

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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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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4 fatty acids in adults with asthma

5

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24

25 **Abstract**

26 Although high dose omega-3 polyunsaturated fatty acid (*n*-3 PUFA) supplementation
27 reduces exercise- and hyperpnoea-induce bronchoconstriction (EIB/HIB), there are concurrent
28 issues with cost, compliance, and gastrointestinal discomfort. It is thus pertinent to establish the
29 efficacy of lower *n*-3 PUFA doses. Eight male adults with asthma and HIB and 8 controls without
30 asthma were randomly supplemented with two *n*-3 PUFA doses (6.2 g/d (3.7g EPA and 2.5g DHA)
31 and 3.1 g/d (1.8g EPA and 1.3g DHA)) and a placebo, each for 21 days followed by 14 days
32 washout. A eucapnic voluntary hyperpnoea (EVH) challenge was performed before and after
33 treatments. Outcome measures remained unchanged in the control group. In the HIB group, the
34 peak fall in forced expiratory volume in 1 s (FEV₁) after EVH at day 0 (-1005 (SD 520) mL, -30
35 (SD 18) %) was unchanged after placebo. The peak fall in FEV₁ was similarly reduced from day 0
36 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
37 15) %) and 3.1 g/d *n*-3 PUFA (-970 (SD 480) mL, -28 (SD 18) % vs. -700 (SD 420) mL, -21 (SD
38 15) %) ($P < 0.001$). Baseline fraction of exhaled nitric oxide was reduced by 24% ($P = 0.020$) and
39 31% ($P = 0.018$) after 6.2 and 3.1 g/d *n*-3 PUFA, respectively. Peak increases in 9 α , 11 β PGF₂ after
40 EVH were reduced by 65% ($P = 0.009$) and 56% ($P = 0.041$) after 6.2 and 3.1 g/d *n*-3 PUFA,
41 respectively. In conclusion, 3.1 g/d *n*-3 PUFA supplementation attenuated HIB and markers of
42 airway inflammation to a similar extent as a higher dose. Lower doses of *n*-3 PUFA thus represent a
43 potentially beneficial adjunct treatment for adults with asthma and EIB.

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54 Introduction

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

64 One potential candidate therapy involves the omega-3 long chain polyunsaturated fatty acids
65 (*n*-3 PUFA) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)⁽⁷⁾. Dietary
66 supplementation of *n*-3 PUFA increases cell membrane EPA and DHA content and reduces *n*-6
67 arachidonic acid content⁽⁸⁾. This subsequently reduces the synthesis of the pro-inflammatory
68 arachidonic acid-derived eicosanoids cysteinyl leukotrienes and prostaglandins. EPA and DHA may
69 also increase synthesis of resolvin and protectin compounds through the cyclooxygenase and
70 lipoxygenase pathways which are involved in the resolution of inflammation. These mechanisms
71 are **thought to be** central to the well-established anti-inflammatory effects of *n*-3 PUFA⁽⁸⁾, which
72 provide a rationale for the use of *n*-3 PUFA in asthma⁽⁷⁾. To date, however, the role of EPA and
73 DHA in the management of asthma and EIB remains uncertain⁽⁹⁻¹⁴⁾. Early research showed that
74 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
75 asthma reduced leukotriene generation and neutrophil chemotactic responsiveness, but did not
76 reduce bronchoconstriction after cycling exercise and a histamine challenge⁽⁹⁾. Similarly,
77 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
78 asthma did not attenuate bronchoconstriction or markers of airway inflammation in response to a
79 mannitol challenge test⁽¹²⁾. Conversely, supplementation of 5.4 g/d *n*-3 PUFA (3.2 g/d EPA and 2.2
80 g/d DHA) for 3 weeks in elite athletes⁽¹⁰⁾ and physically active asthmatic males^(11, 15) abolished EIB.
81 Subsequent studies showed that supplementation of *n*-3 PUFA (3.2 g/d EPA and 2.0 g/d DHA)
82 reduced bronchoconstriction and markers of airway inflammation after eucapnic voluntary
83 hyperpnoea (EVH)⁽¹⁶⁾ and that *n*-3 PUFA (~3.2 g/d EPA and ~2.0 g/d DHA) was as effective as a
84 leukotriene modifier⁽¹⁷⁾.

85 Given the lack of reported risk in taking *n*-3 PUFA, the American Thoracic
86 Society/European Respiratory Society (ATS/ERS) guidelines suggest that the latter studies offer
87 some support for the consumption of *n*-3 PUFA by interested individuals with EIB^(10, 11, 17). In
88 contrast however, recent practice parameter⁽¹⁴⁾ cautions the recommendation for *n*-3 PUFA use in
89 EIB based on more recent findings^(12, 18). When considering the practicalities associated with *n*-3
90 PUFA supplementation for asthma management, previous studies have used doses of 5.2-6.0 g/d.
91 Such doses require individuals to ingest between 8-20 capsules of commercial fish oil daily, which
92 has implications for cost, compliance, and gastrointestinal discomfort^(19, 20). In a single-blind study
93 an alternative flavoured beverage delivery was investigated (3.0 g/d EPA, 3.0 g/d DHA and 30 µg
94 of vitamin D3) but failed to show any attenuation in HIB or markers of airway inflammation
95 (F_ENO, urinary 9α, 11β PGF₂ and cysteinyl leukotriene E4)⁽¹⁸⁾. However, no measure of compliance
96 to the treatment was made and participants included had mild asthma or did not have a physician
97 diagnosis of asthma. It is therefore pertinent to establish if lower doses of *n*-3 PUFA are effective in
98 reducing EIB. With respect to the simulation of EIB under laboratory conditions, an EVH challenge
99 causes a highly reproducible hyperpnoea-induced bronchoconstriction (HIB) (a surrogate for EIB)
100 in adults with asthma⁽²¹⁾ which makes this an attractive challenge test to evaluate the effects of *n*-3
101 PUFA treatments on airway hyper responsiveness. Thus, the aim of the current study was to
102 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
103 markers of airway inflammation in adults with asthma. Provisional data from the study was
104 previously part published in abstract form⁽²²⁾.

