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Marine lipid fraction PCSO-524™ (lyprinol®/omega XL®) of the New Zealand green lipped mussel attenuates hyperpnea-induced bronchoconstriction in asthma

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KEYWORDS
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Summary
Purpose: Evaluate the effect of the marine lipid fraction of the New Zealand green-lipped mussel (*Perna canaliculus*) PCSO-524™ (Lyprinol®/Omega XL®), rich in omega-3 fatty acids, on airway inflammation and the bronchoconstrictor response to eucapnic voluntary hyperpnea (EVH) in asthmatics.
Methods: Twenty asthmatic subjects, with documented HIB, participated in a placebo controlled double-blind randomized crossover trial. Subjects entered the study on their usual diet and were then placed on 3 weeks of PCSO-524™ or placebo supplementation, followed by a 2 week washout period, before crossing over to the alternative diet. Pre- and post-eucapnic voluntary hyperpnea (EVH) pulmonary function, fraction of exhaled nitric oxide (F_ENO), asthma symptom scores, medication use, exhaled breath condensate (EBC) pH, cysteinyl leukotrienes (cyst-LT), 8-isoprostane and urinary 9α, 11β-prostaglandin (PG)F_2 and Clara (CC16) protein concentrations were assessed at the beginning of the trial and at the end of each treatment period.
Results: The PCSO-524™ diet significantly reduced ($p < 0.05$) the maximum fall in post-EVH FEV_1 ($-8.4 \pm 3.2\%$) compared to usual ($-19.3 \pm 5.4\%$) and placebo diet ($-22.5 \pm 13.7\%$). Pre- and post- EVH EBC cyst-LT and 8-isoprostane, and urinary 9α, 11β-PGF_2 and CC16 concentrations were significantly reduced ($p < 0.05$) on the PCSO-524™ diet compared to the usual and placebo diet. EBC pH and asthma symptom scores were significantly improved ($p < 0.05$) and rescue medication use significantly reduced ($p < 0.05$) on the PCSO-524™ diet compared to the usual and placebo diet.

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Conclusion: PCSO-524™ (Lyprinol®/Omega XL®) may have beneficial effects in HIB and asthma by serving as a pro-resolving agonist and/or inflammatory antagonist.

Introduction

Asthma is a multifaceted condition in which multiple environmental and genetic influences can lead to several clinical phenotypes such as exercise-induced bronchoconstriction (EIB), which is a transient deterioration in lung function following exercise,[1] that can occur in patients with asthma[2] and elite athletes.[3] The mechanisms responsible for EIB likely involve multiple mechanistic pathways, however it is generally accepted that exercise or dry air hyperpnea play an important role as an initiating stimulus through airway surface effects of water loss, which include mucosal cooling, dehydration and epithelial disruption.[1] This transient dehydration causes an increase in airway surface liquid osmolality which activates histamine, neuropeptides, and the release of leukotrienes and prostaglandins (arachidonic acid metabolites), from resident airway cells, resulting in bronchial smooth muscle contraction and subsequent airway narrowing.[4]

Although the treatment of EIB almost exclusively involves pharmacotherapy, there is mounting evidence that nutritional supplementation has potential to modify this condition.[5] While the clinical data on the effect of fish oil supplementation in asthma has been equivocal,[6] supplementing the diet with fish oil rich in omega-3 (n-3) polyunsaturated fatty acids (PUFA), such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), in asthmatic individuals[7–9] and elite athletes with EIB[10] has yielded promising results. Two mechanisms of action underpinning the novel anti-inflammatory bioactions of fish oil include the ability of EPA to compete with arachidonic acid as a substrate for cyclooxygenase (COX)-2 and 5-lipoxygenase (5-LO) enzymes and be converted to less inflammatory leukotrienes and prostanoids,[11] and to generate the potent anti-inflammatory E-series resolvins.[12] Less well-characterized mechanisms include the capability of DHA to alter gene transcription and translation via direct or indirect actions on intracellular signaling pathways,[13] and to produce the D-series resolvins and protectins (neuroprotectin D1).[12]

