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Comparable reductions in hyperpnoea-induced bronchoconstriction and markers of airway inflammation after supplementation with 6.2 and 3.1 g/d of long chain omega-3 polyunsaturated fatty acids in adults with asthma

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Title: Comparable reductions in hyperpnoea-induced bronchoconstriction and markers of airway inflammation after supplementation with 6.2 and 3.1 g/d of long chain omega-3 polyunsaturated fatty acids in adults with asthma

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Abstract

Although high dose omega-3 polyunsaturated fatty acid (*n*-3 PUFA) supplementation reduces exercise- and hyperpnoea-induce bronchoconstriction (EIB/HIB), there are concurrent issues with cost, compliance, and gastrointestinal discomfort. It is thus pertinent to establish the efficacy of lower *n*-3 PUFA doses. Eight male adults with asthma and HIB and 8 controls without asthma were randomly supplemented with two *n*-3 PUFA doses (6.2 g/d (3.7g EPA and 2.5g DHA) and 3.1 g/d (1.8g EPA and 1.3g DHA)) and a placebo, each for 21 days followed by 14 days washout. A eucapnic voluntary hyperpnoea (EVH) challenge was performed before and after treatments. Outcome measures remained unchanged in the control group. In the HIB group, the peak fall in forced expiratory volume in 1 s (FEV_1) after EVH at day 0 (-1005 (SD 520) mL, -30 (SD 18) %) was unchanged after placebo. The peak fall in FEV_1 was similarly reduced from day 0 to day 21 of 6.2 g/d *n*-3 PUFA (-1000 (SD 460) mL, -29 (SD 17) % vs. -690 (SD 460) mL, -20 (SD 15) %) and 3.1 g/d *n*-3 PUFA (-970 (SD 480) mL, -28 (SD 18) % vs. -700 (SD 420) mL, -21 (SD 15) %) ($P<0.001$). Baseline fraction of exhaled nitric oxide was reduced by 24% ($P=0.020$) and 31% ($P = 0.018$) after 6.2 and 3.1 g/d *n*-3 PUFA, respectively. Peak increases in 9α, 11ß PGF_2 after EVH were reduced by 65% ($P=0.009$) and 56% ($P=0.041$) after 6.2 and 3.1 g/d *n*-3 PUFA, respectively. In conclusion, 3.1 g/d *n*-3 PUFA supplementation attenuated HIB and markers of airway inflammation to a similar extent as a higher dose. Lower doses of *n*-3 PUFA thus represent a potentially beneficial adjunct treatment for adults with asthma and EIB.

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Introduction

Exercise-induced bronchoconstriction (EIB) is a prominent asthma phenotype affecting an estimated 90% of asthma patients and up to 50% of elite athlete populations[1]. EIB is characterised by transient airway narrowing during and/or after exercise[2] and is ascribed to airway drying leading to degranulation of inflammatory cells and release of inflammatory mediators[3, 4]. Inhaled corticosteroids and short- and long-acting β_2-agonists are effective therapies, but they are not curative and do not modify disease progression[5]. Furthermore, inhaled corticosteroids adherence is notoriously poor and may have undesirable side effects, while chronic β_2-agonist use results in tolerance[5, 6]. Development of therapies that modulate asthma immunopathology without adverse side effects is therefore desirable.

One potential candidate therapy involves the omega-3 long chain polyunsaturated fatty acids (*n*-3 PUFA) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)[7]. Dietary supplementation of *n*-3 PUFA increases cell membrane EPA and DHA content and reduces *n*-6 arachidonic acid content[8]. This subsequently reduces the synthesis of the pro-inflammatory arachidonic acid-derived eicosanoids cysteinyl leukotrienes and prostaglandins. EPA and DHA may also increase synthesis of resolvin and protectin compounds through the cyclooxygenase and lipoxygenase pathways which are involved in the resolution of inflammation. These mechanisms are thought to be central to the well-established anti-inflammatory effects of *n*-3 PUFA[8], which provide a rationale for the use of *n*-3 PUFA in asthma[7]. To date, however, the role of EPA and DHA in the management of asthma and EIB remains uncertain[9-14]. Early research showed that supplementation of 5.4 g/d *n*-3 PUFA (3.2 g/d EPA and 2.2 g/d DHA) for 10 weeks in adults with asthma reduced leukotriene generation and neutrophil chemotactic responsiveness, but did not reduce bronchoconstriction after cycling exercise and a histamine challenge[9]. Similarly, supplementation of 6.0 g/d *n*-3 PUFA (4.0 g/d EPA and 2.0 g/d DHA) for 3 weeks in adults with asthma did not attenuate bronchoconstriction or markers of airway inflammation in response to a mannitol challenge test[12]. Conversely, supplementation of 5.4 g/d *n*-3 PUFA (3.2 g/d EPA and 2.2 g/d DHA) for 3 weeks in elite athletes[10] and physically active asthmatic males[11, 15] abolished EIB. Subsequent studies showed that supplementation of *n*-3 PUFA (3.2 g/d EPA and 2.0 g/d DHA) reduced bronchoconstriction and markers of airway inflammation after eucapnic voluntary hyperpnoea (EVH)[16] and that *n*-3 PUFA (~3.2 g/d EPA and ~2.0 g/d DHA) was as effective as a leukotriene modifier[17].

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Given the lack of reported risk in taking *n*-3 PUFA, the American Thoracic Society/European Respiratory Society (ATS/ERS) guidelines suggest that the latter studies offer some support for the consumption of *n*-3 PUFA by interested individuals with EIB[10, 11, 17]. In contrast however, recent practice parameter [14] cautions the recommendation for *n-3* PUFA use in EIB based on more recent findings[12, 18]. When considering the practicalities associated with *n*-3 PUFA supplementation for asthma management, previous studies have used doses of 5.2-6.0 g/d. Such doses require individuals to ingest between 8-20 capsules of commercial fish oil daily, which has implications for cost, compliance, and gastrointestinal discomfort[19, 20]. In a single-blind study an alternative flavoured beverage delivery was investigated (3.0 g/d EPA, 3.0 g/d DHA and 30 µg of vitamin D3) but failed to show any attenuation in HIB or markers of airway inflammation (F_ENO, urinary 9α, 11ß PGF_2 and cysteinyl leukotriene E4)[18]. However, no measure of compliance to the treatment was made and participants included had mild asthma or did not have a physician diagnosis of asthma. It is therefore pertinent to establish if lower doses of *n*-3 PUFA are effective in reducing EIB. With respect to the simulation of EIB under laboratory conditions, an EVH challenge causes a highly reproducible hyperpnoea-induced bronchoconstriction (HIB) (a surrogate for EIB) in adults with asthma[21] which makes this an attractive challenge test to evaluate the effects of *n*-3 PUFA treatments on airway hyper responsiveness. Thus, the aim of the current study was to compare the effects of a 6.2 g/d *n*-3 PUFA dose with a half dose of 3.1 g/d *n*-3 PUFA on HIB and markers of airway inflammation in adults with asthma. Provisional data from the study was previously part published in abstract form[22].