105

106 **Methods**

107 *Participants*

108 Sixteen non-smoking, recreationally active men (completing ≥6-h of endurance exercise per
109 week) provided written, informed consent to participate in the study (Table 1). Eight participants
110 formed a HIB group and 8 formed a control group. Inclusion criteria for the HIB group were:
111 physician diagnosis of asthma, a baseline FEV₁ >65% of predicted⁽²³⁾, and a ≥10% fall in FEV₁
112 following initial EVH screening^(24, 25). The HIB group were on step 1-3 of the stepwise approach to
113 asthma control, indicating well-controlled asthma using either reliever medication (short acting β2-
114 agonist) alone or in combination with controller medication (low dose inhaled corticosteroid and/or
115 long-acting β2-agonist⁽²⁶⁾ (Table 1). Inclusion criteria for the control group were: a baseline FEV₁

116 >65% of predicted and a <10% fall in FEV₁ following initial EVH screening. Participants avoided
117 exercise for 24-h before an EVH test, and the HIB group ceased their medication as previously
118 described^(21, 24). On EVH test days, participants abstained from caffeine and alcohol and arrived at the
119 laboratory >2-h post-prandial^(27, 28). Participants were instructed to abstain from consumption of *n*-3
120 PUFA supplements and eat no more than 2 oily fish meals per week for 3 weeks prior to the study
121 and throughout the study⁽¹¹⁾. Participants were free from acute upper respiratory tract infections
122 throughout the study.

123 *Experimental Design and Protocol*

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

128 Participants were randomised (block randomisation) to receive three 21 day treatments each
129 separated by a 14 day washout period. All treatments involved daily oral consumption of 8 capsules
130 (CRODA International Plc, Snaith, UK). Four capsules were taken in the morning and four in the
131 afternoon with a recommendation to take with food. The three treatments were: 6.2 g/d *n*-3 PUFA
132 (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™
133 TG4030, CRODA, UK) and placebo (CRODAMOL™ GTCC medium chain triglyceride, CRODA
134 International Plc, Snaith, UK) (Table 2). CRODAMOL™ GTCC was chosen as it is readily
135 oxidised in the liver so has little impact on human health-related biomarkers^(29, 30). Measurements
136 were taken at day 0 and 21 of each treatment period. The 6.2 g/d *n*-3 PUFA dose was comprised of
137 four *n*-3 PUFA capsules in the morning and four *n*-3 PUFA capsules in the afternoon. To ensure an
138 equal number of capsules were taken the 3.1 g/d *n*-3 PUFA comprised of two *n*-3 PUFA capsules
139 and two placebo capsules in the morning and two *n*-3 PUFA capsules and two placebo capsules in
140 the afternoon. **All capsules (placebo and *n*-3 PUFA) were identical in appearance.**

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

142 The EVH test was undertaken at day 0 and day 21 of each treatment and comprised 6 min of
143 breathing dry gas at a target minute ventilation (\dot{V}_E) of 85% of the predicted maximal voluntary
144 ventilation (MVV) (30 x baseline FEV₁). Pulmonary function (forced vital capacity, FVC; FEV₁;
145 peak expiratory flow, PEF; and forced expiratory flow 25-75%, FEF_{25-75%}) was assessed according
146 to ATS/ERS guidelines⁽³¹⁾ in triplicate at baseline and in duplicate at 3, 6, 16, 20, and 30 min after

147 EVH, as previously described. The highest values recorded were used for analyses. Baseline $F_{E}NO$
148 was measured (NIOX MINO; Aerocrine, Solna, Sweden) according to ATS/ERS guidelines⁽³²⁾ in
149 the HIB group only, as it is elevated in asthma patients but not in healthy controls⁽³³⁾.

150 *Urinary 9α , 11β -PGF₂ Analysis*

151 Participants provided a urine sample at baseline and at 12, 60, and 90 min after EVH.
152 Urinary concentration of 9α , 11β -PGF₂, a metabolite of prostaglandin D₂, was subsequently
153 determined by enzyme-linked immunosorbent assay (Caymen Chemicals, Ann Arbor, Michigan,
154 USA) and standardised for urinary creatinine concentration (ABX Pentra 400; Horiba,
155 Northampton, UK) based on a kinetic method using alkaline picrate (Jaffe method)⁽³⁴⁾. The
156 corrected 9α , 11β -PGF₂ was expressed as ng/mmol creatinine. The inter- and intra-assay coefficient
157 of variation was <15%. Two control participants and one HIB participant had samples at day 0
158 below the limit of detection for urinary 9α , 11β -PGF₂ (5 pg/ml) and were subsequently excluded
159 from analysis.

160 *Neutrophil Phospholipid Fatty Acid Analysis*

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

173 *Statistical Analysis*

174 The average minimum perceptible improvement in FEV₁ in adults with asthma is 230
175 mL⁽³⁸⁾, whereas the within participant standard deviation for the fall in FEV₁ after EVH is 100
176 mL⁽²¹⁾. *A priori* sample size calculation revealed that with power = 0.90 and alpha = 0.05, a sample
177 size of 7 in the HIB group would be required to detect a 230 mL improvement in the fall in FEV₁
178 after EVH.