While fish oil has been shown to attenuate airway inflammation and the bronchoconstrictor response to exercise and dry gas hyperpnea,[7,9,10] it is possible that different forms of marine oils in the diet may have varying effects of on these responses, since these oils contain a variety of lipid mediators as well as different amount of n-3 PUFAs.[14] PCSO-524™ (Lyprinol®/Omega XL®) is a patented extract of stabilized lipids from the New Zealand green-lipped mussel (NZGLM), *Perna canaliculus*, combined with olive oil and vitamin E.[15] PCSO-524™ is a multifarious mixture of sterol esters, sterols, polar lipids, triglycerides and free fatty acids (including EPA and DHA),[16] and has been shown to reduce pro-inflammatory leukotriene (LT)B_4 in human monocytes,[17] and to decrease levels of thromboxane B_2, prostaglandin (PG) E_2, and interleukin (IL) 1β with similar potency to low dose n-3 PUFA supplementation.[18] These findings support the potential for PCSO-524™ to attenuate airway inflammation and bronchoconstriction in asthma.

Daily supplementation with PCSO-524™ in human asthmatics has been shown to decrease daytime wheeze and exhaled breath hydrogen peroxide concentration (a marker of airway inflammation),[19] and to suppress the development of allergic inflammation and airway hyperresponsiveness in a mouse model of ovalbumin (OVA)-induced allergic airway disease.[20]

Therefore, the primary aim of this study was to evaluate the effects of PCSO-524™ supplementation on airway inflammation and the bronchoconstrictor response to dry air hyperpnea in individuals with asthma. We hypothesized that PCSO-524™ supplementation would significantly attenuate airway inflammation and hyperpnea-induced bronchoconstriction (HIB) in individuals with asthma.

Methods

Subjects

Twenty subjects (12 males, 8 females, aged 22.6 ± 2.1 yr, height 168.8 ± 11.2 cm) with both physician-diagnosed asthma and documented HIB were recruited from a population of university students and the local community (Fig. 1). All subjects had clinically treated mild to moderate persistent asthma, with a resting forced expiratory volume in 1-sec (FEV_1) of >65% predicted (Table 1), and EIB as demonstrated by a greater than 10% drop in FEV_1 following a eucapnic voluntary hyperventilation (EVH) challenge.[21] A group of non-asthmatic (control) subjects was not included in the present study, as it has been shown that n-3 PUFA supplementation does not alter pulmonary function or inflammatory mediator generation in this population.[10]

All subjects had a history of shortness of breath, chest tightness, and intermittent wheezing following exercise, which was relieved by bronchodilator therapy. No subjects who volunteered for the study were currently taking any maintenance medications (e.g., corticosteroids and leukotriene modifiers) for asthma. Short acting β2-agonists were discontinued 12 h prior to testing. Caffeine/alcohol and physical exercise was not permitted 8 h and 12 h respectively prior to the EVH challenge. Subjects were also excluded if they had a history of taking fish oil supplements and regularly consumed more than one fish meal per week. Subjects were asked not to eat more than one fish meal per week during the course of the study. Subjects were excluded if they were pregnant, had a history of hyperlipidemia, hypertension, diabetes, bleeding disorders, or delayed clotting time. The study was approved by the Indiana University Institutional Review Board for Human