Methods

Participants

Sixteen non-smoking, recreationally active men (completing ≥6-h of endurance exercise per week) provided written, informed consent to participate in the study (Table 1). Eight participants formed a HIB group and 8 formed a control group. Inclusion criteria for the HIB group were: physician diagnosis of asthma, a baseline FEV_1 >65% of predicted[23], and a ≥10% fall in FEV_1 following initial EVH screening[24, 25]. The HIB group were on step 1-3 of the stepwise approach to asthma control, indicating well-controlled asthma using either reliever medication (short acting β2-agonsit) alone or in combination with controller medication (low dose inhaled corticosteroid and/or long-acting β2-agonist[26] (Table 1). Inclusion criteria for the control group were: a baseline FEV_1

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>65% of predicted and a <10% fall in FEV_1 following initial EVH screening. Participants avoided exercise for 24-h before an EVH test, and the HIB group ceased their medication as previously descried[21, 24]. On EVH test days, participants abstained from caffeine and alcohol and arrived at the laboratory >2-h post-prandial[27, 28]. Participants were instructed to abstain from consumption of *n*-3 PUFA supplements and eat no more than 2 oily fish meals per week for 3 weeks prior to the study and throughout the study[11]. Participants were free from acute upper respiratory tract infections throughout the study.

Experimental Design and Protocol

This study was conducted in accordance with the Declaration of Helsinki and all procedures were approved by the Nottingham Trent University Human Ethics Committee (Approval No. 186; Clinical trial No. ISRCTN80857707). The study adopted a counter balanced, double-blind, placebo-controlled crossover design over 14 consecutive weeks (Figure 1).

Participants were randomised (block randomisation) to receive three 21 day treatments each separated by a 14 day washout period. All treatments involved daily oral consumption of 8 capsules (CRODA International Plc, Snaith, UK). Four capsules were taken in the morning and four in the afternoon with a recommendation to take with food. The three treatments were: 6.2 g/d *n*-3 PUFA (3.7 g EPA and 2.5 g DHA), 3.1 g/d *n*-3 PUFA (1.8 g EPA and 1.3 g DHA) (INCROMEGA™ TG4030, CRODA, UK) and placebo (CRODAMOL™ GTCC medium chain triglyceride, CRODA International Plc, Snaith, UK) (Table 2). CRODAMOL™ GTCC was chosen as it is readily oxidised in the liver so has little impact on human health-related biomarkers[29, 30]. Measurements were taken at day 0 and 21 of each treatment period. The 6.2 g/d *n*-3 PUFA dose was comprised of four *n*-3 PUFA capsules in the morning and four *n*-3 PUFA capsules in the afternoon. To ensure an equal number of capsules were taken the 3.1 g/d *n*-3 PUFA comprised of two *n*-3 PUFA capsules and two placebo capsules in the morning and two *n*-3 PUFA capsules and two placebo capsules in the afternoon. All capsules (placebo and *n*-3 PUFA) were identical in appearance.

Measurement of Pulmonary Function, EVH and Fraction of Exhaled Nitric Oxide (F_ENO)

The EVH test was undertaken at day 0 and day 21 of each treatment and comprised 6 min of breathing dry gas at a target minute ventilation ($\dot{V}_E$) of 85% of the predicted maximal voluntary ventilation (MVV) (30 x baseline FEV_1). Pulmonary function (forced vital capacity, FVC; FEV_1; peak expiratory flow, PEF; and forced expiratory flow 25-75%, $FEF_{25-75\%}$) was assessed according to ATS/ERS guidelines[31] in triplicate at baseline and in duplicate at 3, 6, 16, 20, and 30 min after

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EVH, as previously described. The highest values recorded were used for analyses. Baseline F_ENO was measured (NIOX MINO; Aerocrine, Solna, Sweden) according to ATS/ERS guidelines[32] in the HIB group only, as it is elevated in asthma patients but not in healthy controls[33].

Urinary 9α, 11β-PGF_2 Analysis

Participants provided a urine sample at baseline and at 12, 60, and 90 min after EVH. Urinary concentration of 9α, 11β-PGF_2, a metabolite of prostaglandin D_2, was subsequently determined by enzyme-linked immunosorbent assay (Caymen Chemicals, Ann Arbor, Michigan, USA) and standardised for urinary creatinine concentration (ABX Pentra 400; Horiba, Northampton, UK) based on a kinetic method using alkaline picrate (Jaffe method)[34]. The corrected 9α, 11β-PGF_2 was expressed as ng/mmol creatinine. The inter- and intra-assay coefficient of variation was <15%. Two control participants and one HIB participant had samples at day 0 below the limit of detection for urinary 9α, 11β-PGF_2 (5 pg/ml) and were subsequently excluded from analysis.

Neutrophil Phospholipid Fatty Acid Analysis

The neutrophil phospholipid fatty acid composition was assessed as a measure of compliance to the treatments in addition to count of capsules returned after each treatment. Neutrophil cells were isolated from 20 mL of whole venous blood (drawn at baseline from an antecubital vein) through a 3 step purification protocol that consisted of dextran sedimentation (Fisher Scientific, Leicestershire UK), hypotonic lysis and Ficoll Paque sedimentation (GE Healthcare, Buckinghamshire, UK)[35]. This method achieved up to 98% of pure neutrophils which were stored at -80°C under nitrogen until extraction of phospholipids using previously described methods[36]. Fatty acid composition was analysed by gas chromatography as previously described[37]. To identify the fatty acid methyl esters (FAME), retention times were compared against known standards, Supelco 37 component FAME mix and PUFA-3 menhaden oil (Sigma, Dorset, UK). EPA, DHA, arachidonic acid and linoleic acid were expressed as a percentage of total fatty acids.

Statistical Analysis

The average minimum perceptible improvement in FEV_1 in adults with asthma is 230 mL[38], whereas the within participant standard deviation for the fall in FEV_1 after EVH is 100 mL[21]. *A priori* sample size calculation revealed that with power = 0.90 and alpha = 0.05, a sample size of 7 in the HIB group would be required to detect a 230 mL improvement in the fall in FEV_1 after EVH.

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Data were analysed using SPSS (Chicago, IL). Following assessment for normality (Shapiro-Wilks test, skewness and kurtosis), data were analysed using repeated measures ANOVA and Bonferroni adjusted paired t-tests. Statistical significance was set at $P < 0.05$. Data presented are mean (SD) unless otherwise stated. Within treatment percent differences (from day 0 to day 21) for the fall in FEV_1 following EVH were calculated as: ((% fall day 0 - % fall day 21)/ % fall day 0) × 100. For the HIB group, the individual percent protection afforded by the *n*-3 PUFA treatments compared with placebo was calculated for the % fall in FEV_1 following EVH as: ((% fall day 21 placebo - % fall day 21 treatment) / % fall day 21 placebo) × 100. In the HIB group, the overall severity of HIB was determined by calculating the area under the curve for % fall in FEV_1 after EVH ($AUC_{0–30}$) using the trapezoidal rule.