179 Data were analysed using SPSS (Chicago, IL). Following assessment for normality
180 (Shapiro-Wilks test, skewness and kurtosis), data were analysed using repeated measures ANOVA
181 and Bonferroni adjusted paired t-tests. Statistical significance was set at $P < 0.05$. Data presented
182 are mean (SD) unless otherwise stated. Within treatment percent differences (from day 0 to day 21)
183 for the fall in FEV₁ following EVH were calculated as: $((\% \text{ fall day 0} - \% \text{ fall day 21}) / \% \text{ fall day 0})$
184 $\times 100$. For the HIB group, the individual percent protection afforded by the *n*-3 PUFA treatments
185 compared with placebo was calculated for the % fall in FEV₁ following EVH as: $((\% \text{ fall day 21}$
186 $\text{placebo} - \% \text{ fall day 21 treatment}) / \% \text{ fall day 21 placebo}) \times 100$. **In the HIB group, the overall**
187 **severity of HIB was determined by calculating the area under the curve for % fall in FEV₁ after**
188 **EVH (AUC₀₋₃₀) using the trapezoidal rule.**

189 **Results**

190 *Pulmonary Function and Ventilation Rate during EVH*

191 Baseline FEV₁ was lower in the HIB group (3.75 (SD 0.81) L) than the control group (4.63
192 (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
193 control group (5.26 (SD 0.39) L) ($P = 0.051$). There were no within group differences in FEV₁ or
194 FVC measured at day 0 between the three treatments.

195 As expected, at day 0 there was a greater peak fall in FEV₁ after EVH in the HIB group
196 (pooled data: -29 (SD 17) %) than the control group (pooled data: -3 (SD 2) %) ($P = 0.001$). In the
197 control group, there was no effect of treatment or day on the peak fall in FEV₁ after EVH. In the
198 HIB group, there was a treatment \times day interaction for the peak fall in FEV₁ after EVH ($P = 0.011$).
199 Further analyses revealed an effect of treatment for the peak fall in FEV₁ after EVH at day 21 ($P =$
200 0.001). Specifically, the peak fall in FEV₁ after EVH was reduced by 34 (SD 14) % (-690 (SD 460)
201 mL) after 6.2 g/d *n*-3 PUFA (mean difference = 310 (SD 150) mL, 95% CI = 185, 432 mL, $P =$
202 0.001 , effect size = 0.70), and by 30 (SD 11) % (-700 (SD 420) mL) after 3.1 g/d *n*-3 PUFA (mean
203 difference = 270 (SD 120) mL, 95% CI = 170, 377 mL, $P = 0.001$, effect size = 0.58). The reduced
204 peak falls in FEV₁ after 6.2 g/d and 3.1 g/d *n*-3 PUFA were not different ($P = 0.834$) (Figure 2,
205 Table 4). The percent protection afforded by the *n*-3 PUFA treatments compared with placebo is
206 shown in Table 4. **The peak fall in FEV₁ was unchanged from day 0 to day 21 of placebo.**

207 **Figure 3 shows the percent change in FEV₁ during 30 min recovery after EVH in the HIB**
208 **group. At day 0, the percent change in FEV₁ during recovery was not different between treatments.**
209 **At day 21, the percent change in FEV₁ was reduced for up to 20 and 30 min recovery after 6.2 g/d**
210 **and 3.1 g/d *n*-3 PUFA, respectively. Furthermore, compared with placebo at day 21, the percent**

211 change in FEV₁ was lower for up to 6 and 20 min recovery after 6.2 g/d and 3.1 g/d *n*-3 PUFA,
212 respectively.

213 In the HIB group, there was a treatment × day interaction for AUC₀₋₃₀ ($P = 0.004$). Further
214 analysis revealed that at day 21 the AUC₀₋₃₀ was reduced after 6.2 g/d (-415 (SD 382), $P = 0.002$)
215 and 3.1 g/d (-398 (SD 399), $P = 0.001$) *n*-3 PUFA compared with placebo (-595 (SD 424)). The
216 AUC₀₋₃₀ at day 21 of 6.2 g/d and 3.1 g/d *n*-3 PUFA was not different ($P = 0.751$).

217 Consistent with our previous findings^(21, 39) the peak fall in FEV₁ after EVH in the HIB
218 group was reproducible with no differences occurring between day 0 of the three treatments (within
219 participant CV = 7 (SD 4) %; measurement error = 84 mL; reproducibility = 231 mL; smallest
220 meaningful change = 115 mL). The peak fall in FVC showed similar outcomes. At day 0 of the
221 three treatments the peak fall in FVC was greater in the HIB group (pooled data: -992 (SD 604) mL;
222 -21 (SD 15) %) than the control group (pooled data: -140 (SD 90) mL; -3 (SD 2) %) ($P = 0.004$). In
223 the control group, the peak fall in FVC was unchanged after placebo and both doses of *n*-3 PUFA.
224 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
225 by 29 (SD 24) % after 3.1 g/d *n*-3 PUFA but was unchanged after placebo in the HIB group. The
226 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
227 HIB group, the peak fall in PEF was unchanged after placebo and both *n*-3 PUFA treatments
228 (pooled data: -2.52 (SD 1.68) L/s). In the HIB group, the peak fall in FEF_{25-75%} was reduced from
229 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 =
230 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
231 peak fall in FEF_{25-75%} was unchanged after 3.1g/d *n*-3 PUFA and placebo in the HIB group, and
232 after all treatments in the control group ($P > 0.05$).