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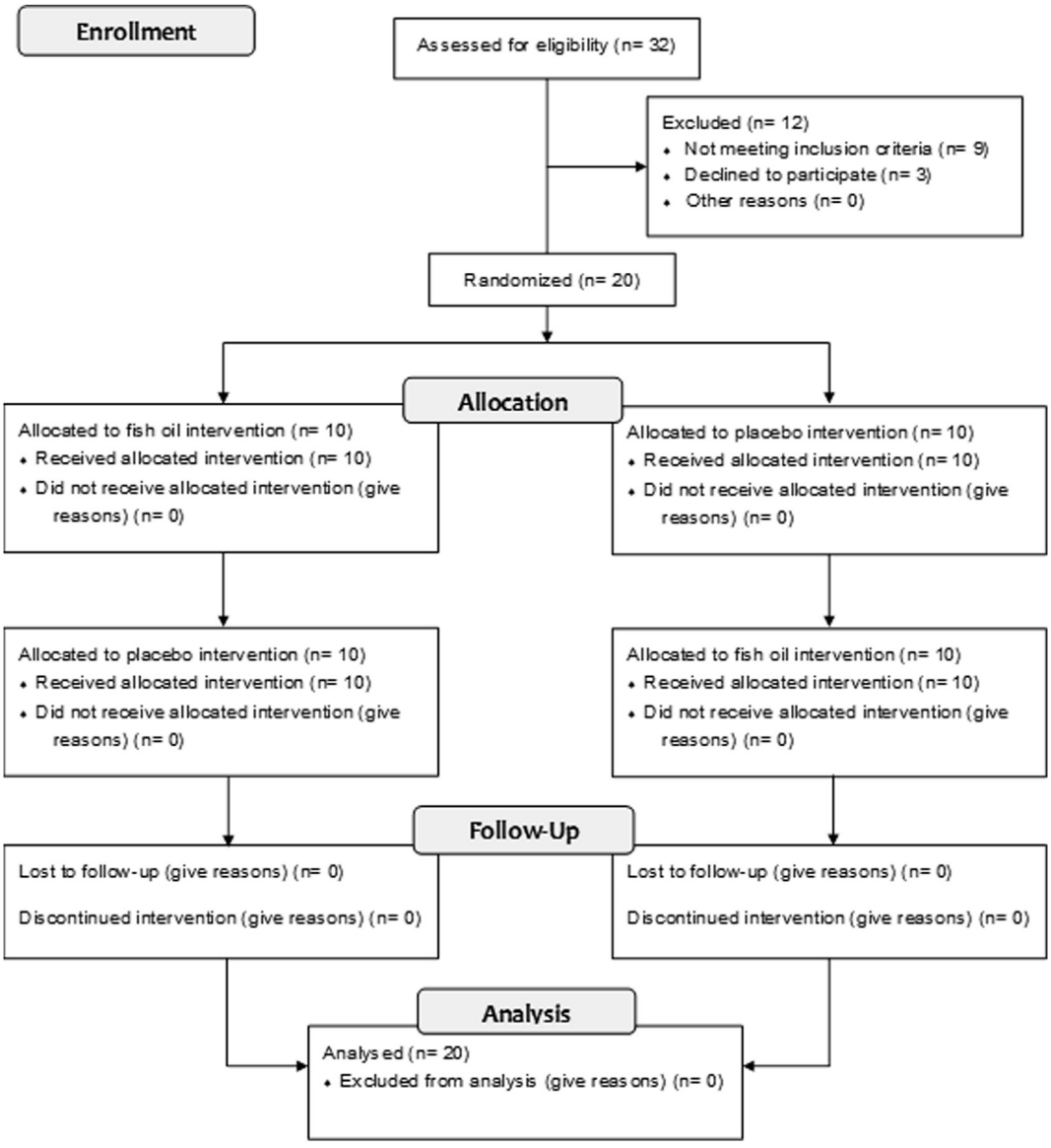

Figure 1 CONSORT flow of participants through the study diagram.

Table 1 Pre-hyperpnea (baseline) pulmonary function.

	Diet		
	PCSO-524™	Usual	Placebo
FVC (L)	4.34 + 0.74	4.45 + 0.82	4.29 + 0.92
% predicted	92.4 ± 5.2%	93.6 ± 5.6%	90.1 ± 6.1%
FEV_1 (L)	3.60 + 0.71	3.69 + 0.72	3.59 + 0.87
% predicted	93.8 ± 7.9%	95.7 ± 7.4%	92.2 ± 6.5%
$FEF_{25-75\%}$(L/min)	3.58 + 0.88	3.72 ± 1.10	3.59 ± 1.13
% predicted	97.1 ± 7.6%	98.6 ± 8.1%	97.9 ± 8.4%
PEF (L)	7.43 ± 1.31	7.52 ± 1.65	7.61 ± 1.91
% predicted	94.5 ± 8.6%	95.6 ± 9.1%	96.3 ± 8.3%

Definition of abbreviations: FVC, forced vital capacity; FEV_1, forced expiratory volume in 1-s; $FEF_{25-75\%}$, forced expiratory flow at 25–75% of FVC; PEF: peak expiratory flow. Values are mean ± SD. There was no significant difference ($p > 0.05$) for any variables between diets.

