

## Comparable reductions in hyperpnoea-induced bronchoconstriction and markers of airway inflammation after supplementation with 6·2 and 3·1 g/d of long-chain n-3 PUFA in adults with asthma

Neil C. Williams<sup>1\*</sup>, Kirsty A. Hunter<sup>1</sup>, Dominick E. Shaw<sup>2</sup>, Kim G. Jackson<sup>3</sup>, Graham R. Sharpe<sup>1</sup> and Michael A. Johnson<sup>1</sup>

<sup>1</sup>Exercise and Health Research Group, Department of Sport Science, Sport, Health and Performance Enhancement (SHAPE) Research Centre, Nottingham Trent University, Nottingham NG11 8NS, UK

<sup>2</sup>Respiratory Research Unit, University of Nottingham, Nottingham NG5 1PB, UK

<sup>3</sup>Hugh Sinclair Unit of Human Nutrition, Department of Food and Nutritional Sciences, University of Reading, Reading RG6 6AP, UK

(Submitted 27 October 2016 – Final revision received 2 May 2017 – Accepted 3 May 2017)

### Abstract

Although high dose n-3 PUFA supplementation reduces exercise- and hyperpnoea-induced 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 eight controls without asthma were randomly supplemented with two n-3 PUFA doses (6·2 g/d (3·7 g EPA and 2·5 g DHA) and 3·1 g/d (1·8 g EPA and 1·3 g DHA)) and a placebo, each for 21 d followed by 14 d 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<sub>1</sub>) after EVH at day 0 (−1005 (sd 520) ml, −30 (sd 18)%) was unchanged after placebo. The peak fall in FEV<sub>1</sub> was similarly reduced from day 0 to day 21 of 6·2 g/d n-3 PUFA (−1000 (sd 460) ml, −29 (sd 17) % v. −690 (sd 460) ml, −20 (sd 15)%) and 3·1 g/d n-3 PUFA (−970 (sd 480) ml, −28 (sd 18) % v. −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, 11β PGF<sub>2</sub> 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.

**Key words:** n-3 PUFA; Asthma; Exercise; Inflammation

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

One potential candidate therapy involves the long-chain n-3 PUFA, EPA and DHA<sup>(7)</sup>. Dietary supplementation of n-3 PUFA increases cell membrane EPA and DHA content and reduces n-6 arachidonic acid content<sup>(8)</sup>. This subsequently reduces the synthesis of the pro-inflammatory arachidonic acid-derived eicosanoids cysteinyl leukotrienes and PG. EPA and DHA may also increase synthesis of resolvin and protectin compounds through the cyclo-oxygenase 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<sup>(8)</sup>, which provide a rationale for the use of n-3 PUFA in asthma<sup>(7)</sup>. To date, however, the role of EPA and DHA in the management of asthma and EIB remains uncertain<sup>(9–14)</sup>. Early research showed

**Abbreviations:** EIB, exercise-induced bronchoconstriction; EVH, eucapnic voluntary hyperpnoea; F<sub>E</sub>NO, fraction of exhaled nitric oxide; FEV<sub>1</sub>, forced expiratory volume in 1 s; FVC, forced vital capacity; HIB, hyperpnoea-induced bronchoconstriction; ppb, parts per billion.

\* **Corresponding author:** Dr N. C. Williams, email neil.williams@ntu.ac.uk

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<sup>(9)</sup>. 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<sup>(12)</sup>. 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<sup>(10)</sup> and physically active asthmatic males<sup>(11,15)</sup> 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)<sup>(16)</sup> and that *n*-3 PUFA 3·2 g/d EPA and 2·0 g/d DHA was as effective as a leukotriene modifier<sup>(17)</sup>.

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<sup>(10,11,17)</sup>. In contrast however, recent practice parameter<sup>(14)</sup> cautions the recommendation for *n*-3 PUFA use in EIB based on more recent findings<sup>(12,18)</sup>. 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 eight and twenty capsules of commercial fish oil daily, which has implications for cost, compliance and gastrointestinal discomfort<sup>(19,20)</sup>. 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 D<sub>3</sub>) but failed to show any attenuation in hyperpnoea-induced bronchoconstriction (HIB) or markers of airway inflammation (fraction of exhaled nitric oxide (F<sub>ENO</sub>), urinary 9α, 11β PGF<sub>2</sub> and cysteinyl leukotriene E4)<sup>(18)</sup>. 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 HIB (a surrogate for EIB) in adults with asthma<sup>(21)</sup> which makes this an attractive challenge test to evaluate the effects of *n*-3 PUFA treatments on airway hyperresponsiveness. 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<sup>(22)</sup>.

## Methods

### Participants

In all, 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 eight formed a control group. Inclusion criteria for the HIB group were: physician diagnosis of asthma, a baseline forced expiratory volume in 1 s

(FEV<sub>1</sub>) >65% of predicted<sup>(23)</sup> and a ≥10% fall in FEV<sub>1</sub> following initial EVH screening<sup>(24,25)</sup>. The HIB group were on steps 1–3 of the stepwise approach to asthma control, indicating well-controlled asthma using either reliever medication (short-acting β<sub>2</sub>-agonist) alone or in combination with controller medication (low-dose inhaled corticosteroid and/or long-acting β<sub>2</sub>-agonist)<sup>(26)</sup> (Table 1). Inclusion criteria for the control group were: a baseline FEV<sub>1</sub> >65% of predicted and a <10% fall in FEV<sub>1</sub> following initial EVH screening. Participants avoided exercise for 24 h before an EVH test, and the HIB group ceased their medication as previously described<sup>(21,24)</sup>. On EVH test days, participants abstained from caffeine and alcohol and arrived at the laboratory >2 h post-prandial<sup>(27,28)</sup>. Participants were instructed to abstain from consumption of *n*-3 PUFA supplements and eat no more than two oily fish meals per week for 3 weeks before the study and throughout the study<sup>(11)</sup>. 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 (Fig. 1).