Results

Pulmonary Function and Ventilation Rate during EVH

Baseline FEV_1 was lower in the HIB group (3.75 (SD 0.81) L) than the control group (4.63 (SD 0.37) L) ($P = 0.017$), and FVC tended to be lower in the HIB group (4.66 (SD 0.06) L) than the control group (5.26 (SD 0.39) L) ($P = 0.051$). There were no within group differences in FEV_1 or FVC measured at day 0 between the three treatments.

As expected, at day 0 there was a greater peak fall in FEV_1 after EVH in the HIB group (pooled data: -29 (SD 17) %) than the control group (pooled data: -3 (SD 2) %) ($P = 0.001$). In the control group, there was no effect of treatment or day on the peak fall in FEV_1 after EVH. In the HIB group, there was a treatment × day interaction for the peak fall in FEV_1 after EVH ($P = 0.011$). Further analyses revealed an effect of treatment for the peak fall in FEV_1 after EVH at day 21 ($P = 0.001$). Specifically, the peak fall in FEV_1 after EVH was reduced by 34 (SD 14) % (-690 (SD 460) mL) after 6.2 g/d *n*-3 PUFA (mean difference = 310 (SD 150) mL, 95% CI = 185, 432 mL, $P = 0.001$, effect size = 0.70), and by 30 (SD 11) % (-700 (SD 420) mL) after 3.1 g/d *n*-3 PUFA (mean difference = 270 (SD 120) mL, 95% CI = 170, 377 mL, $P = 0.001$, effect size = 0.58). The reduced peak falls in FEV_1 after 6.2 g/d and 3.1 g/d *n*-3 PUFA were not different ($P = 0.834$) (Figure 2, Table 4). The percent protection afforded by the *n*-3 PUFA treatments compared with placebo is shown in Table 4. The peak fall in FEV_1 was unchanged from day 0 to day 21 of placebo.

Figure 3 shows the percent change in FEV_1 during 30 min recovery after EVH in the HIB group. At day 0, the percent change in FEV_1 during recovery was not different between treatments. At day 21, the percent change in FEV_1 was reduced for up to 20 and 30 min recovery after 6.2 g/d and 3.1 g/d *n*-3 PUFA, respectively. Furthermore, compared with placebo at day 21, the percent

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change in FEV_1 was lower for up to 6 and 20 min recovery after 6.2 g/d and 3.1 g/d *n*-3 PUFA, respectively.

In the HIB group, there was a treatment x day interaction for $AUC_{0–30}$ ($P = 0.004$). Further analysis revealed that at day 21 the $AUC_{0–30}$ was reduced after 6.2 g/d (-415 (SD 382), $P = 0.002$) and 3.1 g/d (-398 (SD 399), $P = 0.001$) *n*-3 PUFA compared with placebo (-595 (SD 424)). The $AUC_{0–30}$ at day 21 of 6.2 g/d and 3.1 g/d *n*-3 PUFA was not different ($P = 0.751$).

Consistent with our previous findings[21, 39] the peak fall in FEV_1 after EVH in the HIB group was reproducible with no differences occurring between day 0 of the three treatments (within participant CV = 7 (SD 4) %; measurement error = 84 mL; reproducibility = 231 mL; smallest meaningful change = 115 mL). The peak fall in FVC showed similar outcomes. At day 0 of the three treatments the peak fall in FVC was greater in the HIB group (pooled data: -992 (SD 604) mL; -21 (SD 15) %) than the control group (pooled data: -140 (SD 90) mL; -3 (SD 2) %) ($P = 0.004$). In the control group, the peak fall in FVC was unchanged after placebo and both doses of *n*-3 PUFA. The peak fall in FVC after EVH at day 0 was reduced by 30 (SD 21) % after 6.2 g/d *n*-3 PUFA and by 29 (SD 24) % after 3.1 g/d *n*-3 PUFA but was unchanged after placebo in the HIB group. The reduced peak fall in FVC after 6.2 g/d and 3.1 g/d *n*-3 PUFA was not different ($P = 0.847$). In the HIB group, the peak fall in PEF was unchanged after placebo and both *n*-3 PUFA treatments (pooled data: -2.52 (SD 1.68) L/s). In the HIB group, the peak fall in $FEF_{25-75\%}$ was reduced from day 0 (-1.55 (SD 0.37) L/s) to day 21 (-1.24 (SD 0.45) L/s) of 6.2 g/d *n*-3 PUFA (mean difference = 0.31 (SD 0.23) L/s, 95% CI = 0.12, 0.50 L/s, $P = 0.006$, effect size = 0.77). In the HIB group, the peak fall in $FEF_{25-75\%}$ was unchanged after 3.1g/d *n*-3 PUFA and placebo in the HIB group, and after all treatments in the control group ($P > 0.05$).

Minute ventilation achieved during each of the six EVH trials did not differ in the HIB group ($P > 0.05$; between trial CV = 0.23) (pooled data: 109.5 ± 21.4 L/min; 78 ± 18% of MVV target) which was less compared with the control group ($P = 0.001$; between trial CV = 0.09) (pooled data: 133.4 ± 10.8 L/min; 60 ± 6% of MVV target).

Fraction of Exhaled Nitric Oxide

In the HIB group, there was a treatment × day interaction for F_ENO ($P = 0.004$). After 6.2 g/d *n*-3 PUFA, F_ENO was reduced by 24% from day 0 (48 (SD 33) ppb) to day 21 (35 (SD 28) ppb) (mean difference = 13 (SD 12) ppb, 95% CI = 3, 24 ppb, $P = 0.020$, effect size = 0.41). Similarly, after 3.1 g/d *n*-3 PUFA, F_ENO was reduced by 31% from day 0 (49 (SD 33) ppb) to day 21 (34 (SD

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28) ppb) (mean difference = 15 (SD 14) ppb, 95% CI = 4, 27 ppb, $P = 0.018$, effect size = 0.46) (Figure 4). The reduced F_ENO after 6.2 g/d and 3.1 g/d *n*-3 PUFA was not different ($P = 0.491$) (Figure 4). F_ENO remained unchanged from day 0 (47 (SD 26) ppb) to day 21 (47 (SD 27) ppb) of placebo.

Urinary 9α, 11ß PGF_2

Baseline urinary 9α, 11ß PGF_2 did not differ between the HIB group (day 0 pooled data: 40.85 (SD 21.49) ng/mmol creatinine) and the control group (day 0 pooled data: 27.05 (SD 14.01) ng/mmol creatinine) ($P = 0.133$). There were no within-group differences in urinary 9α, 11ß PGF_2 measured before EVH at day 0 and day 21 of the three treatments.

