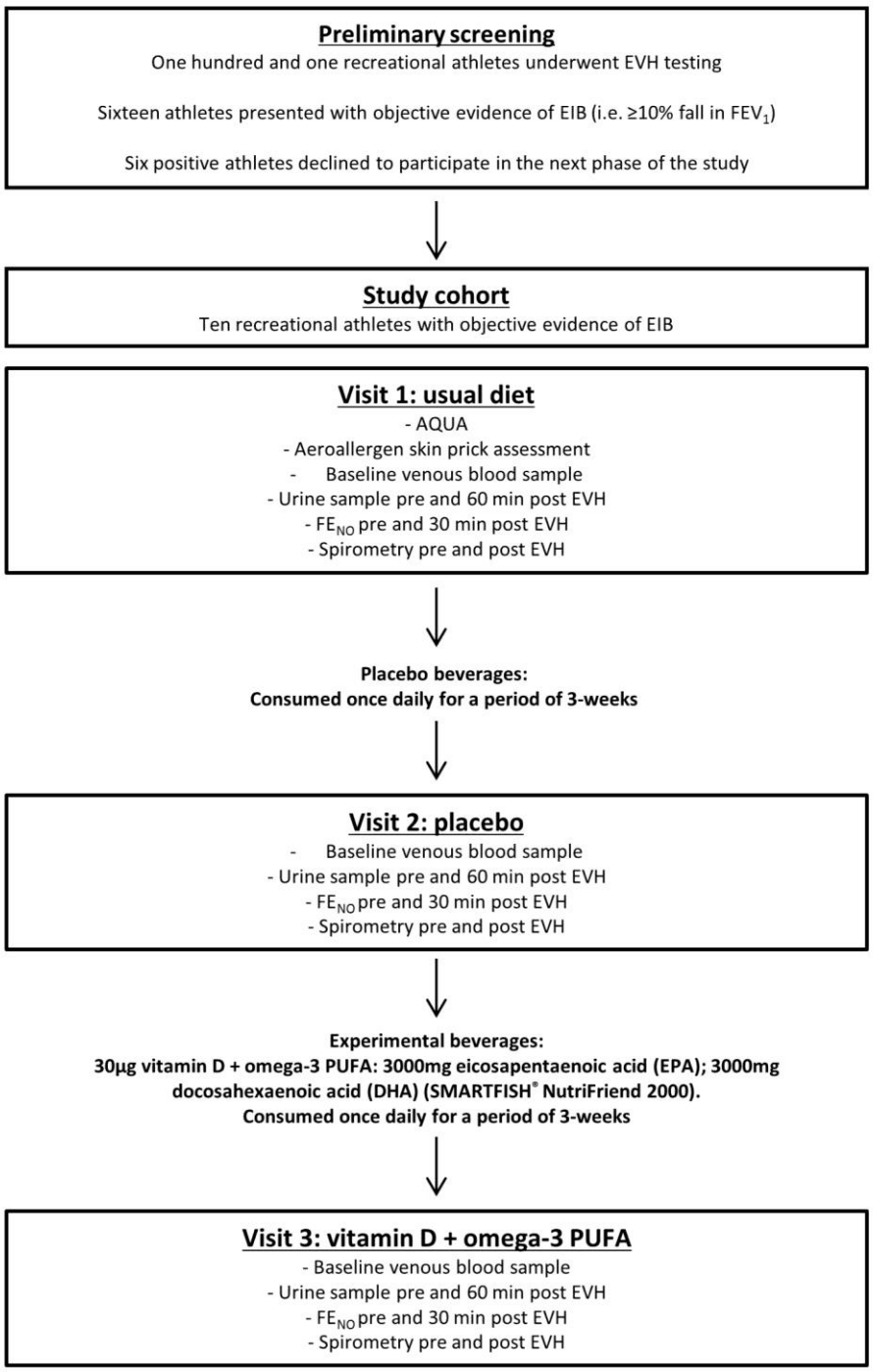
Figure 1. Schematic depicting the experimental design.