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

237 *Fraction of Exhaled Nitric Oxide*

238 In the HIB group, there was a treatment × day interaction for F_ENO ($P = 0.004$). After 6.2
239 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)
240 (mean difference = 13 (SD 12) ppb, 95% CI = 3, 24 ppb, $P = 0.020$, effect size = 0.41). Similarly,
241 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

242 28) ppb) (mean difference = 15 (SD 14) ppb, 95% CI = 4, 27 ppb, $P = 0.018$, effect size = 0.46)
243 (Figure 4). The reduced $F_{E}NO$ after 6.2 g/d and 3.1 g/d $n-3$ PUFA was not different ($P = 0.491$)
244 (Figure 4). $F_{E}NO$ remained unchanged from day 0 (47 (SD 26) ppb) to day 21 (47 (SD 27) ppb) of
245 placebo.

246 *Urinary 9 α , 11 β PGF₂*

247 Baseline urinary 9 α , 11 β PGF₂ did not differ between the HIB group (day 0 pooled data:
248 40.85 (SD 21.49) ng/mmol creatinine) and the control group (day 0 pooled data: 27.05 (SD 14.01)
249 ng/mmol creatinine) ($P = 0.133$). There were no within-group differences in urinary 9 α , 11 β PGF₂
250 measured before EVH at day 0 and day 21 of the three treatments.

251 At day 0, urinary 9 α , 11 β PGF₂ increased by 31.53 (SD 22.77) ng/mmol creatinine (pooled
252 data) after EVH in the HIB group ($P = 0.030$) but not in the control group (pooled data: 11.11 (SD
253 16.30) ng/mmol creatinine). At day 0, changes in 9 α , 11 β PGF₂ after EVH were different between
254 groups ($P = 0.003$). In the HIB group, subsequent analyses revealed an effect of treatment on the
255 peak increase in 9 α , 11 β PGF₂ after EVH at day 21 ($P = 0.014$). Specifically, the peak increase in
256 9 α , 11 β PGF₂ after EVH was reduced after 6.2 g/d $n-3$ PUFA (mean difference = 11.75 (SD 7.42)
257 ng/mmol creatinine, 95% CI = 5.54, 17.96 ng/mmol creatinine, $P = 0.009$, effect size = 0.50) and
258 3.1 g/d $n-3$ PUFA (mean difference = 12.86 (SD 14.90) ng/mmol creatinine, 95% CI = 0.40, 25.33
259 ng/mmol creatinine, $P = 0.041$, effect size = 0.40). The reduced peak increase in 9 α , 11 β PGF₂ after
260 EVH following 6.2 g/d and 3.1 g/d $n-3$ PUFA was not different ($P = 0.377$) (Figure 5). There was
261 no effect of placebo on the increase in urinary 9 α , 11 β PGF₂ after EVH.

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

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

273

274 **Discussion**

275 Previous research has shown that high doses of *n*-3 PUFA (5.2-5.4 g/d) reduce the severity
276 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
277 equally effective in reducing HIB in adult men with asthma. The 6.2 and 3.1 g/d *n*-3 PUFA
278 treatments also resulted in similar reductions in baseline F_ENO, and comparable suppression of
279 urinary 9 α , 11 β -PGF₂ after EVH. These findings suggest that 3.1 g/d *n*-3 PUFA could be used as an
280 adjunct therapy for physically active adults with asthma and EIB. The percent protection afforded
281 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
282 of *n*-3 PUFA as an adjunct therapy. The percent protection afforded by *n*-3 PUFA is comparable to
283 montelukast (50mg twice daily)⁽⁴⁰⁾ but less than the 60-70% protection afforded by short-acting β -
284 agonists procaterol (10 micrograms/inhalation) and albuterol (90 micrograms/inhalation)⁽⁴¹⁾. Long-
285 acting β -agonists also provide effective treatment for EIB,⁽⁴²⁻⁴⁴⁾ although chronic treatment with
286 both long- and short-acting β -agonists can result in tolerance^(45, 46). Furthermore, compliance to the
287 lower 3.1 g/d *n*-3 PUFA dose may be improved due to reduced capsule numbers, reduce cost and
288 risk of gastrointestinal distress, which is commonly associated with bloating, and stomach upset.
289 Indeed, two participants withdrew from the study when on the 6.2 g/d *n*-3 PUFA treatment due to
290 gastrointestinal distress, whereas full compliance and no participant withdrawals (capsule counts)
291 was observed with 3.1 g/d *n*-3 PUFA.

292 The EVH test is an indirect bronchial provocation test that is a suitable objective surrogate
293 for identifying EIB⁽²⁴⁾. We have previously reported that the EVH protocol used in the present study
294 elicits a highly reproducible fall in FEV₁^(21, 39) which is crucial when assessing treatment efficacy.
295 The ~290 mL (~32%) decrease in the post-EVH fall in FEV₁ after 6.2 g/d and 3.1 g/d *n*-3 PUFA
296 supplementation exceeds the minimum perceptible change of 230 mL⁽³⁸⁾ and is therefore clinically
297 relevant. Our findings are in agreement with previous studies showing an attenuation of HIB⁽¹⁷⁾ and
298 EIB⁽¹¹⁾ after *n*-3 PUFA supplementation in adults with asthma. These findings collectively support
299 the ATS/ERS guidance⁽²⁵⁾ suggesting *n*-3 PUFA supplementation could be of benefit in individuals
300 with EIB. Our findings are also in broad agreement with work on *n*-3 PUFA supplementation in
301 elite athletes with EIB⁽¹⁰⁾, and asthmatic patients with EIB and HIB^(11, 15-17). An interesting
302 difference is that previous work showed abolition of EIB after *n*-3 PUFA supplementation⁽¹⁰⁾,
303 whereas in the present study only 3 participants became non-diagnostic (Figure 2). **This may be**
304 **partly due to inter-study differences in the severity of EIB: the peak fall in FEV₁ in the present**