At day 0, urinary 9α, 11ß PGF_2 increased by 31.53 (SD 22.77) ng/mmol creatinine (pooled data) after EVH in the HIB group ($P = 0.030$) but not in the control group (pooled data: 11.11 (SD 16.30) ng/mmol creatinine). At day 0, changes in 9α, 11ß PGF_2 after EVH were different between groups ($P = 0.003$). In the HIB group, subsequent analyses revealed an effect of treatment on the peak increase in 9α, 11ß PGF_2 after EVH at day 21 ($P = 0.014$). Specifically, the peak increase in 9α, 11ß PGF_2 after EVH was reduced after 6.2 g/d *n*-3 PUFA (mean difference = 11.75 (SD 7.42) ng/mmol creatinine, 95% CI = 5.54, 17.96 ng/mmol creatinine, $P = 0.009$, effect size = 0.50) and 3.1 g/d *n*-3 PUFA (mean difference = 12.86 (SD 14.90) ng/mmol creatinine, 95% CI = 0.40, 25.33 ng/mmol creatinine, $P = 0.041$, effect size = 0.40). The reduced peak increase in 9α, 11ß PGF_2 after EVH following 6.2 g/d and 3.1 g/d *n*-3 PUFA was not different ($P = 0.377$) (Figure 5). There was no effect of placebo on the increase in urinary 9α, 11ß PGF_2 after EVH.

Compliance Assessment - Neutrophil Phospholipid Fatty Acid Content, and Capsule Counts

Good compliance to all treatments was shown from the capsule counts in both the HIB and control groups (Table 3). Further compliance evidence was provided from the neutrophil phospholipid fatty acid content. In the HIB group, 6.2 g/d *n*-3 PUFA increased EPA ($P = 0.002$) and DHA ($P = 0.045$) content, and reduced arachidonic acid ($P = 0.005$). In the HIB group, 3.1 g/d *n*-3 PUFA increased EPA content ($P = 0.018$) and reduced arachidonic acid ($P = 0.009$); there was a trend for an increase in DHA content ($P = 0.074$). In the HIB group, linoleic acid content was unchanged after 6.2 g/d and 3.1 g/d *n*-3 PUFA. Phospholipid fatty acid content was unchanged after placebo in both groups. In the control group, 6.2 g/d *n*-3 PUFA tended to increase EPA ($P = 0.054$) and DHA content ($P = 0.087$), whereas 3.1 g/d *n*-3 PUFA increased DHA content ($P = 0.007$) and reduced arachidonic acid ($P = 0.038$); EPA content was unchanged (Table 3).

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Discussion

Previous research has shown that high doses of *n*-3 PUFA (5.2-5.4 g/d) reduce the severity of HIB and EIB[10, 11, 15-17]. The present study demonstrates that a lower dose of 3.1 g/d *n*-3 PUFA is equally effective in reducing HIB in adult men with asthma. The 6.2 and 3.1 g/d *n*-3 PUFA treatments also resulted in similar reductions in baseline F_ENO, and comparable suppression of urinary 9α, 11β-PGF_2 after EVH. These findings suggest that 3.1 g/d *n*-3 PUFA could be used as an adjunct therapy for physically active adults with asthma and EIB. The percent protection afforded by 6.2 g/d *n*-3 PUFA (35 (SD 14) %) and 3.1 g/d *n*-3 PUFA (33 (SD 12) %) highlights the efficacy of *n*-3 PUFA as an adjunct therapy. The percent protection afforded by *n*-3 PUFA is comparable to montelukast (50mg twice daily)[40] but less than the 60-70% protection afforded by short-acting β_2-agonists procaterol (10 micrograms/inhalation) and albuterol (90 micrograms/inhalation)[41]. Long-acting β_2-agonists also provide effective treatment for EIB,[42-44] although chronic treatment with both long- and short-acting β_2-agonists can result in tolerance[45, 46]. Furthermore, compliance to the lower 3.1 g/d *n*-3 PUFA dose may be improved due to reduced capsule numbers, reduce cost and risk of gastrointestinal distress, which is commonly associated with bloating, and stomach upset. Indeed, two participants withdrew from the study when on the 6.2 g/d *n*-3 PUFA treatment due to gastrointestinal distress, whereas full compliance and no participant withdrawals (capsule counts) was observed with 3.1 g/d *n*-3 PUFA.

The EVH test is an indirect bronchial provocation test that is a suitable objective surrogate for identifying EIB[24]. We have previously reported that the EVH protocol used in the present study elicits a highly reproducible fall in FEV_1[21, 39] which is crucial when assessing treatment efficacy. The ~290 mL (~32%) decrease in the post-EVH fall in FEV_1 after 6.2 g/d and 3.1 g/d *n*-3 PUFA supplementation exceeds the minimum perceptible change of 230 mL[38] and is therefore clinically relevant. Our findings are in agreement with previous studies showing an attenuation of HIB[17] and EIB[11] after *n*-3 PUFA supplementation in adults with asthma. These findings collectively support the ATS/ERS guidance[25] suggesting *n*-3 PUFA supplementation could be of benefit in individuals with EIB. Our findings are also in broad agreement with work on *n*-3 PUFA supplementation in elite athletes with EIB[10], and asthmatic patients with EIB and HIB[11, 15-17]. An interesting difference is that previous work showed abolition of EIB after *n*-3 PUFA supplementation[10], whereas in the present study only 3 participants became non-diagnostic (Figure 2). This may be partly due to inter-study differences in the severity of EIB: the peak fall in FEV_1 in the present

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study (-30%) was ~50% greater than that reported in previous *n*-3 PUFA supplementation studies demonstrating a reduction in EIB/HIB[11, 15-17].