Participants were randomised (block randomisation) to receive three 21 d treatments each separated by a 14-d washout period. All treatments involved daily oral consumption of eight capsules (Corda International Plc). 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 International Plc) and placebo (CRODAMOL™ GTCC medium chain TAG; Croda International Plc) (Table 2). CRODAMOL™ GTCC was chosen as it is readily oxidised in the liver so has little impact on human health-related biomarkers<sup>(29,30)</sup>. Measurements were taken at days 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, eucapnic voluntary hyperpnoea and fraction of exhaled nitric oxide

The EVH test was undertaken at days 0 and 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 × baseline FEV<sub>1</sub>). Pulmonary function (forced vital capacity (FVC), FEV<sub>1</sub>, peak expiratory flow (PEF) and forced expiratory flow 25–75% (FEF<sub>25–75%</sub>)) was assessed according to ATS/ERS guidelines<sup>(31)</sup> in triplicate at baseline

**Table 1.** Individual anthropometric data, baseline pulmonary function and medication (Individual values; mean values and standard deviations; percentage predicted)

	Age (years)	Height (cm)	Body mass (kg)	FVC (litres)		FEV <sub>1</sub> (litres)		Medications
				Volume	%	Volume	%	
<b>HIB</b>								
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	30	177	76	4.66	95	3.75*	91	
SD	9	3	9	0.68	10	0.84	16	
<b>Control</b>								
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	25	181	79	5.26	98	4.63	103	
SD	4	6	9	0.39	7	0.38	7	

FVC, forced vital capacity; FEV<sub>1</sub>, forced expiratory volume in 1 s; HIB, hyperpnoea-induced bronchoconstriction; S, salbutamol; BUD, budesonide; FORM, formoterol; BEC, beclomethasone.

\* Difference between the HIB and control groups ( $P=0.017$ ).

and in duplicate at 3, 6, 16, 20 and 30 min after EVH, as previously described. The highest values recorded were used for analyses. Baseline F<sub>ENO</sub> was measured (NOX MINO; Aerocrine) according to ATS/ERS guidelines<sup>(32)</sup> in the HIB group only, as it is elevated in asthma patients but not in healthy controls<sup>(33)</sup>.

### Urinary 9 $\alpha$ , 11 $\beta$ -PGF<sub>2</sub> analysis

Participants provided a urine sample at baseline and at 12, 60 and 90 min after EVH. Urinary concentration of 9 $\alpha$ , 11 $\beta$ -PGF<sub>2</sub>, a metabolite of PGD<sub>2</sub>, was subsequently determined by ELISA (Caymen Chemicals) and standardised for urinary creatinine concentration (ABX Pentra 400; Horiba) based on a kinetic method using alkaline picrate (Jaffe method)<sup>(34)</sup>. The corrected 9 $\alpha$ , 11 $\beta$ -PGF<sub>2</sub> was expressed as ng/mmol creatinine. The inter- and intra-assay CV was <15%. Two control participants and one HIB participant had samples at day 0 below the limit of detection for urinary 9 $\alpha$ , 11 $\beta$ -PGF<sub>2</sub> (5 pg/ml) and were subsequently excluded from analysis.

### Neutrophil phospholipid fatty acid analysis

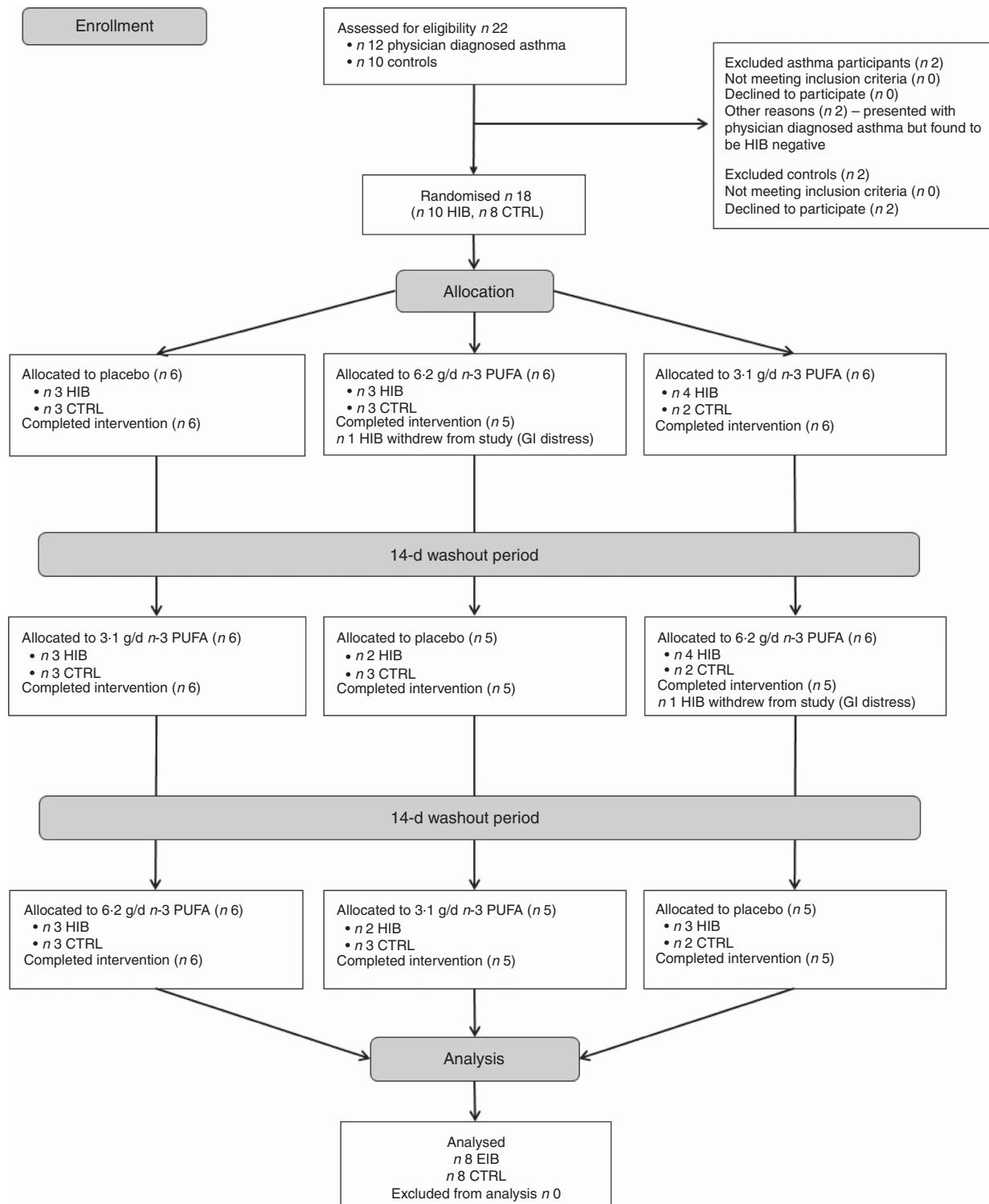
The neutrophil phospholipid fatty acid composition was assessed as a measure of compliance to the treatments in addition to count of capsules returned after each treatment. Neutrophil cells were isolated from 20 ml of whole venous blood (drawn at baseline from an antecubital vein) through a three-step purification protocol that consisted of dextran sedimentation (Fisher Scientific), hypotonic lysis and Ficoll-Paque sedimentation (GE Healthcare)<sup>(35)</sup>. This method achieved up to 98% of pure neutrophils which were stored at

–80°C under N until extraction of phospholipids using previously described methods<sup>(36)</sup>. Fatty acid composition was analysed by GC as previously described<sup>(37)</sup>. 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). 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<sub>1</sub> in adults with asthma is 230 ml<sup>(38)</sup>, whereas the within participant SD for the fall in FEV<sub>1</sub> after EVH is 100 ml<sup>(21)</sup>. *A priori* sample size calculation revealed that with power = 0.90 and  $\alpha$  = 0.05, a sample size of seven in the HIB group would be required to detect a 230 ml improvement in the fall in FEV<sub>1</sub> after EVH.