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Subjects, and written informed consent for all subjects was obtained prior to participation in the study. The study was registered as a phase 1 clinical trial with clinical trials.gov (study # NCT01504646).

Study design

The study was conducted as a randomized, double-blind, placebo-controlled crossover trial over 8 consecutive weeks, with each subject serving as their own control. Subjects were enrolled while on their usual diet. The order of supplementation was randomly assigned with the use of a computerized random number generator (http://www.randomizer.org/form.htm). The randomization sequence was created using a fixed random block size of two to correspond to the two treatments (i.e. PCSO-524™ and placebo). Sealed capsule bottles labeled with one of two material numbers were provided. Data collection and initial data analysis was completed before the principle investigator was informed which material number corresponded to each treatment. The active and placebo capsules were identical in appearance and taste so that subjects were not aware of which treatment they received.

All subjects ($n = 20$) underwent a 3 −week run period (usual diet; phase 1) preceding the start of the trial in which asthma symptoms, bronchodilator use and peak flow measurements were recorded, after which they were randomly assigned to receive either 8 capsules per day of PCSO-524™ (Lyprinol®/Omega-XL®; Pharmalink International Ltd, Hong Kong) ($n = 10$) containing approximately 72 mg EPA and 48 mg DHA (1 capsule contains 50 mg n-3 PUFAs and 100 mg olive oil or identical placebo ($n = 10$, placebo diet) capsules containing 150 mg of olive oil for 3 weeks (phase 2). Thereafter, they followed a 2-week washout period (usual diet; phase 3) and then switched to the alternative diet for the remaining 3 weeks (phase 4). All subjects were asked to record bronchodilator use, symptom scores and peak flow measurements during each dietary treatment period.

A eucapnic voluntary hyperventilation (EVH) challenge (surrogate for an exercise challenge test) was performed at the beginning of the study and at the end of each treatment period. Pulmonary function measurements were conducted pre-EVH and post-EVH at 5, 10, 15, and 20 min. Exhaled fraction of nitric oxide (F_ENO), a non-invasive measure of airway inflammation, was measured pre-EVH and at 30 min post-EVH. Exhaled breath condensate (EBC) was collected pre-EVH and post-EVH from 0 to 10 min and analyzed for the presence of the cysteinyl (Cyst)-leukotrienes (LTs) (marker of airway inflammation[22]), 8-isoprostane (marker of oxidative stress[23] and airway pH. Urine samples were collected pre-EVH and 60 min post-EVH for 9α, 11β-Prostaglandin F_2 (sensitive marker of mast cell activation and airway inflammation[24]), and pneumoprotein Clara cell (CC16; marker of airway epithelial stress[25]) analysis. Food frequency questionnaires were administered at the beginning of each testing session. Pre- and post-EVH pulmonary function and F_ENO measurements were conducted at the end of the 2-week washout period in order to verify that lung function and exhaled nitric oxide values had returned to baseline levels. Subjects were asked to record daily peak flow and asthma symptom scores throughout the course of the study.

Eucapnic voluntary hyperventilation

The EVH protocol required subjects to breathe compressed dry air (<3 mg $H_2O.L^{-1}$ air and 21%O_2, 5%CO_2, balance N_2) at a predetermined rate of 85% of maximal voluntary ventilation (estimated from 30 $\times$ the volume of resting FEV_1) for 6 min. Gas flowed from a cylinder to a reservoir bag through high-pressure tubing. From the reservoir bag gas was directed to the subject through a tube connected to a two way breathing valve and mouthpiece. Expired gases passed through a flow sensor and ventilation was measured and recorded as verification of respiration intensity (V_{max} 22 Metabolic Measurement Cart, SensorMedics, Yorba Linda, CA).