Unlike the rather consistent finding that pharmacological therapy reduces EIB[42-44], the efficacy of *n*-3 PUFA remains controversial. Our findings contrast those of Arm et al.[9] and Brannan et al.[12] who despite using relatively high *n*-3 PUFA doses (5.4-6.0 g/d), reported no change in bronchial hyperresponsiveness to inhaled histamine and mannitol, respectively. Arm et al.[9] reported no change in airway resistance (fall in FEV_1 was not measured) after a cycling exercise challenge, although their data are confounded by: (1) the suggestion that low-grade, non-pharmaceutical *n*-3 PUFA was used[47]; (2) low statistical power due to the small number of participants (n = 6) performing the exercise; (3) exercise being performed at ambient temperature and humidity, which may result in insufficient environmental stress; and (4) the low exercise intensity (60-130 W) which likely (data not measured/reported) elicited only a modest increase in $\dot{V}_E$. The efficacy of *n*-3 PUFA supplementation may also partly depend on the choice of bronchial provocation test. Direct bronchial provocation tests such as inhaled histamine act directly on receptors on the airway smooth muscle causing contraction[48] and may not, therefore, fully reveal the potential anti-inflammatory effects of *n*-3 PUFA. Indirect bronchial provocation tests such as mannitol and EVH both alter the tonicity and volume of the airway surface liquid thereby promoting the release of bronchoconstrictive inflammatory mediators[49, 50]. However, mannitol and EVH may exert different levels of osmotic and mechanical stress on the airways. Specifically, mannitol induces dehydration stress primarily in the proximal airways[50, 51], whereas the high $\dot{V}_E$ during EVH results in the dehydration stress being extended to peripheral airways[52] which are increasingly recruited to heat and humidify inspired air. Furthermore, EVH also elicits airflow-induced shear stress and high transepithelial pressure gradients[53]. These differences may predispose the airway epithelium to greater damage after EVH than mannitol; indeed, EVH tends to result in greater increases in urinary Clara cell protein 16 concentration, a marker of airway epithelial damage[54, 55]. This is significant because *n*-3 PUFA supplementation attenuates the increase in urinary Clara cell protein concentration after EVH, which suggests that reduced HIB may be partly due to reduced airway epithelial damage[56]. Differences in the intensity of the stimulus to the airways may thus explain the divergent effects of *n*-3 PUFA supplementation on bronchial hyperresponsiveness to EVH and mannitol.

Recently, Price et al.[18] reported no change in the severity of HIB or markers of airway inflammation (F_ENO, urinary 9α, 11ß PGF_2 and cysteinyl leukotriene E4) in recreationally active

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adults supplemented daily with a combined treatment of 30 µg vitamin D3 with *n*-3 PUFA (3.0 g EPA and 2.0 g DHA). Unfortunately, objective treatment compliance measures (e.g. neutrophil phospholipid DHA and EPA content) were not taken in this study. The fall in FEV_1 after EVH was also modest (~16%) compared with the ~30% fall observed at day 0 in the present study, and 6 from 10 of the participants did not have physician diagnosed asthma (and consequently no asthma medication). Two participants in Price et al.[18] also became non-diagnostic for HIB after the placebo treatment. It may be that *n*-3 PUFA supplementation is thus more effective in individuals with diagnosed asthma and more severe HIB, as observed in the present study.

The increase in urinary 9α, 11β-PGF_2 after EVH at day 0 in the HIB group is similar in magnitude to that reported previously in adults with asthma performing exercise or EVH[10, 57]. The reduced increase in 9α, 11β-PGF_2 after EVH was comparable after 6.2 g/d and 3.1 g/d *n*-3 PUFA supplementation. Previous work also reports a reduced increase in 9α, 11β-PGF_2 after EVH following *n*-3 PUFA supplementation[10]. The increased EPA and DHA cell membrane content following *n*-3 PUFA supplementation is likely to result in dual inhibition of arachidonic acid-dependent cyclooxygenase and 5-lipoxygenase pathways which are responsible for eicosanoid generation[7]. This may explain the decrease in 9α, 11β-PGF_2, a metabolite of the arachidonic acid-derived pro-inflammatory eicosanoid prostaglandin-D_2. The osmotic changes in the airways following EVH results in mast cell activation and release of PGD2 during bronchoconstriction[49, 50]. Suppression of mast cell activation, and reduced eicosanoid generation, may thus partially explain reduced HIB after *n*-3 PUFA supplementation.

The comparable reduction in baseline F_ENO after 6.2 g/d and 3.1 g/d *n*-3 PUFA supplementation concurs with previous work[17, 58] and is indicative of reduced baseline eosinophilic airway inflammation. Increased F_ENO can also result from elevated expression of inducible nitric oxide synthase in T-cells, macrophages, airway epithelial cells, and other inflammatory cells within the airways[59, 60]. Elevated expression of inducible nitric oxide synthase can be induced by certain pro-inflammatory cytokines such as TNF-α[59, 60] probably via the activation of nuclear factor-kappa β transcription factor[61]. The *n*-3 and *n*-6 PUFAs modulate nuclear factor-kappa β transcription factor activation[62] and presumably therefore TNF-α expression, which may explain the observed fall in F_ENO after *n*-3 PUFA supplementation in the present study. Support for this argument comes from work showing that 5.4 g/d *n*-3 PUFA suppresses circulating plasma TNF-α in athletes with EIB[10]. The comparable reduction in both baseline F_ENO and the post-EVH increase in 9α, 11β-PGF_2 after 6.2 g/d and 3.1 g/d *n*-3 PUFA supplementation suggests that both doses increased the *n*-

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3 PUFA content of the phospholipid bilayer of cell membranes to influence the inflammatory response. Furthermore, and consistent with previous reports[17, 56], the lower F_ENO observed after *n*-3 PUFA supplementation in the present study may have resulted from an increase in airway pH, which is considered a determinant of F_ENO and airway inflammation[63].

Limitations of the current study include no run-in period to assess habitual *n*-3 PUFA intake, the exclusion of females, the comparatively low participant numbers, and the wide range of asthma phenotypes suggested by the heterogeneous fall in FEV_1 and large variation in inflammatory markers after EVH. However, our cohort provides evidence for the use of *n*-3 PUFA in more severe HIB than previous literature[10, 11]. Future work may explore whether particular asthma phenotypes and HIB/EIB severity respond preferentially to *n*-3 PUFA supplementation. Furthermore, given the existing inter-study differences regarding the efficacy of *n*-3 PUFA for the management of EIB, further studies using larger sample sizes are warranted.

In conclusion, 3.1 g/d *n*-3 PUFA supplementation effectively reduced HIB in men with asthma to a similar extent as a higher dose. Lower doses of *n*-3 PUFA thus represent a potentially beneficial adjunct treatment for adults with asthma and EIB, whilst also reducing the burden of cost, compliance and potential gastrointestinal distress. Further studies are needed to elucidate if the bronchial provocation test used and the severity of asthma and HIB/EIB affect the degree of protection afforded by *n*-3 PUFA supplementation.

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TABLE 1. Individual and mean (SD) anthropometric data, baseline pulmonary function, and medication.

				FVC (L)		FEV_1 (L)		
	Age (y)	Height (cm)	Body Mass (kg)	*n*	%	*n*	%	Medications
HIB								
1	21	177	75	5.54	106	4.54	103	S
2	44	178	87	4.22	94	3.17	87	S, BUD+FORM
3	21	173	58	3.78	76	2.77	65	S, BUD+FORM
4	38	180	77	4.62	95	3.80	95	S
5	38	173	83	4.43	91	3.49	90	S
6	28	177	68	4.91	96	4.31	100	S
7	22	181	82	5.70	107	5.10	114	S
8	31	173	75	4.10	91	2.84	76	S, BEC
Mean (SD)	30 (9)	177 (3)	76 (9)	4.66 (0.68)	95 (10)	3.75 (0.84)*	91 (16)	
Control								
1	30	183	80	5.30	98	4.15	92	
2	20	184	80	5.93	106	5.18	110	
3	20	178	69	4.61	88	4.49	101	
4	28	170	84	4.87	103	4.18	104	
5	26	184	70	5.23	93	4.90	104	
6	23	189	92	5.34	91	5.00	102	
7	27	177	69	5.31	103	4.45	103	
8	27	181	88	5.46	101	4.69	104	
Mean (SD)	25 (4)	181 (6)	79 (9)	5.26 (0.39)	98 (7)	4.63 (0.38)	103 (7)	

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BEC = beclomethasone; BUD = budesonide; FORM = formoterol; FEV_1 = forced expiratory volume in 1 second; FVC = forced vital capacity; S = salbutamol. *Difference between HIB and control group ($P = 0.017$).