Data were analysed using SPSS. Following assessment for normality (Shapiro-Wilk 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 values and standard deviations unless otherwise stated. Within treatment percentage differences (from day 0 to day 21) for the fall in FEV<sub>1</sub> following EVH were calculated as: ((% fall day 0 – % fall day 21)/% fall day 0) × 100. For the HIB group, the individual percentage protection afforded by the n-3 PUFA treatments compared with placebo was calculated for the % fall in FEV<sub>1</sub> 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 AUC for % fall in FEV<sub>1</sub> after EVH (AUC<sub>0–30</sub>) using the trapezoidal rule.



**Fig. 1.** Participant flow diagram. HIB, hyperpnoea-induced bronchoconstriction; CTRL, controls; GI, gastrointestinal.

## Results

### Pulmonary function and ventilation rate during eucapnic voluntary hyperpnoea

Baseline FEV<sub>1</sub> was lower in the HIB group (3.75 (*sd* 0.81) litres) than the control group (4.63 (*sd* 0.37) litres) (*P*=0.017) and FVC

tended to be lower in the HIB group (4.66 (*sd* 0.06) litres) than the control group (5.26 (*sd* 0.39) litres) (*P*=0.051). There were no within-group differences in FEV<sub>1</sub> or FVC measured at day 0 between the three treatments.

As expected, at day 0 there was a greater peak fall in FEV<sub>1</sub> after EVH in the HIB group (pooled data: -29 (*sd* 17) %) than

**Table 2.** Fatty acid composition (% total fatty acids) of the *n*-3 PUFA (INCROMEGA™ TG4030) and placebo (CRODAMOL™ GTCC, medium-chain TAG) treatments\*

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

\* Individual fatty acids making up  $\geq 1\%$  are shown.

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<sub>1</sub> after EVH. In the HIB group, there was a treatment  $\times$  day interaction for the peak fall in FEV<sub>1</sub> after EVH ( $P=0.011$ ). Further analyses revealed an effect of treatment for the peak fall in FEV<sub>1</sub> after EVH at day 21 ( $P=0.001$ ). Specifically, the peak fall in FEV<sub>1</sub> 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<sub>1</sub> after 6.2 and 3.1 g/d *n*-3 PUFA were not different ( $P=0.834$ ) (Fig. 2, Table 3). The percentage protection afforded by the *n*-3 PUFA treatments compared with placebo is shown in Table 3. The peak fall in FEV<sub>1</sub> was unchanged from day 0 to day 21 of placebo.

Fig. 3 shows the percentage change in FEV<sub>1</sub> during 30 min recovery after EVH in the HIB group. At day 0, the percentage change in FEV<sub>1</sub> during recovery was not different between treatments. At day 21, the percentage change in FEV<sub>1</sub> was reduced for up to 20 and 30 min recovery after 6.2 and 3.1 g/d *n*-3 PUFA, respectively. Furthermore, compared with placebo at day 21, the percentage change in FEV<sub>1</sub> was lower for up to 6 and 20 min recovery after 6.2 and 3.1 g/d *n*-3 PUFA, respectively.

In the HIB group, there was a treatment  $\times$  day interaction for AUC<sub>0–30</sub> ( $P=0.004$ ). Further analysis revealed that at day 21 the AUC<sub>0–30</sub> 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<sub>0–30</sub> at day 21 of 6.2 and 3.1 g/d *n*-3 PUFA was not different ( $P=0.751$ ).

Consistent with our previous findings<sup>(21,39)</sup> the peak fall in FEV<sub>1</sub> 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 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) litre/s). In the HIB group, the peak fall in FEF<sub>25–75%</sub> was reduced from day 0 ( $-1.55$  (sd 0.37) litre/s) to day 21 ( $-1.24$  (sd 0.45) litre/s) of 6.2 g/d *n*-3 PUFA (mean difference = 0.31 (sd 0.23) litre/s, 95% CI 0.12, 0.50 litre/s,  $P=0.006$ , effect size = 0.77). In the HIB group, the peak fall in FEF<sub>25–75%</sub> was unchanged after 3.1 g/d *n*-3 PUFA and placebo in the HIB group, and after all treatments in the control group ( $P>0.05$ ).

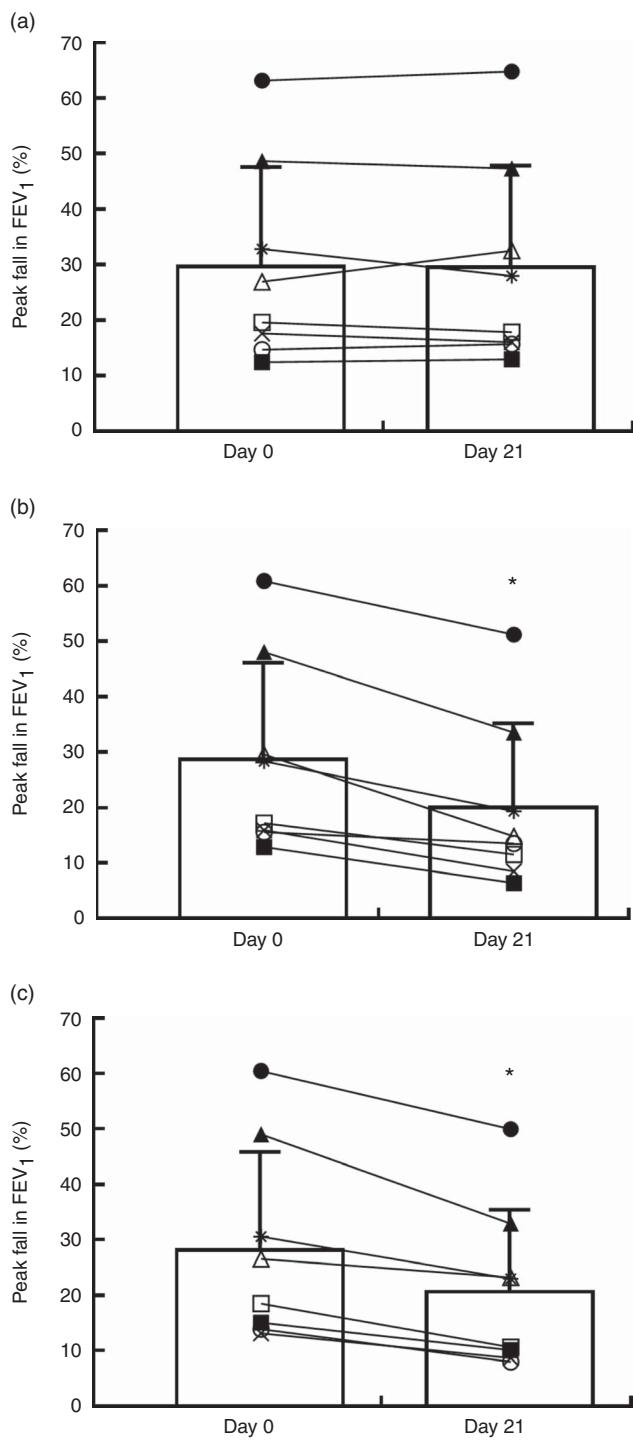
Minute ventilation achieved during each of the six EVH trials did not differ in the HIB group ( $P>0.05$ ; between trial CV = 0.23) (pooled data: 109.5 (sd 21.4) litre/min; 78 (sd 18)% of MVV target) which was less compared with the control group ( $P=0.001$ ; between trial CV = 0.09) (pooled data: 133.4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