Definitions of abbreviations: AQUA, The Allergy Questionnaire for Athletes; **EIB,** exercise-induced bronchoconstriction; **FEV_1,** forced expiratory volume in 1^{-s}; **EVH**; Eucapnic voluntary hyperpnea; **FE_{NO}**, fractional exhaled nitric oxide.

Figure 2. Percentage change in FEV_1 post EVH between visits. Usual diet (*open circles*); placebo (*closed circles*); vitamin D + omega-3 PUFA (*closed triangles*). Broken horizontal line represents abnormal lung function (i.e. ≥10% fall in FEV_1). Placebo SD error lines omitted to improve clarity of graph.

Figure 3. Fractional exhaled nitric oxide (FE_{NO}) concentration (ppb) pre-EVH (*closed bar*) and 30 min post-EVH (*open bar*) between visits. * denotes significant difference within condition between pre- and post-EVH ($P<0.05$)

Figure 4. Panel a). Urinary LTE_4 concentration pre EVH (*closed bar*) and 60 min post EVH (*open bar*) between visits. Panel b). Urinary 9α, 11β- prostaglandin F_2 pre EVH (*closed bar*) and 60 min post EVH (*open bar*) between visits. * denotes significant difference pre-EVH between condition ($P<0.05$). # denotes significant difference post-EVH between condition ($P<0.05$).



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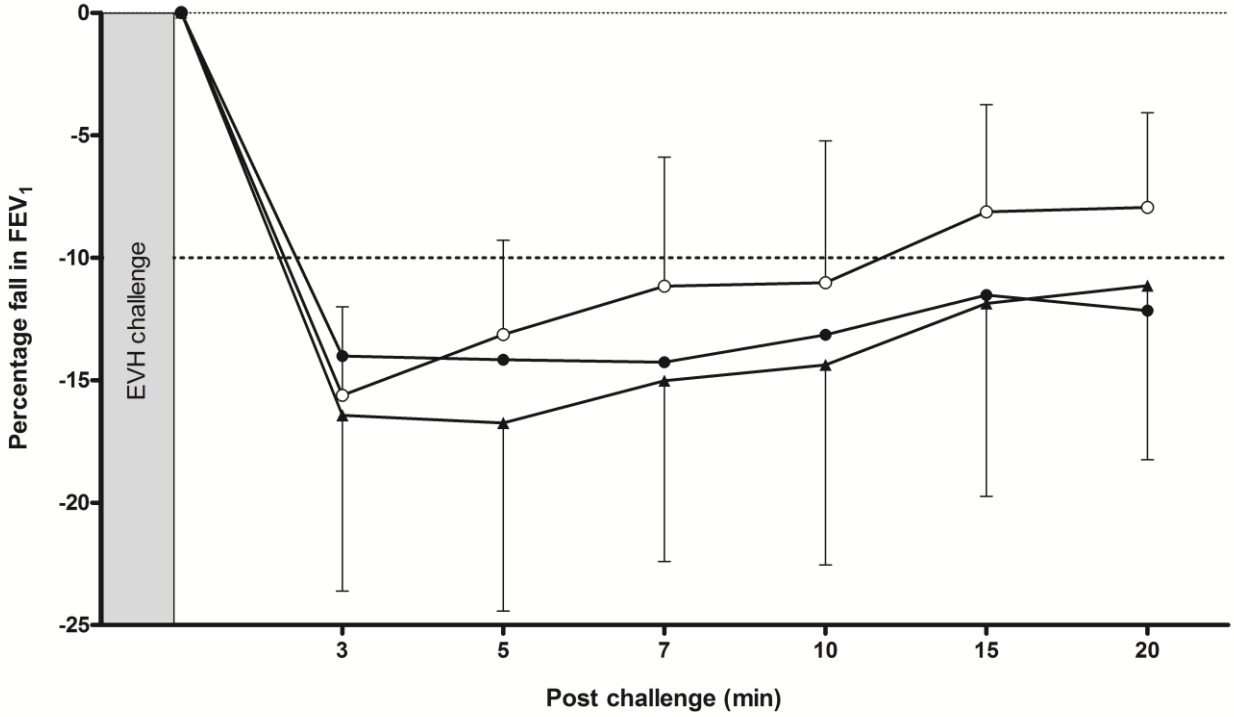
415 **Figure 1.**