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

307 Unlike the rather consistent finding that pharmacological therapy reduces EIB⁽⁴²⁻⁴⁴⁾, the
308 efficacy of *n*-3 PUFA remains controversial. Our findings contrast those of Arm et al.⁽⁹⁾ and
309 Brannan et al.⁽¹²⁾ who despite using relatively high *n*-3 PUFA doses (5.4-6.0 g/d), reported no
310 change in bronchial hyperresponsiveness to inhaled histamine and mannitol, respectively. Arm et
311 al.⁽⁹⁾ reported no change in airway resistance (fall in FEV₁ was not measured) after a cycling
312 exercise challenge, although their data are confounded by: (1) the suggestion that low-grade, non-
313 pharmaceutical *n*-3 PUFA was used⁽⁴⁷⁾; (2) low statistical power due to the small number of
314 participants (*n* = 6) performing the exercise; (3) exercise being performed at ambient temperature
315 and humidity, which may result in insufficient environmental stress; and (4) the low exercise
316 intensity (60-130 W) which likely (data not measured/reported) elicited only a modest increase in
317 \dot{V}_E . The efficacy of *n*-3 PUFA supplementation may also partly depend on the choice of bronchial
318 provocation test. Direct bronchial provocation tests such as inhaled histamine act directly on
319 receptors on the airway smooth muscle causing contraction⁽⁴⁸⁾ and may not, therefore, fully reveal
320 the potential anti-inflammatory effects of *n*-3 PUFA. Indirect bronchial provocation tests such as
321 mannitol and EVH both alter the tonicity and volume of the airway surface liquid thereby
322 promoting the release of bronchoconstrictive inflammatory mediators^(49, 50). However, mannitol and
323 EVH may exert different levels of osmotic and mechanical stress on the airways. Specifically,
324 mannitol induces dehydration stress primarily in the proximal airways^(50, 51), whereas the high \dot{V}_E
325 during EVH results in the dehydration stress being extended to peripheral airways⁽⁵²⁾ which are
326 increasingly recruited to heat and humidify inspired air. Furthermore, EVH also elicits airflow-
327 induced shear stress and high transepithelial pressure gradients⁽⁵³⁾. These differences may
328 predispose the airway epithelium to greater damage after EVH than mannitol; indeed, EVH tends to
329 result in greater increases in urinary Clara cell protein 16 concentration, a marker of airway
330 epithelial damage^(54, 55). This is significant because *n*-3 PUFA supplementation attenuates the
331 increase in urinary Clara cell protein concentration after EVH, which suggests that reduced HIB
332 may be partly due to reduced airway epithelial damage⁽⁵⁶⁾. Differences in the intensity of the
333 stimulus to the airways may thus explain the divergent effects of *n*-3 PUFA supplementation on
334 bronchial hyperresponsiveness to EVH and mannitol.

335 Recently, Price et al.⁽¹⁸⁾ reported no change in the severity of HIB or markers of airway
336 inflammation (F_ENO, urinary 9 α , 11 β PGF₂ and cysteinyl leukotriene E4) in recreationally active

337 adults supplemented daily with a combined treatment of 30 μ g vitamin D3 with *n*-3 PUFA (3.0 g
338 EPA and 2.0 g DHA). Unfortunately, objective treatment compliance measures (e.g. neutrophil
339 phospholipid DHA and EPA content) were not taken in this study. The fall in FEV₁ after EVH was
340 also modest (~16%) compared with the ~30% fall observed at day 0 in the present study, and 6
341 from 10 of the participants did not have physician diagnosed asthma (and consequently no asthma
342 medication). Two participants in Price et al.⁽¹⁸⁾ also became non-diagnostic for HIB after the
343 placebo treatment. It may be that *n*-3 PUFA supplementation is thus more effective in individuals
344 with diagnosed asthma and more severe HIB, as observed in the present study.

345 The increase in urinary 9 α , 11 β -PGF₂ after EVH at day 0 in the HIB group is similar in
346 magnitude to that reported previously in adults with asthma performing exercise or EVH^(10, 57). The
347 reduced increase in 9 α , 11 β -PGF₂ after EVH was comparable after 6.2 g/d and 3.1 g/d *n*-3 PUFA
348 supplementation. Previous work also reports a reduced increase in 9 α , 11 β -PGF₂ after EVH
349 following *n*-3 PUFA supplementation⁽¹⁰⁾. The increased EPA and DHA cell membrane content
350 following *n*-3 PUFA supplementation is likely to result in dual inhibition of arachidonic acid-
351 dependent cyclooxygenase and 5-lipoxygenase pathways which are responsible for eicosanoid
352 generation⁽⁷⁾. This may explain the decrease in 9 α , 11 β -PGF₂, a metabolite of the arachidonic acid-
353 derived pro-inflammatory eicosanoid prostaglandin-D₂. The osmotic changes in the airways
354 following EVH results in mast cell activation and release of PGD₂ during bronchoconstriction^(49, 50).
355 Suppression of mast cell activation, and reduced eicosanoid generation, may thus partially explain
356 reduced HIB after *n*-3 PUFA supplementation.