#### Fraction of exhaled nitric oxide

In the HIB group, there was a treatment  $\times$  day interaction for F<sub>ENO</sub> ( $P=0.004$ ). After 6.2 g/d *n*-3 PUFA, F<sub>ENO</sub> was reduced by 24% from day 0 (48 (sd 33) parts per billion (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<sub>ENO</sub> was reduced by 31% from day 0 (49 (sd 33) ppb) to day 21 (34 (sd 28) ppb) (mean difference = 15 (sd 14) ppb, 95% CI 4, 27 ppb,  $P=0.018$ , effect size = 0.46) (Fig. 4). The reduced F<sub>ENO</sub> after 6.2 and 3.1 g/d *n*-3 PUFA was not different ( $P=0.491$ ) (Fig. 4). F<sub>ENO</sub> remained unchanged from day 0 (47 (sd 26) ppb) to day 21 (47 (sd 27) ppb) of placebo.

#### Urinary 9 $\alpha$ , 11 $\beta$ PGF<sub>2</sub>

Baseline urinary 9 $\alpha$ , 11 $\beta$  PGF<sub>2</sub> did not differ between the HIB group (day 0 pooled data: 40.85 (sd 21.49) ng/mmol



**Fig. 2.** Peak falls in forced expiratory volume in 1 s (FEV<sub>1</sub>) before and after placebo (a), 6.2 g/d n-3 PUFA (b), and 3.1 g/d n-3 PUFA (c) in the hypernoea-induced bronchoconstriction (HIB) group ( $n=8$ ). Values are means, and standard deviations represented by vertical bars and identical symbols represent the same HIB participant. \* Day 0 v. day 21 ( $P=0.001$ ).

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 9 $\alpha$ , 11 $\beta$ -PGF<sub>2</sub> measured before EVH at days 0 and 21 of the three treatments.

At day 0, urinary 9 $\alpha$ , 11 $\beta$ -PGF<sub>2</sub> 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 9 $\alpha$ , 11 $\beta$ -PGF<sub>2</sub> 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 9 $\alpha$ , 11 $\beta$ -PGF<sub>2</sub> after EVH at day 21 ( $P=0.014$ ). Specifically, the peak increase in 9 $\alpha$ , 11 $\beta$ -PGF<sub>2</sub> 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 9 $\alpha$ , 11 $\beta$ -PGF<sub>2</sub> after EVH following 6.2 and 3.1 g/d n-3 PUFA was not different ( $P=0.377$ ) (Fig. 5). There was no effect of placebo on the increase in urinary 9 $\alpha$ , 11 $\beta$ -PGF<sub>2</sub> 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 4). 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 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 4).

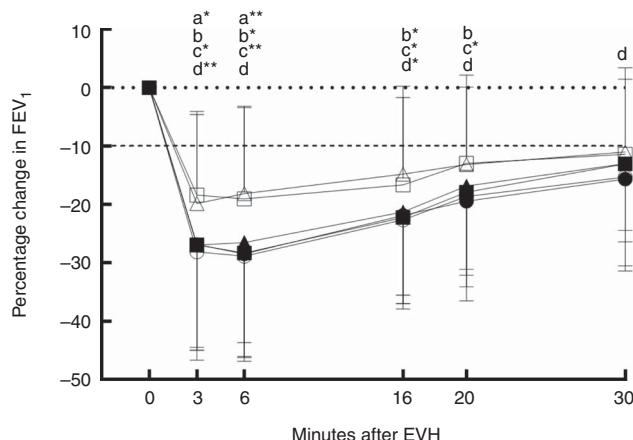
#### Discussion

Previous research has shown that high doses of n-3 PUFA (5.2–5.4 g/d) reduce the severity of HIB and EIB<sup>(10,11,15–17)</sup>. 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<sub>E</sub>NO, and comparable suppression of urinary 9 $\alpha$ , 11 $\beta$ -PGF<sub>2</sub> 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 percentage 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 percentage protection afforded by n-3 PUFA is comparable with montelukast (50 mg twice daily)<sup>(40)</sup> but less than the 60–70% protection afforded by short-acting  $\beta_2$ -agonists procaterol (10 micrograms/inhalation) and albuterol (90 micrograms/inhalation)<sup>(41)</sup>. Long-acting  $\beta_2$ -agonists also provide effective treatment for EIB<sup>(42–44)</sup>, although chronic treatment with both long- and short-acting

**Table 3.** Individual peak % fall in forced expiratory volume in 1 s (FEV<sub>1</sub>) at days 0 and 21 of each treatment and percentage protection afforded by n-3 PUFA in the hyperpnoea-induced bronchoconstriction (HIB) group

HIB	Peak % fall in FEV <sub>1</sub>						Percentage protection afforded by n-3 PUFA treatments for peak % fall in FEV <sub>1</sub> *			
	Placebo		6.2 g/d n-3 PUFA		3.1 g/d n-3 PUFA					
	Day 0	Day 21	Day 0	Day 21	Day 0	Day 21				
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	-30	-30	-29	-20	-28	-21	35	33		
SD	18	18	17	15	18	15	14	12		