Pulmonary function tests

Pulmonary function tests were conducted on all subjects using a calibrated computerized pneumotachograph spirometer (V_{max} 22, SensorMedics, Yorba Linda, CA) according to according to American Thoracic Society (ATS) recommendations.[26] The maximum percentage fall in FEV_1 from the baseline (pre-EVH) value was calculated using the following equation: (Pre-EVH FEV_1 − lowest post-EVH FEV_1)/(Pre-EVH FEV_1). In addition, the bronchoconstrictor response to EVH was assessed as the area under the curve of the percentage fall in post-EVH FEV_1 plotted against time for 20 min (AUC0−20), using trapezoidal integration.

Fraction of exhaled nitric oxide

Fraction of exhaled nitric oxide (F_ENO) was measured with an online measurement of resting values using a restricted exhaled breath protocol (NOA 280i Nitric Oxide Analyzer, Accurate NO Breath Kit, Thermal Mass Flowmeter, NO Analysis software Version 3.21, Sievers Instruments, Boulder, CO). Measurements were conducted as outlined by American Thoracic Society guidelines.[27] Three exhalations were performed with nose clips at each test with at least 30 s between exhalations.[27] The procedure entailed maximal inhalation to total lung capacity and immediate exhalation against expiratory resistance for at least 6 s to obtain a NO plateau lasting at least 3 s. Subjects were instructed to maintain a flow rate of 50 $\pm$ 10 mL/s as monitored by a visual computer display.

Quantification of exhaled breath condensate markers

EBC samples were collected with a specially designed condensing chamber (ECoScreen, Jaeger, Hoechberg, Germany) using ATS/ERS recommendations.[28] The EBC protocol required subjects to breathe normally, wearing nose clips, through a mouthpiece connected to a non-rebreathing valve, whereby exhaled breath entered a condenser system. A temperature of −20 °C inside the condensing chamber, which was maintained throughout the collection time, produced immediate sample freezing. Exhaled breath was collected for 10 min prior to and intervals (0−10 min) following the EVH challenge.

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The pH of the non-deaerated EBC was measured immediately following collection (Orion 2 star pH meter, Thermo Scientific, Beverly, MA). It has been shown that pH measurements in EBC collected by ECoScreen are repeatable and reproducible.[29]

The remainder of the condensate was stored at $-80\ ^{\circ}C$ for later analysis of Cyst-LTs and 8-isoprostane using enzyme immunoassay techniques (Cayman Chemicals, Ann Arbor, MI) as previously discussed.[9] Cross-reactivity of the Cyst-LT antibody against an array of related compounds is: LTC_4 (100%), LTD_4 (100%), LTE_4 (67%), LTD_5 (61%), LTC_5 (54%), LTE_5 (41%), N-acetyl-LTE_4 (10.5%) and below 0.01% for other primary eicosanoid metabolites. The intra- and inter-assay coefficient of variation (CV) for the Cyst-LT enzyme immunoassay kit is reported to be $<10\%$ respectively. Cross-reactivity of the 8-isoprostane antibody against an array of related compounds is: 8-isoprostane (100%), 8-*iso* Prostaglandin $F_{2\alpha}$ ethanolamide (100%), and 8-*iso* Prostaglandin $F_{3\alpha}$ (20.6%).

Urinary 9α, 11β-prostaglandin (PG) F_2 and clara cell (CC16) quantification

Urine collection containers were provided to each subject during each visit to the laboratory. The urine was subsequently pipetted into microfuge tubes and stored at $-80\ ^{\circ}C$ until analysis. Urine was assayed for 9α, 11β-PGF_2 (Cayman Chemicals, Ann Arbor, MI) and Clara Cell protein (CC16) (BioVendor LLC, Karasek, Czech Republic) using enzyme immunoassay techniques as discussed previously.[9,25,30] The 9α, 11β-PGF_2 antibody cross-reacts with 2,3 dinor-11β-$PGF_{2\alpha}$ (10%), 11β-13,14-dihydro-15-keto-$PGF_{2\alpha}$ (0.5%) and below 0.01% for all other primary eicosanoid metabolites. The antibodies used in the CC16 ELISA are specific for human Clara cell protein with no detectable cross reactivity's to the cytokines that may be present in human serum. Inter- and intra-assay CV for the 9α, 11β-PGF_2 and CC16 assay is $<15\%$, and 2.2% and 3.7% respectively. The concentration of 9α, 11β-PGF_2 and CC16 was adjusted for creatinine concentration (Cayman Chemicals, Ann Arbor, MI). The intra- and inter assay CV for creatinine is reported to be 2.7% and 3.0% respectively.