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417 **Figure 2.**

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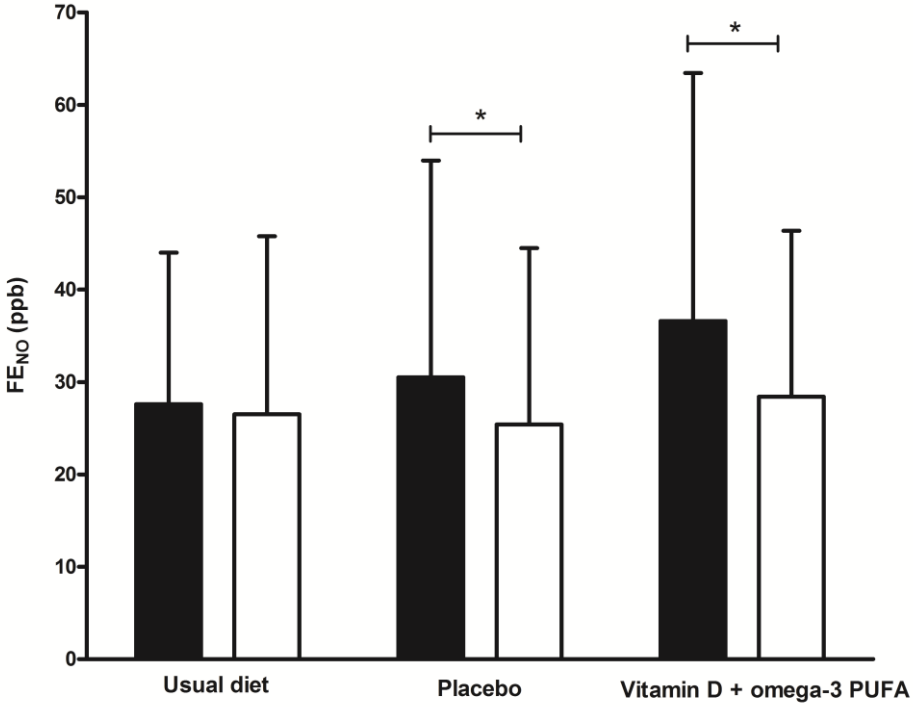