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



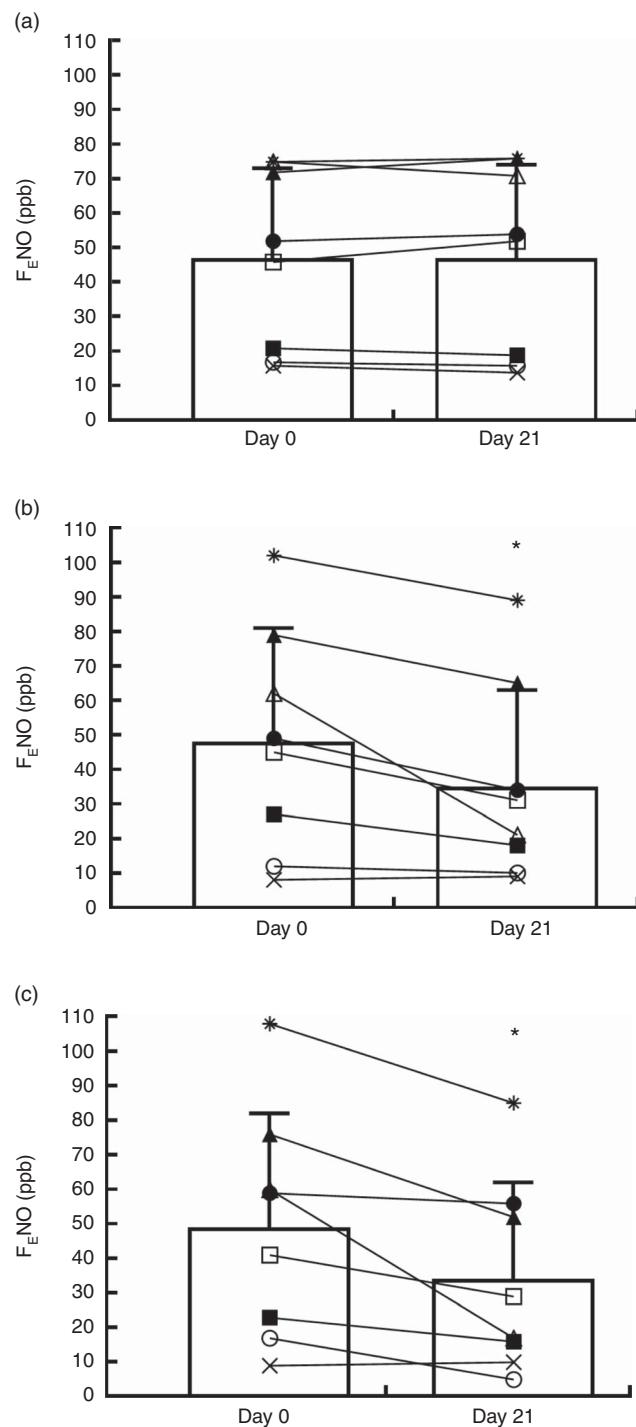
**Fig. 3.** Percentage change in forced expiratory volume in 1 s (FEV<sub>1</sub>) after eucapnic voluntary hyperpnoea (EVH) in participants with hyperpnoea-induced bronchoconstriction (HIB). A 10% fall in FEV<sub>1</sub> (shown by the -----) is diagnostic of HIB. ●, Day 0 of placebo treatment; ○, day 21 of placebo treatment; ■, day 0 of 6.2 g/d n-3 PUFA treatment; □, day 21 of 6.2 g/d n-3 PUFA; ▲, day 0 of 3.1 g/d n-3 PUFA; △, day 21 of 3.1 g/d n-3 PUFA. Mean values with unlike letters were significantly different: <sup>a</sup>, 6.2 g/d n-3 PUFA v. placebo; <sup>b</sup>, 3.1 g/d n-3 PUFA v. placebo; <sup>c</sup>, day 0 v. day 21 of 6.2 g/d n-3 PUFA; <sup>d</sup>, day 0 v. day 21 of 3.1 g/d n-3 PUFA.  $P < 0.05$ , \*  $P < 0.01$ , \*\*  $P < 0.001$ .

$\beta_2$ -agonists can result in tolerance<sup>(45,46)</sup>. 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<sup>(24)</sup>. We have previously reported that the EVH protocol used in the present study elicits a highly reproducible fall in FEV<sub>1</sub><sup>(21,39)</sup> which is crucial when assessing treatment efficacy. The approximately 290 ml (32%) decrease in the post-EVH fall in FEV<sub>1</sub> after 6.2 and 3.1 g/d n-3 PUFA supplementation exceeds the minimum

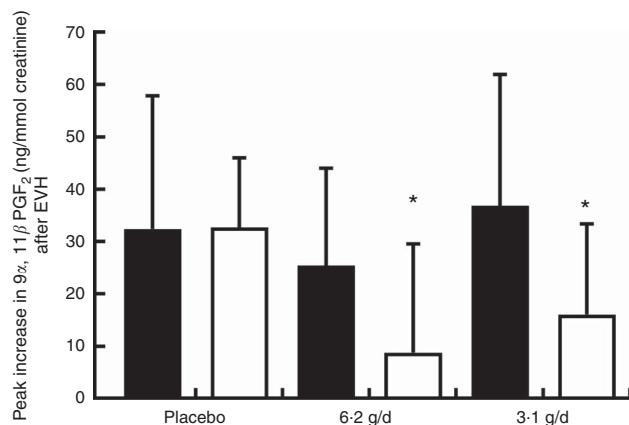
perceptible change of 230 ml<sup>(38)</sup> and is therefore clinically relevant. Our findings are in agreement with previous studies showing an attenuation of HIB<sup>(17)</sup> and EIB<sup>(11)</sup> after n-3 PUFA supplementation in adults with asthma. These findings collectively support the ATS/ERS guidance<sup>(25)</sup> 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<sup>(10)</sup>, and asthmatic patients with EIB and HIB<sup>(11,15–17)</sup>. An interesting difference is that previous work showed abolition of EIB after n-3 PUFA supplementation<sup>(10)</sup>, whereas in the present study only three participants became non-diagnostic (Fig. 2). This may be partly due to inter-study differences in the severity of EIB: the peak fall in FEV<sub>1</sub> in the present study (-30%) was approximately 50% greater than that reported in previous n-3 PUFA supplementation studies demonstrating a reduction in EIB/HIB<sup>(11,15–17)</sup>.