Nutrient intake and compliance

Nutrient intake was monitored to ensure that dietary factors that could potentially affect asthma or EIB did not change through the course of the study. Nutrient data was collected using the GSEL food frequency questionnaire developed by the Nutrition Assessment Shared Resource (NASR) of Fred Hutchinson Cancer Research Center. This questionnaire has been shown to be valid and reliable in the collection of dietary data.[31] Subjects completed the GSEL version of the questionnaire at the first testing session and at the end of each supplementation period. Analysis of GSEL for nutrient intake was conducted by the Fred Hutchinson Cancer Research Center. Nutrients of interest obtained from the GSEL analysis included macronutrient composition, antioxidants (α-tocopherol, β-carotene, lycopene, Vitamin C), certain minerals (magnesium, sodium, zinc), and types of dietary fatty acids (omega-3, total polyunsaturated fatty acids, saturated fatty acids). Adherence to the treatment regimen was monitored by asking the subjects to document the dose of capsules consumed daily and to return any unused capsules.[32] For the purpose of the study a compliance of $\geq$90% was considered acceptable.

Symptoms, rescue β-agonist use and peak flow measurements

Symptoms such as wheeze, shortness of breath, chest tightness, and cough were recorded by each subject once daily with the use of a logbook. The symptom severity rating scale was defined as follows: 0: absent, no symptoms; 1: mild, symptom was minimally troublesome (not sufficient to interfere with normal daily activity or sleep); 2: moderate, symptom was sufficiently troublesome to interfere with normal daily activity or sleep; 3: severe, symptom was so severe as to prevent normal activity and/or sleep. All subjects were instructed to record each use of their rescue medication throughout the study duration, entering each medication use and the number of puffs used per occasion. Subjects were asked to perform 3 peak flow maneuvers at home in the morning and evening 2 weeks prior to the start of study, and throughout the course of the study. The subjects were provided with a peak flowmeter (Piko-1, Ferraris, Louisville, CO) and a log to record the best of 3 trials.

Data analysis

The primary analysis was on an intention-to-treat basis and involved all subjects who were randomly assigned. Data were analyzed using the SPSS version 20.0 statistical software (SPSS Inc., Chicago, USA). Normality of data was assessed using a Kolmogorov–Smirnov test and Levene's test was used to check for homogeneity of variance between groups. A two-way repeated measures analysis of variance (ANOVA) was used to analyze the data, with both treatment and time as "within-subject" effects, whereas a two-way analysis of variance was used to analyze "between-subject" effects. Mauchly's test was conducted to determine if sphericity was violated. If sphericity was violated, the repeated measures ANOVA were corrected using a Greenhouse-Geisser adjustment factor. Where a significant F ratio was found ($p < 0.05$), a Fisher protected least-square difference *post-hoc* test was used to detect differences in group means ($p < 0.05$). The Wilcoxon signed rank test was used to compare symptom scores and bronchodilator use during the trial. Data were analyzed for the presence of carryover effects between treatments using a 2×2 ANOVA. Statistical significance was set at $p < 0.05$. Data are expressed as mean $\pm$ SD, and their 95% confidence interval (CI).