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421 **Figure 3.**

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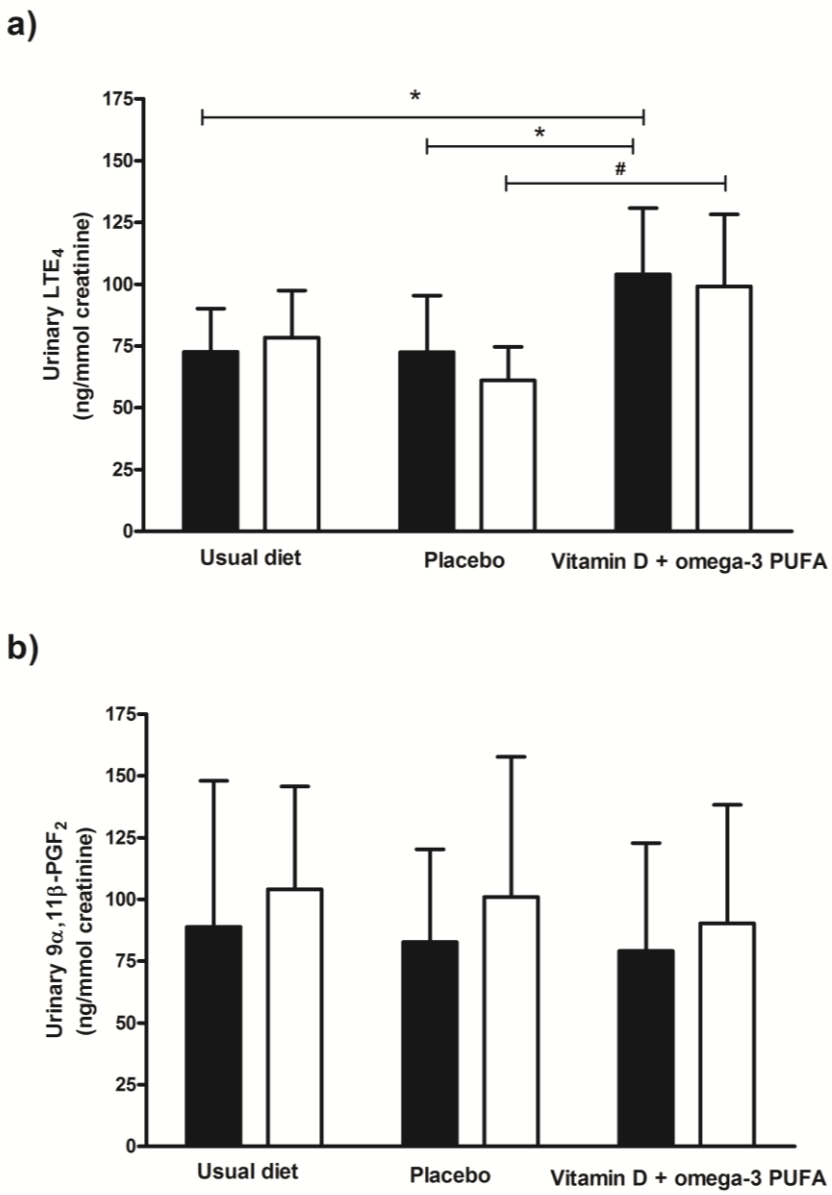

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425 **Figure 4.**

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**Mickleborough TD, Murray RL, Ionescu AA, Lindley MR. Fish oil supplementatio reduces severity of exercise-induced bronchoconstriction in elite athletes. *Am J Respir Crit Care Med*,168(10), 1181-1189 (2003).

Fish oil supplementation (i.e. omega-3 PUFA) provides a protective effect in suppressing EIB in elite athletes due to their anti-inflammatory properties.

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Fish oil supplementation (i.e. omega-3 PUFA) provides a protective effect in suppressing EIB in elite athletes with asthma.

**Brannan JD, Bood J, Alkhabaz A *et al.* The effect of omega-3 fatty acids on bronchial hyperresponsiveness, sputum eosinophilia and mast cell mediators in asthma. *CHEST Journal*, (2014).

Omega-3 supplementation does not improve bronchial hyper-responsivesness to mannitol or inhibit urinary inflammatory mediator excretion in adults with mild-moderate asthma.

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Bronchoconstrictor response to EVH attenuated following omega-3 PUFA supplementation derived from New Zealand green lipped mussel (Perna canaliculus)in asthmatic patients with EIB.

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OP confirms full responsibility for the content of the manuscript, including data and analysis.

CONTRIBUTION STATEMENT

OP was involved in the conception and design of the study, acquisition, interpretation of data, drafting and critical revision of manuscript and final approval of the version to be published.

JH was involved in the conception and design of the study, interpretation of data, drafting and critical revision of manuscript and final approval of the version to be published

GH was involved in the conception and design of the study, drafting and critical revision of manuscript and final approval of the version to be published.

PA was involved in the conception and design of the study, drafting and critical revision of manuscript and final approval of the version to be published.

LA was involved in the conception and design of the study, interpretation of data, drafting and critical revision of manuscript and final approval of the version to be published