Unlike the rather consistent finding that pharmacological therapy reduces EIB<sup>(42–44)</sup>, the efficacy of n-3 PUFA remains controversial. Our findings contrast those of Arm *et al.*<sup>(9)</sup> and Brannan *et al.*<sup>(12)</sup> 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.*<sup>(9)</sup> reported no change in airway resistance (fall in FEV<sub>1</sub> 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<sup>(47)</sup>; (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<sup>(48)</sup> 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



**Fig. 4.** Baseline fraction of exhaled nitric oxide ( $F_{E\text{NO}}$ ) before and after placebo (a), 6.2 g/d n-3 PUFA (b) and 3.1 g/d n-3 PUFA (c) in the hyperpnoea-induced bronchoconstriction (HIB) group ( $n=8$ ). Values are means, and standard deviations represented by vertical bars and identical symbols represent the same HIB participant. \* Day 0 v. day 21 ( $P<0.05$ ) ppb, Parts per billion.

promoting the release of bronchoconstrictive inflammatory mediators<sup>(49,50)</sup>. 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<sup>(50,51)</sup>, whereas the high  $\dot{V}_E$  during EVH



**Fig. 5.** Peak increase in urinary  $9\alpha, 11\beta\text{-PGF}_2$  after eucapnic voluntary hyperpnoea (EVH) in the hyperpnoea-induced bronchoconstriction group ( $n=8$ ). Values are means, and standard deviations represented by vertical bars. ■ and □, Day 0 and day 21, respectively, of the treatment. \* Day 0 v. day 21 ( $P<0.05$ ).

results in the dehydration stress being extended to peripheral airways<sup>(52)</sup> which are increasingly recruited to heat and humidify inspired air. Furthermore, EVH also elicits airflow-induced shear stress and high transepithelial pressure gradients<sup>(53)</sup>. 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<sup>(54,55)</sup>. 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<sup>(56)</sup>. 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.*<sup>(18)</sup> reported no change in the severity of HIB or markers of airway inflammation ( $F_{E\text{NO}}$ , urinary  $9\alpha, 11\beta\text{-PGF}_2$  and cysteinyl leukotriene E4) in recreationally active adults supplemented daily with a combined treatment of 30 µg vitamin D<sub>3</sub> 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  $\text{FEV}_1$  after EVH was also modest (approximately 16%) compared with the approximately 30% fall observed at day 0 in the present study, and six from ten of the participants did not have physician diagnosed asthma (and consequently no asthma medication). Two participants in Price *et al.*<sup>(18)</sup> 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  $9\alpha, 11\beta\text{-PGF}_2$  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<sup>(10,57)</sup>. The reduced increase in  $9\alpha, 11\beta\text{-PGF}_2$  after EVH was comparable after 6.2 and 3.1 g/d n-3 PUFA supplementation. Previous work also reports a reduced increase in  $9\alpha, 11\beta\text{-PGF}_2$  after EVH following n-3 PUFA supplementation<sup>(10)</sup>. The increased EPA and DHA cell membrane content following n-3 PUFA

**Table 4.** Treatment adherence (%) based on capsule count, and fatty acid composition of neutrophil extracts (%total fatty acids) at days 0 and 21 of each treatment in hyperpnoea-induced bronchoconstriction (HIB) and control groups  
(Mean values and standard deviations)

	Placebo				6.2 g/d n-3 PUFA				3.1 g/d n-3 PUFA			
	Day 0		Day 21		Day 0		Day 21		Day 0		Day 21	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
<b>HIB</b>												
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
<b>Control</b>												
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

Difference between day 0 and day 21 within treatment: \*  $P < 0.05$ , \*\*  $P < 0.01$ .

supplementation is likely to result in dual inhibition of arachidonic acid-dependent cyclo-oxygenase and 5-lipoxygenase pathways which are responsible for eicosanoid generation<sup>(7)</sup>. This may explain the decrease in 9 $\alpha$ , 11 $\beta$ -PGF<sub>2</sub>, a metabolite of the arachidonic acid-derived pro-inflammatory eicosanoid PGD<sub>2</sub>. The osmotic changes in the airways following EVH results in mast cell activation and release of PGD<sub>2</sub> during bronchoconstriction<sup>(49,50)</sup>. 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<sub>E</sub>NO after 6.2 and 3.1 g/d n-3 PUFA supplementation concurs with previous work<sup>(17,58)</sup> and is indicative of reduced baseline eosinophilic airway inflammation. Increased F<sub>E</sub>NO 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<sup>(59,60)</sup>. Elevated expression of inducible nitric oxide synthase can be induced by certain pro-inflammatory cytokines such as TNF- $\alpha$ <sup>(59,60)</sup> probably via the activation of NF- $\kappa$ B transcription factor<sup>(61)</sup>. The n-3 and n-6 PUFA modulate NF- $\kappa$ B transcription factor activation<sup>(62)</sup> and presumably therefore TNF- $\alpha$  expression, which may explain the observed fall in F<sub>E</sub>NO 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- $\alpha$  in athletes with EIB<sup>(10)</sup>. The comparable reduction in both baseline F<sub>E</sub>NO and the post-EVH increase in 9 $\alpha$ , 11 $\beta$ -PGF<sub>2</sub> after 6.2 and 3.1 g/d n-3 PUFA supplementation suggests that both doses increased the n-3 PUFA content of the phospholipid bilayer of cell membranes to influence the inflammatory response. Furthermore, and consistent with previous reports<sup>(17,56)</sup>, the lower F<sub>E</sub>NO 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<sub>E</sub>NO and airway inflammation<sup>(63)</sup>.

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<sub>1</sub> 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<sup>(10,11)</sup>. 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.

## Acknowledgements

In-kind support of the n-3 PUFA and placebo supplements was provided by Croda Healthcare Ltd (Croda International Plc, Cowick Hall, Snaith, Goole, East Yorkshire, DN14 9AA, UK). The company was not involved in the design or implementation of the study.

N. C. W., K. A. H., D. E. S., G. R. S. and M. A. J. designed the research; N. C. W., K. A. H., G. R. S. and M. A. J. conducted research; N. C. W. and K. G. J. provided essential reagents and conducted analysis of urine and blood samples; N. C. W., G. R. S. and M. A. J. analysed data. N. C. W., K. A. H., G. R. S. and M. A. J. wrote paper; N. C. W., K. A. H., D. E. S., K. G. J., G. R. S. and M. A. J. contributed to reviewing and approval of the final manuscript.

None of the authors has any conflicts of interest to declare.