Results

Subjects

Bronchodilator use (average number of doses/puffs per day) was significantly reduced during the last 2 weeks of

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the PCSO-524™ diet (1.6 ± 0.7 puffs) compared to the normal diet [9.8 ± 2.5 puffs; $p < 0.001$; Δ (mean difference), 8.2 ± 2.3 puffs; 95% CI, 6.3 to 8.8 puffs] and placebo diet (8.5 ± 2.5 puffs; $p < 0.001$; Δ, 6.9 ± 2.8 puffs; 95% CI, 5.4 to 8.4 puffs). There was no significant difference ($p = 0.456$) in bronchodilator use between the normal and placebo diet. A significant improvement in mean asthma symptoms scores during the 3 week treatment period was observed on the PCSO-524™ diet (0.8 ± 0.5) compared to the normal diet (2.6 ± 0.5; $p < 0.001$; Δ, 1.8 ± 0.9; 95% CI, 1.3−2.3) and placebo diet (2.8 ± 0.4; $p < 0.001$; Δ, 2.0 ± 0.6; 95% CI, 1.6−2.3). No significant difference ($p = 0.423$) in mean asthma symptom scores was observed between the normal and placebo diet. The combined mean morning and evening peak flow was significantly increased during the 3 week treatment period on the PCSO-524™ diet (386.3 ± 22.8 L/min) compared to the normal diet (370.4 ± 23.6 L/min; $p = 0.001$; Δ, 15.9 ± 16.2 L/min; 95% CI, 7.3 to 24.5 L/min) and placebo diet (364.5 + 17.2 L/min; $p < 0.000$; Δ, 21.8 + 16.1 L/min; 95% CI, 13.2 to 30.4 L/min). There was no significant difference ($p = 0.221$) between the normal and placebo diet for the combined mean morning and evening peak flow. A 2 × 2 ANOVA, used to test for the presence of carryover effects between diets, indicated that none were present for all measured variables ($p < 0.05$); this was further supported by post-EVH pulmonary function and F_ENO values measured at the end of the 2-week washout period returning to baseline levels. In addition, no statistical difference ($p > 0.05$) was observed between sex for all dependent measures.

Pulmonary function

No significant difference was observed ($p > 0.05$) was observed in baseline (pre-EVH) pulmonary function between groups (PCSO-524™, placebo and usual diet) (Table 1). The percentage change in the pre-EVH to post-EVH FEV_1, as a consequence of diet, is shown in Fig. 2. The maximum percentage drop in post-EVH FEV_1 on the PCSO-524™ diet (−8.4 ± 3.2%), which is indicative of an attenuated HIB response, was significantly less than the usual diet (−19.3 + 5.4%; $p < 0.001$; Δ, −10.9 ± 5.6%; 95% CI, −13.9 to −7.9%) and placebo diet (−22.5 ± 13.7; $p < 0.001$; Δ, −14.1 ± 13.1%; 95% CI, −21.1 to −7.1%). Similar significant ($p < 0.05$) changes a result of diet were observed for the present drop in post-EVH FVC, $FEF_{25-75\%}$ and PEF. The bronchoconstrictor response to EVH as determined by the AUC_{0-20} for FEV_1 was significantly less on the PCSO-524™ diet (−112.3 + 28.5) compared to the usual diet (−296.8 ± 33.4; $p < 0.001$; Δ, −184.5 ± 26.4; 95% CI, −124.7 to −234.3) and placebo diet (−338.6 ± 38.6; $p < 0.001$, Δ, −226.3 ± 31.2; 95% CI, −154.8 to −314.2). A similar pattern was observed for the AUC_{0-20} for post-EVH FVC, $FEF_{25-75\%}$ and PEF.

Fraction of exhaled nitric oxide

Pre- and post-EVH F_ENO levels were significantly reduced on the PCSO-524™ diet (pre-EVH: 15.3 + 10.7 ppb; post-EVH (11.5 + 8.2 ppb) compared to the usual diet (pre-EVH: 33.6 ± 33.7 ppb; $p = 0.046$; Δ, 14.7 ± 23.8 ppb; 95% CI, 0.04 to 30.4; post-EVH: 27.8 ± 28.0 ppb; $p = 0.035$; Δ, 16.3 ± 22.7 ppb; 95% CI, 0.9−28.4 ppb) and placebo diet (pre-EVH: 25.2 ± 19.1 ppb; $p = 0.048$; Δ, 13.9 ± 19.1 ppb; 95% CI, 0.2 to 27.6; post-EVH: 22.9 ± 17.4 ppb; $p = 0.049$; Δ, 11.4 ± 17.3 ppb; 95% CI, 0.1−26.7 ppb) (Fig. 3).