TABLE 2. Fatty acid composition (% total fatty acids) of the *n*-3 PUFA (INCROMEGA™ TG4030) and placebo (CRODAMOL™ GTCC, medium chain triglycerides) treatments. Individual fatty acids making up ≥ 1% are shown.

		% total fatty acids	
Fatty Acid	Name	*n*-3 PUFA (INCROMEGA™ TG4030)	Placebo (CRODAMOL™ GTCC)
8:0	Caprylic acid		56
10:0	Capric acid		43
18:4 *n*-3	Octadecatetraenoic acid	3	
20:4 *n*-6	Arachidonic acid	3	
20:4 *n*-3	Eicosatetraenoic acid	2	
20:5 *n*-3	Eicosapentaenoic acid	45	
21:5 *n*-3	Heneicosapentaenoic acid	2	
22:5 *n*-3	Docosapentaenoic acid	6	
22:6 *n*-3	Docosahexaenoic acid	31	
Total		92	99

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TABLE 3. Treatment adherence (%) based on capsule count, and fatty acid composition of neutrophil extracts expressed as a percent weight of total fatty acids at day 0 and day 21 of each treatment in HIB and control groups. Data are mean (SD).

	Placebo		6.2 g/d *n*-3 PUFA		3.1 g/d *n*-3 PUFA	
HIB	Day 0	Day 21	Day 0	Day 21	Day 0	Day 21
20:4 *n*-6 Arachidonic Acid	6.52 (0.82)	6.34 (0.62)	6.03 (0.78)	5.37 (0.80)**	6.35 (0.92)	5.26 (0.99)**
20:5 *n*-3 EPA	0.72 (0.17)	0.75 (0.19)	0.77 (0.19)	1.39 (0.38)**	0.59 (0.29)	1.05 (0.31)*
22:6 *n*-3 DHA	2.15 (0.33)	2.20 (0.37)	2.06 (0.33)	2.39 (0.31)*	2.14 (0.40)	2.29 (0.35)
Adherence (%)		93 (4)		94 (4)		95 (4)
Control						
20:4 *n*-6 Arachidonic Acid	5.18 (1.02)	5.09 (1.34)	5.20 (0.63)	4.94 (0.56)	5.73 (1.29)	5.02 (1.01)*
20:5 *n*-3 EPA	0.80 (0.19)	0.79 (0.22)	0.70 (0.20)	0.93 (0.12)	0.73 (0.21)	0.84 (0.10)
22:6 *n*-3 DHA	1.98 (0.31)	2.14 (0.45)	2.02 (0.28)	2.30 (0.30)	1.83 (1.40)	2.22 (0.26)**
Adherence (%)		93 (6)		92 (5)		93 (4)

DHA = Docosahexaenoic acid; EPA = Eicosapentaenoic acid; HIB = Hyperpnoea-induced bronchoconstriction.

Difference between day 0 and day 21 within treatment (* $P < 0.05$; ** $P < 0.01$).

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TABLE 4. Individual and mean (SD) peak % fall in FEV_1 at day 0 and day 21 of each treatment and percent protection afforded by *n*-3 PUFA. Data shown for HIB group only.

	Peak % fall in FEV_1						Percent protection afforded by *n*-3 PUFA treatments for peak % fall in FEV_1	
	Placebo		6.2 g/day *n*-3 PUFA		3.1 g/day *n*-3 PUFA			
HIB	Day 0	Day 21	Day 0	Day 21	Day 0	Day 21	6.2 g/day *n*-3 PUFA	3.1 g/day *n*-3 PUFA
1	-12.58	-13.10	-13.02	-6.54	-14.95	-9.98	50	24
2	-63.22	-64.81	-61.04	-51.36	-60.32	-49.84	21	23
3	-48.73	-47.40	-48.20	-33.70	-48.92	-32.83	29	31
4	-32.90	-28.10	-28.50	-19.54	-30.45	-22.91	30	18
5	-27.02	-32.64	-29.71	-14.98	-26.44	-23.12	54	29
6	-19.72	-18.01	-17.33	-11.67	-18.41	-10.50	35	42
7	-17.74	-16.21	-16.05	-8.64	-13.04	-8.63	47	47
8	-14.83	-15.82	-15.71	-13.67	-13.78	-7.90	14	50
Mean (SD)	-30 (18)	-30 (18)	-29 (17)	-20 (15)	-28 (18)	-21 (15)	35 (14)	33 (12)

Percent protection afforded by the *n*-3 PUFA treatments calculated as: ((% fall day 21 placebo - % fall day 21 treatment) / % fall day 21 placebo) × 100.

Figure Captions

FIGURE 1 – Participant flow diagram. HIB = hyperpnoea-induced bronchoconstriction; CTRL = controls; PUFA = polyunsaturated fatty acid; GI = gastrointestinal.

FIGURE 2 – Peak falls in forced expiratory volume in 1 s (FEV1) before and after placebo (A), 6.2 g/d *n*-3 PUFA (B), and 3.1 g/d *n*-3 PUFA (C) in the HIB group (n = 8). Bars represent mean (SD) and identical symbols represent the same HIB participant. *Day 0 v. day 21 (P = 0.001).

FIGURE 3 – The percent change in forced expiratory volume in 1 second (FEV_1) after eucapnic voluntary hyperpnoea in participants with hyperpnoea-induced bronchoconstriction (HIB). A 10% fall in FEV_1 (shown by the dashed line) is diagnostic of HIB. *Closed circles*, day 0 of placebo treatment; *open circles*, day 21 of placebo treatment; *closed squares*, day 0 of 6.2 g/d *n*-3 PUFA treatment; *open squares*, day 21 of 6.2 g/d *n*-3 PUFA; *closed triangles*, day 0 of 3.1 g/d *n*-3 PUFA; *open triangles*, day 21 of 3.1 g/d *n*-3 PUFA. Significant differences indicated by letters: a, 6.2 g/d *n*-3 PUFA vs. placebo; b, 3.1 g/d *n*-3 PUFA vs. placebo; c, day 0 vs. day 21 of 6.2 g/d *n*-3 PUFA; d, day 0 vs. day 21 of 3.1 g/d *n*-3 PUFA. Single letters, $P < 0.05$; *$P < 0.01$; **$P < 0.001$.