## References

- Carlsen K, Anderson S, Bjermer L, et al. (2008) Exercise-induced asthma, respiratory and allergic disorders in elite athletes: epidemiology, mechanisms and diagnosis: part I of the report from the Joint Task Force of the European Respiratory

- Society (ERS) and the European Academy of Allergy and Clinical Immunology (EAACI) in cooperation with GA2LEN. *Allergy* **63**, 387–403.
- 2. Weiler JM, Anderson SD, Randolph C, *et al.* (2010) Pathogenesis, prevalence, diagnosis, and management of exercise-induced bronchoconstriction: a practice parameter. *Ann Allergy Asthma Immunol* **105**, S1–S47.
  - 3. Hallstrand TS (2012) New insights into pathogenesis of exercise-induced bronchoconstriction. *Curr Opin Allergy Clin Immunol* **12**, 42–48.
  - 4. Hallstrand TS, Altemeier WA, Aitken ML, *et al.* (2013) Role of cells and mediators in exercise-induced bronchoconstriction. *Immunol Allergy Clin North Am* **33**, 313–328.
  - 5. Barnes PJ (2010) New therapies for asthma: is there any progress? *Trends Pharmacol Sci* **31**, 335–343.
  - 6. Ramage L, Lipworth B, Ingram C, *et al.* (1994) Reduced protection against exercise induced bronchoconstriction after chronic dosing with salmeterol. *Respir Med* **88**, 363–368.
  - 7. Kumar A, Mastana SS & Lindley MR (2016) n-3 Fatty acids and asthma. *Nutr Research Rev* **29**, 1–16.
  - 8. Calder PC (2010) Omega-3 fatty acids and inflammatory processes. *Nutrients* **2**, 355–374.
  - 9. Arm JP, Horton CE, Mencia-Huerta JM, *et al.* (1988) Effect of dietary supplementation with fish oil lipids on mild asthma. *Thorax* **43**, 84–92.
  - 10. Mickleborough TD, Murray RL, Ionescu AA, *et al.* (2003) Fish oil supplementation reduces severity of exercise-induced bronchoconstriction in elite athletes. *Am J Respir Crit Care Med* **168**, 1181–1189.
  - 11. Mickleborough TD, Lindley MR, Ionescu AA, *et al.* (2006) Protective effect of fish oil supplementation on exercise-induced bronchoconstriction in asthma. *Chest* **129**, 39–49.
  - 12. Brannan JD, Bood J, Alkhabaz A, *et al.* (2015) The effect of omega-3 fatty acids on bronchial hyperresponsiveness, sputum eosinophilia, and mast cell mediators in asthma. *Chest* **147**, 397–405.
  - 13. Reisman J, Schachter H, Dales R, *et al.* (2006) Treating asthma with omega-3 fatty acids: where is the evidence? A systematic review. *BMC Complement Altern Med* **6**, 1.
  - 14. Weiler JM, Brannan JD, Randolph CC, *et al.* (2016) Exercise-induced bronchoconstriction update – 2016. *J Allergy Clin Immunol* **138**, 1292–1295. e36.
  - 15. Kumar A, Mastana SS & Lindley MR (2016) EPA/DHA dietary supplementation attenuates exercise-induced bronchoconstriction in physically active asthmatic males. *Cogent Med* **3**, 1172696.
  - 16. Mickleborough T & Lindley M (2014) The effect of combining fish oil and vitamin C on airway inflammation and hyperpneainduced bronchoconstriction in asthma. *J Allergy Ther* **5**, 2.
  - 17. Teeklenburg-Lund S, Mickleborough TD, Turner LA, *et al.* (2010) Randomized controlled trial of fish oil and montelukast and their combination on airway inflammation and hyperpneainduced bronchoconstriction. *PLoS ONE* **5**, e13487.
  - 18. Price OJ, Hull JH, Howatson G, *et al.* (2015) Vitamin D and omega-3 polyunsaturated fatty acid supplementation in athletes with exercise-induced bronchoconstriction: a pilot study. *Expert Rev Respir Med* **9**, 369–378.
  - 19. Burns CP, Halabi S, Clamon G, *et al.* (2004) Phase II study of high-dose fish oil capsules for patients with cancer-related cachexia. *Cancer* **101**, 370–378.
  - 20. Walser B & Stebbins CL (2008) Omega-3 fatty acid supplementation enhances stroke volume and cardiac output during dynamic exercise. *Eur J Appl Physiol* **104**, 455–461.
  - 21. Williams NC, Johnson MA, Hunter KA, *et al.* (2015) Reproducibility of the bronchoconstrictive response to eucapnic voluntary hyperpnoea. *Respir Med* **109**, 1262–1267.
  - 22. Williams N, Hunter K, Johnson M, *et al.* (2013) A randomised placebo controlled trial to compare the effects of two dosages of omega-3 PUFA on exercise-induced bronchoconstriction (EIB). *Br J Sports Med* **47**, e4–e4.
  - 23. Argyros GJ, Roach JM, Hurwitz KM, *et al.* (1996) Eucapnic voluntary hyperventilation as a bronchoprovocation technique: development of a standardized dosing schedule in asthmatics. *Chest* **109**, 1520–1524.
  - 24. Anderson SD, Argyros GJ, Magnussen H, *et al.* (2001) Provocation by eucapnic voluntary hyperpnoea to identify exercise induced bronchoconstriction. *Br J Sports Med* **35**, 344–347.
  - 25. Parsons JP, Hallstrand TS, Mastronarde JG, *et al.* (2013) An official American Thoracic Society clinical practice guideline: exercise-induced bronchoconstriction. *Am J Respir Crit Care Med* **187**, 1016–1027.
  - 26. Boulet LP, FitzGerald JM & Reddel HK (2015) The revised 2014 GINA strategy report: opportunities for change. *Curr Opin Pulm Med* **21**, 1–7.
  - 27. Duffy P & Phillips Y (1991) Caffeine consumption decreases the response to bronchoprovocation challenge with dry gas hyperventilation. *Chest* **99**, 1374–1377.
  - 28. Vally H & Thompson P (2002) Alcoholic drinks and asthma. *Clin Exp Allergy* **32**, 186–191.
  - 29. Yusof HM, Miles EA & Calder P (2008) Influence of very long-chain n-3 fatty acids on plasma markers of inflammation in middle-aged men. *Prostaglandins Leukot Essent Fatty Acids* **78**, 219–228.
  - 30. Bahal SM, Romansky JM & Alvarez FJ (2003) Medium chain triglycerides as vehicle for palatable oral liquids: TECHNICAL NOTE. *Pharm Dev Technol* **8**, 111–115.
  - 31. Miller MR, Hankinson J, Brusasco V, *et al.* (2005) Standardisation of spirometry. *Eur Respir J* **26**, 319–338.
  - 32. Dweik RA, Boggs PB, Erzurum SC, *et al.* (2011) An official ATS clinical practice guideline: interpretation of exhaled nitric oxide levels (FENO) for clinical applications. *Am J Respir Crit Care Med* **184**, 602–615.
  - 33. Alving K, Weitzberg E & Lundberg JM (1993) Increased amount of nitric oxide in exhaled air of asthmatics. *Eur Respir J* **6**, 1368–1370.
  - 34. Junge W, Wilke B, Halabi A, *et al.* (2004) Determination of reference intervals for serum creatinine, creatinine excretion and creatinine clearance with an enzymatic and a modified Jaffe method. *Clin Chim Acta* **344**, 137–148.
  - 35. Nauseef WM (2007) Isolation of human neutrophils from venous blood. *Methods Mol Biol* **412**, 15–20.
  - 36. Bligh EG & Dyer WJ (1959) A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* **37**, 911–917.
  - 37. Jackson KG, Bateman PA, Yaqoob P, *et al.* (2009) Impact of saturated, polyunsaturated and monounsaturated fatty acid-rich micelles on lipoprotein synthesis and secretion in Caco-2 cells. *Lipids* **44**, 1081–1089.
  - 38. Santanello N, Zhang J, Seidenberg B, *et al.* (1999) What are minimal important changes for asthma measures in a clinical trial? *Eur Respir J* **14**, 23–27.
  - 39. Williams NC, Johnson MA, Shaw DE, *et al.* (2016) A prebiotic galactooligosaccharide mixture reduces severity of hyperpnoea-induced bronchoconstriction and markers of airway inflammation. *Br J Nutr* **116**, 798–804.
  - 40. Reiss TF, Hill JB, Harman E, *et al.* (1997) Increased urinary excretion of LTE4 after exercise and attenuation of exercise-induced bronchospasm by montelukast, a cysteinyl leukotriene receptor antagonist. *Thorax* **52**, 1030–1035.
  - 41. Shapiro GG, Kemp JP, DeJong R, *et al.* (1990) Effects of albuterol and procaterol on exercise-induced asthma. *Ann Allergy* **65**, 273–276.