357 The comparable reduction in baseline F_ENO after 6.2 g/d and 3.1 g/d *n*-3 PUFA
358 supplementation concurs with previous work^(17, 58) and is indicative of reduced baseline eosinophilic
359 airway inflammation. Increased F_ENO can also result from elevated expression of inducible nitric
360 oxide synthase in T-cells, macrophages, airway epithelial cells, and other inflammatory cells within
361 the airways^(59, 60). Elevated expression of inducible nitric oxide synthase can be induced by certain
362 pro-inflammatory cytokines such as TNF- α ^(59, 60) probably via the activation of nuclear factor-kappa
363 β transcription factor⁽⁶¹⁾. The *n*-3 and *n*-6 PUFAs modulate nuclear factor-kappa β transcription
364 factor activation⁽⁶²⁾ and presumably therefore TNF- α expression, which may explain the observed
365 fall in F_ENO after *n*-3 PUFA supplementation in the present study. Support for this argument comes
366 from work showing that 5.4 g/d *n*-3 PUFA suppresses circulating plasma TNF- α in athletes with
367 EIB⁽¹⁰⁾. The comparable reduction in both baseline F_ENO and the post-EVH increase in 9 α , 11 β -
368 PGF₂ after 6.2 g/d and 3.1 g/d *n*-3 PUFA supplementation suggests that both doses increased the *n*-

369 3 PUFA content of the phospholipid bilayer of cell membranes to influence the inflammatory
370 response. Furthermore, and consistent with previous reports^(17, 56), the lower F_ENO observed after *n*-
371 3 PUFA supplementation in the present study may have resulted from an increase in airway pH,
372 which is considered a determinant of F_ENO and airway inflammation⁽⁶³⁾.

373 Limitations of the current study include no run-in period to assess habitual *n*-3 PUFA intake,
374 the exclusion of females, the comparatively low participant numbers, and the wide range of asthma
375 phenotypes suggested by the heterogeneous fall in FEV₁ and large variation in inflammatory
376 markers after EVH. However, our cohort provides evidence for the use of *n*-3 PUFA in more severe
377 HIB than previous literature^(10, 11). Future work may explore whether particular asthma phenotypes
378 and HIB/EIB severity respond preferentially to *n*-3 PUFA supplementation. **Furthermore, given the**
379 **existing inter-study differences regarding the efficacy of *n*-3 PUFA for the management of EIB,**
380 **further studies using larger sample sizes are warranted.**

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

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395 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.,
396 and M.A.J conducted research; N.C.W., and K.G.J provided essential reagents and conducted
397 analysis of urine and blood samples; N.C.W., G.R.S., and M.A.J analysed data. N.C.W., K.A.H.,
398 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
399 reviewing and approval of the final manuscript.

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401 None of the other authors had a personal or financial conflict of interest.

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

	Age (y)	Height (cm)	Body Mass (kg)	FVC (L)		FEV ₁ (L)		Medications
				<i>n</i>	%	<i>n</i>	%	
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)	

407 BEC = beclomethasone; BUD = budesonide; FORM = formoterol; FEV₁ = forced expiratory volume in 1 second; FVC
 408 = forced vital capacity; S = salbutamol. *Difference between HIB and control group ($P = 0.017$).

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

Fatty Acid	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

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

HIB	Placebo		6.2 g/d <i>n</i> -3 PUFA		3.1 g/d <i>n</i> -3 PUFA	
	Day 0	Day 21	Day 0	Day 21	Day 0	Day 21
20:4 <i>n</i> -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 <i>n</i> -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 <i>n</i> -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 <i>n</i> -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 <i>n</i> -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 <i>n</i> -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)

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

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

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445 **TABLE 4.** Individual and mean (SD) peak % fall in FEV₁ at day 0 and day 21 of each treatment
446 and percent protection afforded by *n*-3 PUFA. Data shown for HIB group only.

HIB	Peak % fall in FEV ₁						Percent protection afforded by <i>n</i> -3 PUFA	
	Placebo		6.2 g/day <i>n</i> -3 PUFA		3.1 g/day <i>n</i> -3 PUFA		treatments for peak % fall in FEV ₁	
	Day 0	Day 21	Day 0	Day 21	Day 0	Day 21	6.2 g/day <i>n</i> -3 PUFA	3.1 g/day <i>n</i> -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)

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

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450 Figure Captions

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

453 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),
454 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
455 HIB participant. *Day 0 v. day 21 (P = 0.001).

456 FIGURE 3 – The percent change in forced expiratory volume in 1 second (FEV₁) after eucapnic voluntary hyperpnoea
457 in participants with hyperpnoea-induced bronchoconstriction (HIB). A 10% fall in FEV₁ (shown by the dashed line) is
458 diagnostic of HIB. *Closed circles*, day 0 of placebo treatment; *open circles*, day 21 of placebo treatment; *closed*
459 *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
460 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
461 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
462 g/d *n*-3 PUFA. Single letters, P < 0.05; *P < 0.01; **P < 0.001.

463 FIGURE 4 – Baseline fraction of exhaled nitric oxide (FENO) before and after placebo (A), 6.2 g/d *n*-3 PUFA (B), and
464 3.1 g/d *n*-3 PUFA (C) in the HIB group (n = 8). Bars represent mean (SD) and identical symbols represent the same
465 HIB participant. *Day 0 v. day 21 (P < 0.05).