FIGURE 4 – Baseline fraction of exhaled nitric oxide (FENO) before and after placebo (A), 6.2 g/d n-3 PUFA (B), and 3.1 g/d n-3 PUFA (C) in the HIB group (n = 8). Bars represent mean (SD) and identical symbols represent the same HIB participant. *Day 0 v. day 21 ($P < 0.05$).

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FIGURE 5 - Peak increase in urinary 9α, 11ß PGF2 after EVH in the HIB group (n = 8). Filled and open bars represent day 0 and day 21, respectively, of the treatment. Data are mean (SD). *Day 0 v. day 21 ($P < 0.05$

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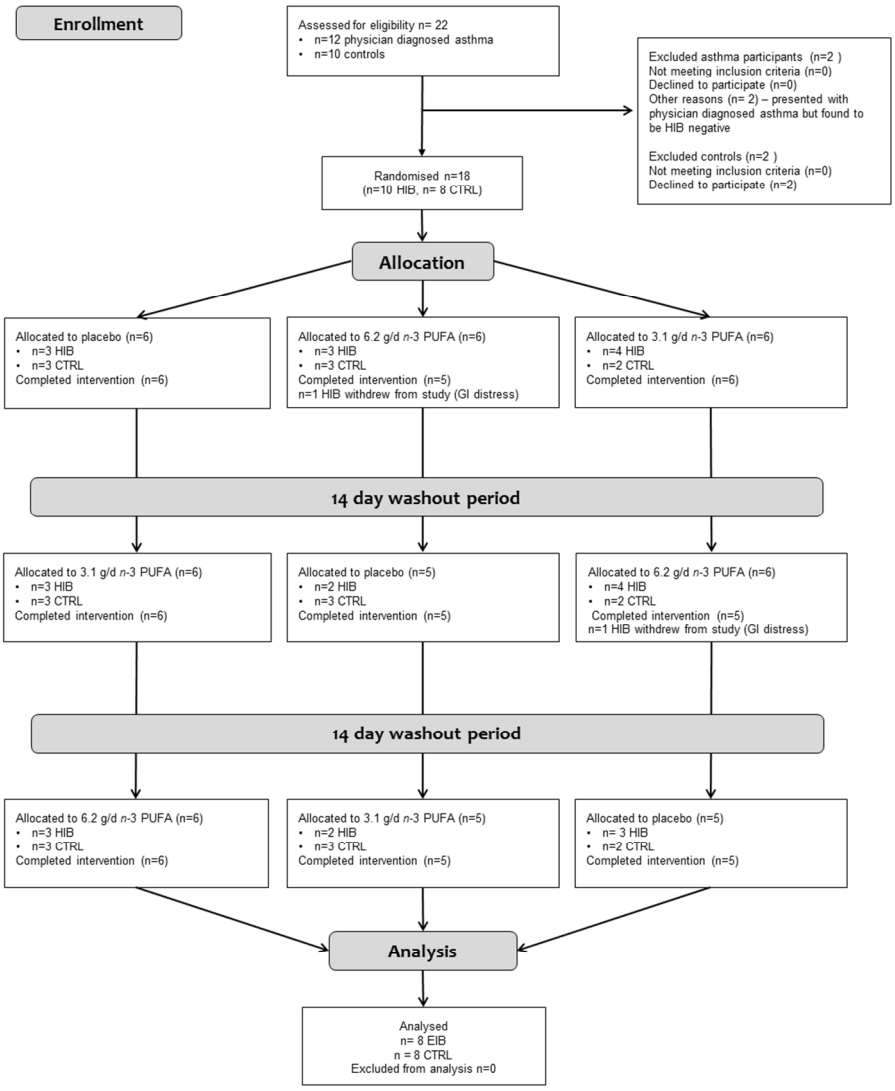

FIGURE 1 – Participant flow diagram. HIB = hyperpnoea-induced bronchoconstriction; CTRL = controls; PUFA = polyunsaturated fatty acid; GI = gastrointestinal.

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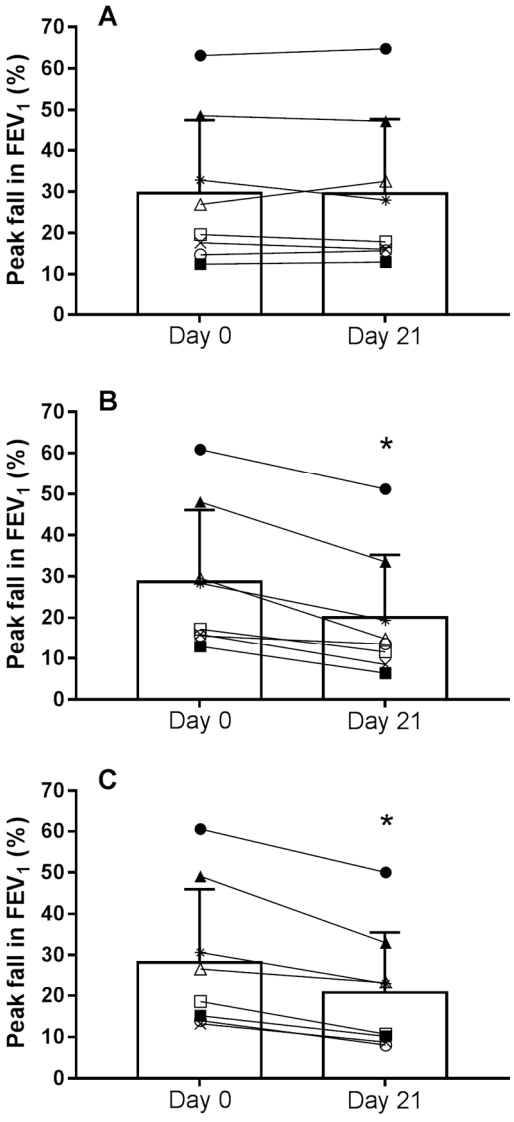

FIGURE 2 – Peak falls in forced expiratory volume in 1 s (FEV1) before and after placebo (A), 6.2 g/d n-3 PUFA (B), and 3.1 g/d n-3 PUFA (C) in the HIB group (n = 8). Bars represent mean (SD) and identical symbols represent the same HIB participant. *Day 0 v. day 21 (P = 0.001).