42. Nelson JA, Strauss L, Skowronski M, *et al.* (1998) Effect of long-term salmeterol treatment on exercise-induced asthma. *N Engl J Med* **339**, 141–146.
43. Anderson S, Rodwell L, Du Toit J, *et al.* (1991) Duration of protection by inhaled salmeterol in exercise-induced asthma. *Chest* **100**, 1254–1260.
44. Bronsky EA, Yegen Ü, Yeh CM, *et al.* (2002) Formoterol provides long-lasting protection against exercise-induced bronchospasm. *Ann Allergy Asthma Immunol* **89**, 407–412.
45. Cheung D, Timmers MC, Zwiderman AH, *et al.* (1992) Long-term effects of a long-acting  $\beta_2$ -adrenoceptor agonist, salmeterol, on airway hyperresponsiveness in patients with mild asthma. *N Engl J Med* **327**, 1198–1203.
46. Simons FE, Gerstner TV & Cheang MS (1997) Tolerance to the bronchoprotective effect of salmeterol in adolescents with exercise-induced asthma using concurrent inhaled glucocorticoid treatment. *Pediatrics* **99**, 655–659.
47. Mickleborough T & Rundell K (2005) Dietary polyunsaturated fatty acids in asthma-and exercise-induced bronchoconstriction. *Eur J Clin Nutr* **59**, 1335–1346.
48. Brannan JD & Lougheed MD (2012) Airway hyperresponsiveness in asthma: mechanisms, clinical significance, and treatment. *Front Physiol* **3**, 460.
49. O'Sullivan S, Roquet A, Dahlen B, *et al.* (1998) Evidence for mast cell activation during exercise-induced bronchoconstriction. *Eur Respir J* **12**, 345–350.
50. Brannan JD, Gulliksson M, Anderson SD, *et al.* (2003) Evidence of mast cell activation and leukotriene release after mannitol inhalation. *Eur Respir J* **22**, 491–496.
51. Daviskas E, Anderson SD, Brannan JD, *et al.* (1997) Inhalation of dry-powder mannitol increases mucociliary clearance. *Eur Respir J* **10**, 2449–2454.
52. Daviskas E, Anderson SD, Gonda I, *et al.* (1995) Changes in mucociliary clearance during and after isocapnic hyperventilation in asthmatic and healthy subjects. *Eur Respir J* **8**, 742–751.
53. Kippelen P, Fitch KD, Anderson SD, *et al.* (2012) Respiratory health of elite athletes – preventing airway injury: a critical review. *Br J Sports Med* **46**, 471–476.
54. Kippelen P, Tufvesson E, Ali L, *et al.* (2013) Urinary CC16 after challenge with dry air hyperpnoea and mannitol in recreational summer athletes. *Respir Med* **107**, 1837–1844.
55. Romberg K, Bjermer L & Tufvesson E (2011) Exercise but not mannitol provocation increases urinary Clara cell protein (CC16) in elite swimmers. *Respir Med* **105**, 31–36.
56. Mickleborough TD, Vaughn CL, Shei R, *et al.* (2013) Marine lipid fraction PCSO-524<sup>TM</sup>(lyprinol®/omega XL®) of the New Zealand green lipped mussel attenuates hyperpneainduced bronchoconstriction in asthma. *Respir Med* **107**, 1152–1163.
57. Simpson AJ, Bood JR, Anderson SD, *et al.* (2016) A standard, single dose of inhaled terbutaline attenuates hyperpneainduced bronchoconstriction and mast cell activation in athletes. *J Appl Physiol* (1985) **120**, 1011–1017.
58. Schubert R, Kitz R, Beermann C, *et al.* (2009) Effect of n-3 polyunsaturated fatty acids in asthma after low-dose allergen challenge. *Int Arch Allergy Immunol* **148**, 321–329.
59. Hamid Q, Springall DR, Polak J, *et al.* (1993) Induction of nitric oxide synthase in asthma. *Lancet* **342**, 1510–1513.
60. Robbins R, Springall D, Warren J, *et al.* (1994) Inducible nitric oxide synthase is increased in murine lung epithelial cells by cytokine stimulation. *Biochem Biophys Res Commun* **198**, 835–843.
61. Barnes PJ (1995) Nitric oxide and airway disease. *Ann Med* **27**, 389–393.
62. Schmitz G & Ecker J (2008) The opposing effects of n-3 and n-6 fatty acids. *Prog Lipid Res* **47**, 147–155.
63. Accordini R, Visentin A, Bordin A, *et al.* (2008) Long-term repeatability of exhaled breath condensate pH in asthma. *Respir Med* **102**, 377–381.