466 FIGURE 5 - Peak increase in urinary 9α , 11β PGF₂ after EVH in the HIB group (n = 8). Filled and open bars represent
467 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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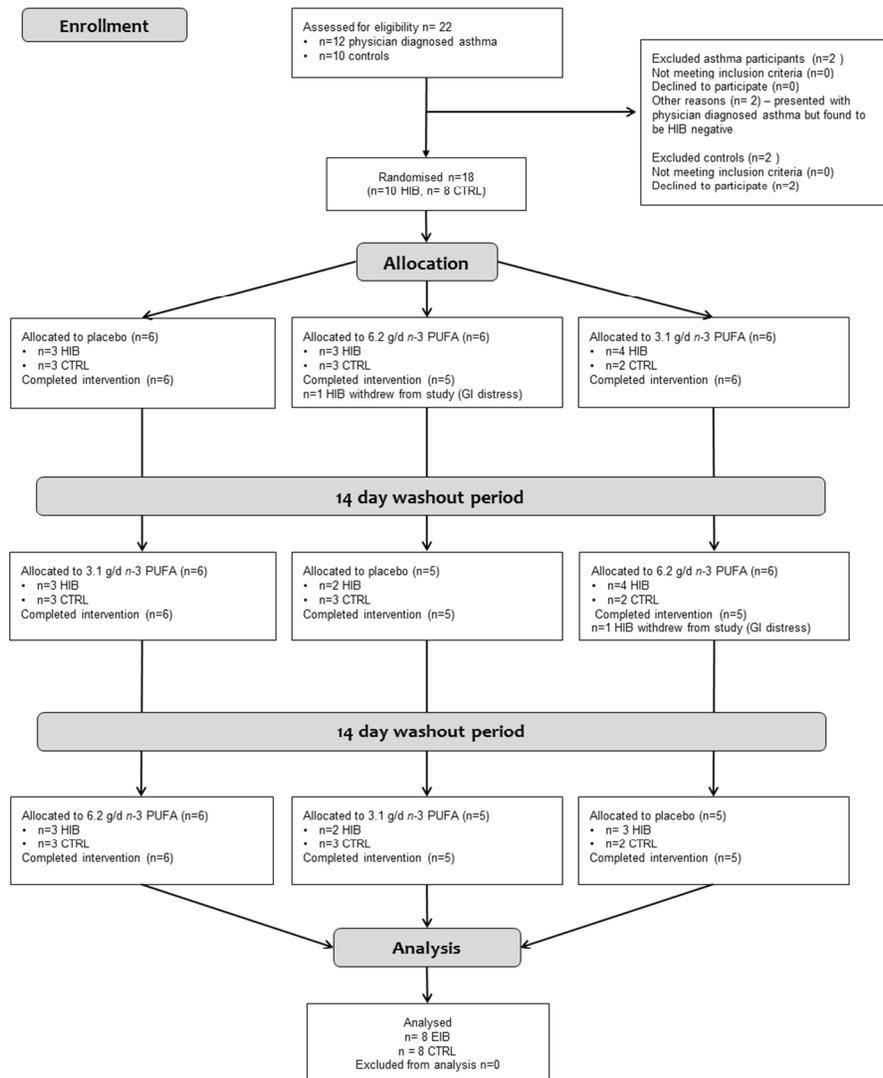


FIGURE 1 – Participant flow diagram. HIB = hyperpnoea-induced bronchoconstriction; CTRL = controls; PUFA = polyunsaturated fatty acid; GI = gastrointestinal.

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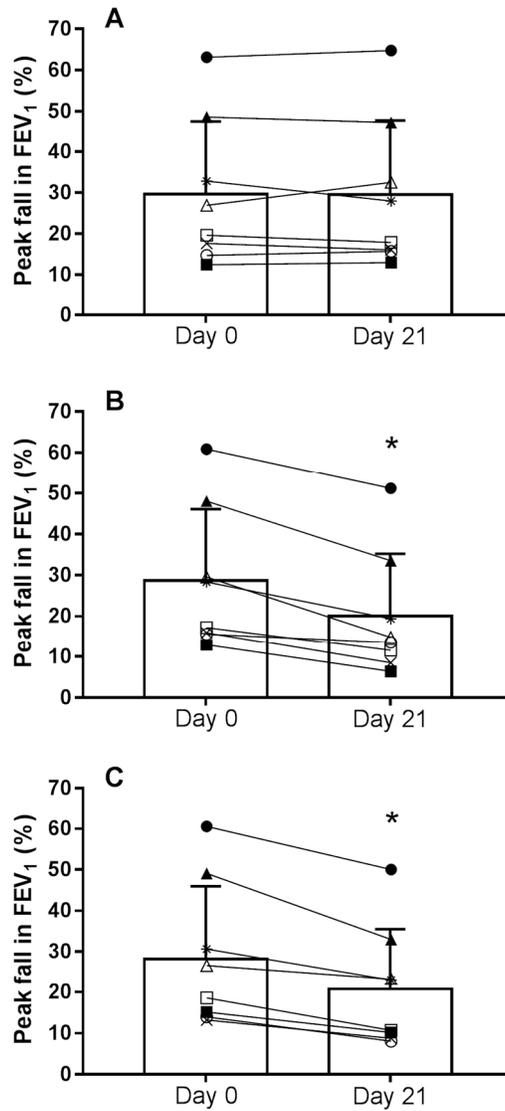


FIGURE 2 – Peak falls in forced expiratory volume in 1 s (FEV₁) before and after placebo (A), 6.2 g/d n-3 PUFA (B), and 3.1 g/d n-3 PUFA (C) in the HIB group (n = 8). Bars represent mean (SD) and identical symbols represent the same HIB participant. *Day 0 v. day 21 (P = 0.001).