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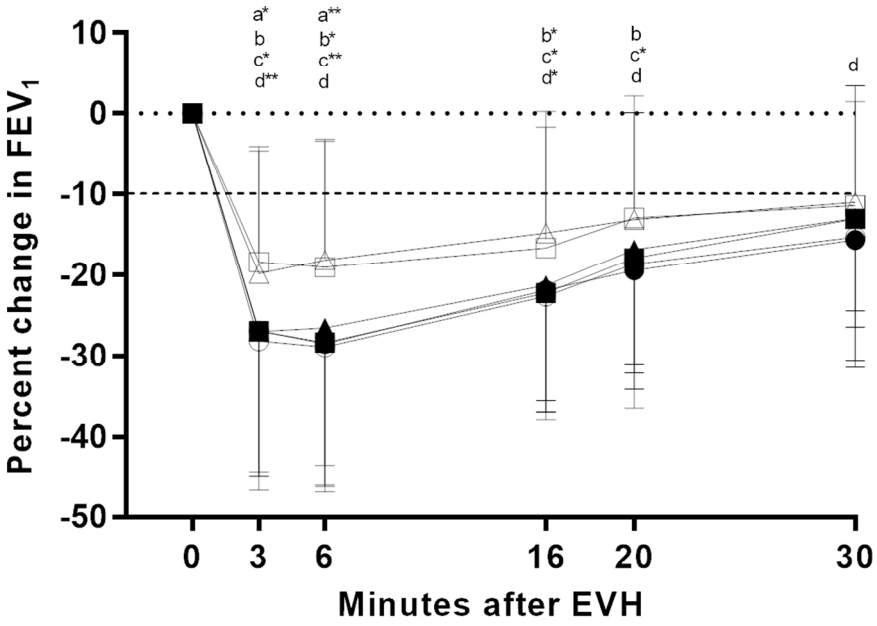

FIGURE 3 – The percent change in forced expiratory volume in 1 second (FEV1) after eucapnic voluntary hyperpnoea in participants with hyperpnoea-induced bronchoconstriction (HIB). A 10% fall in FEV1 (shown by the dashed line) is diagnostic of HIB. Closed circles, day 0 of placebo treatment; open circles, day 21 of placebo treatment; closed squares, day 0 of 6.2 g/d n-3 PUFA treatment; open squares, day 21 of 6.2 g/d n-3 PUFA; closed triangles, day 0 of 3.1 g/d n-3 PUFA; open triangles, day 21 of 3.1 g/d n-3 PUFA. Significant differences indicated by letters: a, 6.2 g/d n-3 PUFA vs. placebo; b, 3.1 g/d n-3 PUFA vs. placebo; c, day 0 vs. day 21 of 6.2 g/d n-3 PUFA; d, day 0 vs. day 21 of 3.1 g/d n-3 PUFA. Single letters, $P < 0.05$; *$P < 0.01$; **$P < 0.001$.

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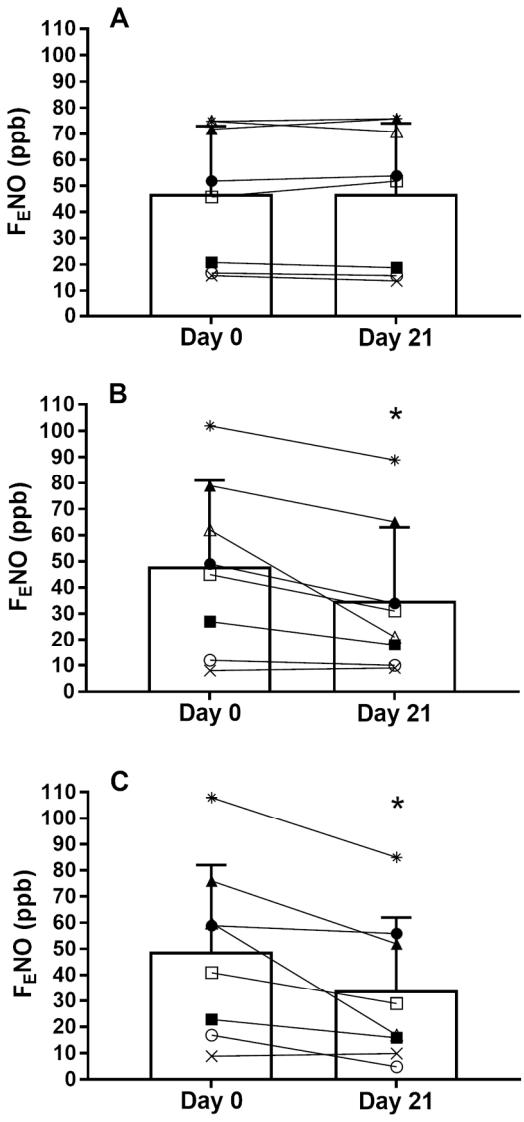

FIGURE 4 – Baseline fraction of exhaled nitric oxide (FENO) before and after placebo (A), 6.2 g/d n-3 PUFA (B), and 3.1 g/d n-3 PUFA (C) in the HIB group (n = 8). Bars represent mean (SD) and identical symbols represent the same HIB participant. *Day 0 v. day 21 ($P < 0.05$).

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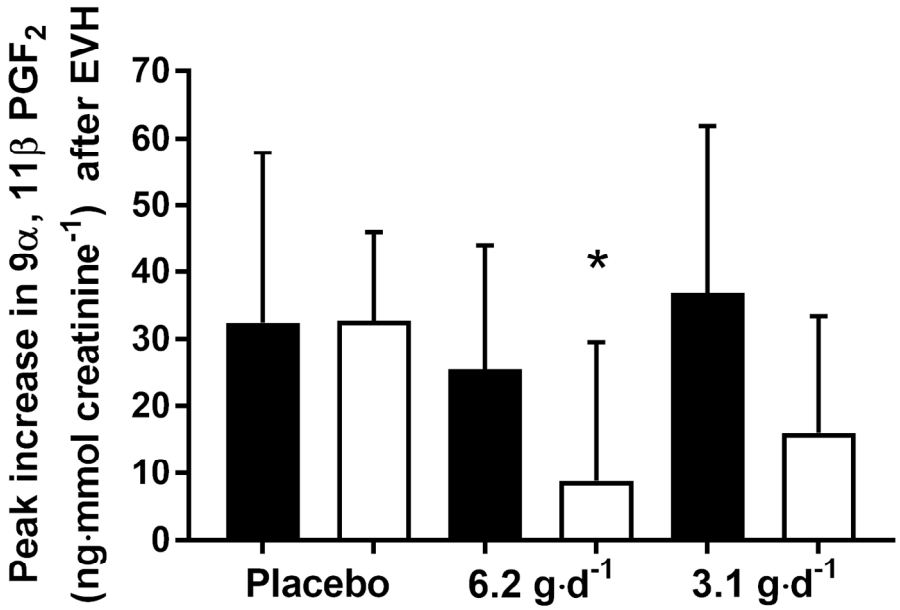

FIGURE 5 - Peak increase in urinary 9α, 11ß PGF2 after EVH in the HIB group (n = 8). Filled and open bars represent day 0 and day 21, respectively, of the treatment. Data are mean (SD). *Day 0 v. day 21 ($P < 0.05$

145x104mm (300 x 300 DPI)

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