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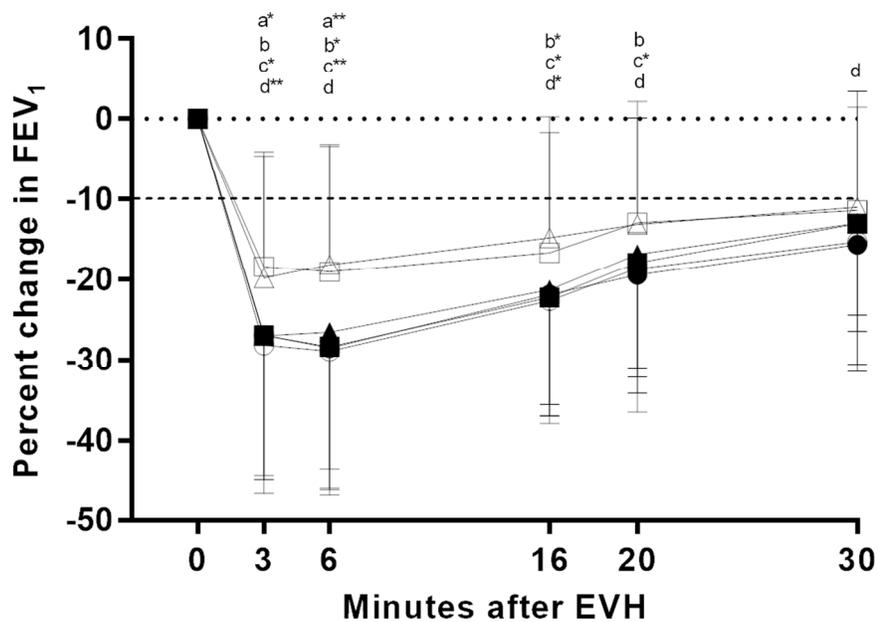


FIGURE 3 – The percent change in forced expiratory volume in 1 second (FEV₁) after eucapnic voluntary hyperpnoea in participants with hyperpnoea-induced bronchoconstriction (HIB). A 10% fall in FEV₁ (shown by the dashed line) is diagnostic of HIB. Closed circles, day 0 of placebo treatment; open circles, 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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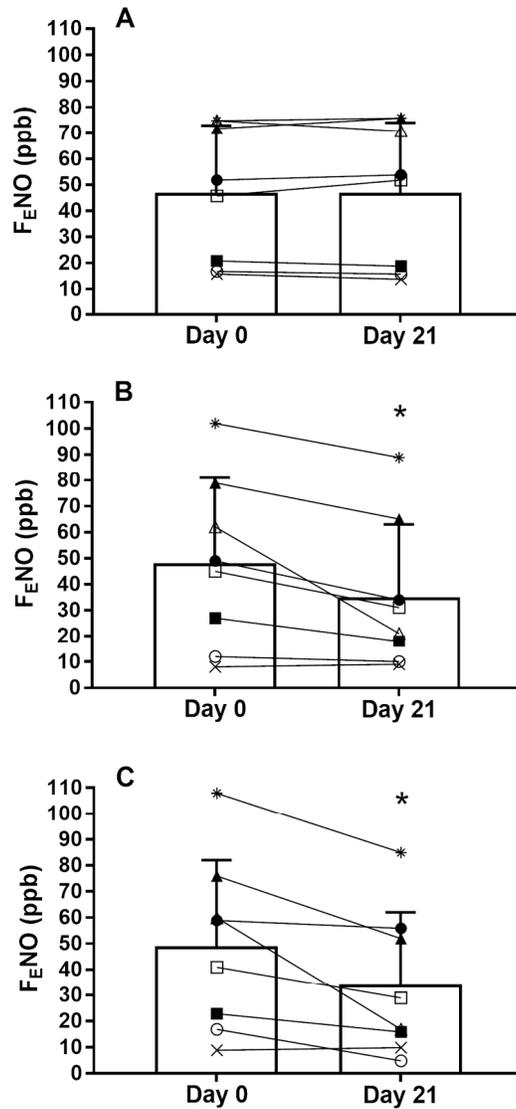


FIGURE 4 – Baseline fraction of exhaled nitric oxide (FENO) before and after placebo (A), 6.2 g/d n-3 PUFA (B), and 3.1 g/d n-3 PUFA (C) in the HIB group (n = 8). Bars represent mean (SD) and identical symbols represent the same HIB participant. *Day 0 v. day 21 (P < 0.05).

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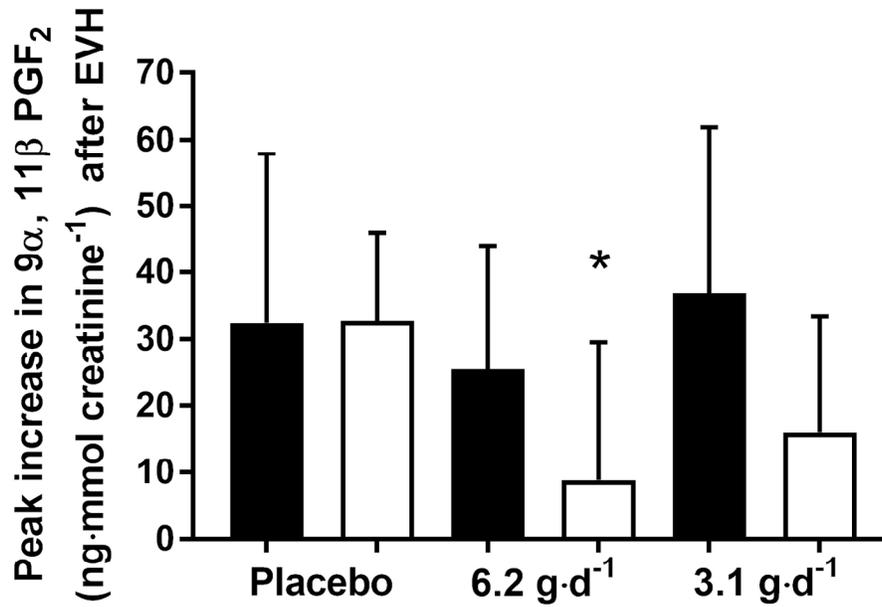


FIGURE 5 - Peak increase in urinary 9α , 11β PGF₂ after EVH in the HIB group (n = 8). Filled and open bars represent day 0 and day 21, respectively, of the treatment. Data are mean (SD). *Day 0 v. day 21 (P < 0.05)

145x104mm (300 x 300 DPI)

Only