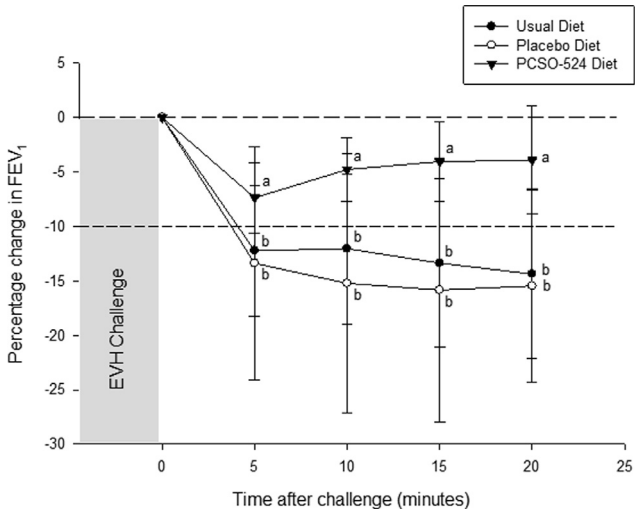

Figure 2 The percentage change in FEV_1 from pre- to post-EVH across the three treatments. Reductions in post-EVH in excess of 10% represent abnormal pulmonary function. Letters a and b refer to comparisons by treatment within respective time period. Different letters designate a significant difference ($p < 0.05$).

Exhaled breath condensate and urinary inflammatory markers

The EBC pre-EVH pH was significantly higher on the PCSO-524™ diet (6.81 ± 0.31) compared to the usual diet

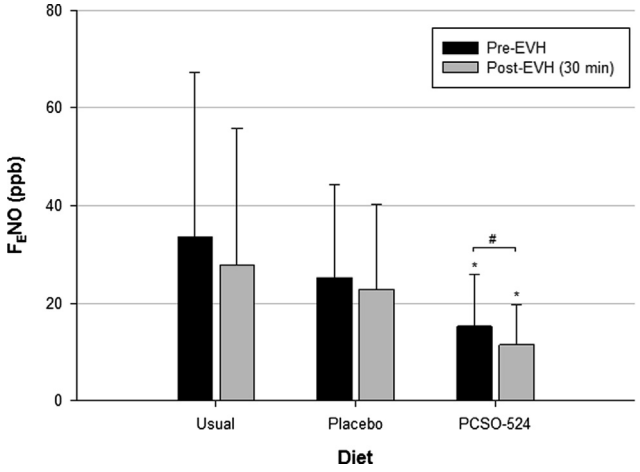

Figure 3 Mean fraction of exhaled nitric oxide (F_ENO) concentration (ppb). # designates a statistical difference ($p < 0.05$) from pre-EVH value within diet. * designates a statistical difference ($p < 0.05$) compared to respective time point between diet.

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(6.57 + 0.29; $p = 0.016$; Δ, 0.24 + 0.35; 95% CI, 0.05—0.42) and placebo diet (6.60 ± 0.25; $p = 0.042$; Δ, 0.21 ± 0.38; 95% CI, 0.00845—0.41) (Fig. 4, Panel A). In addition, EBC pH was significantly increased on the PCSO-524™ diet at 5 min (6.99 ± 0.47) and 10 min (7.11 ± 0.51) post-EVH compared to 5 min (6.66 ± 0.29; $p = 0.037$; Δ, 0.33 + 0.59; 95% CI, 0.184—0.534) and 10 min (6.57 ± 0.41; $p = 0.003$; 0.54 ± 0.55; 95% CI, 0.250—0.945) post-EVH on the usual diet and compared to 5 min (6.56 ± 0.36; $p = 0.046$; Δ, 0.43 ± 0.62; 95% CI, 0.214—0.844) and 10 min (6.71 ± 0.31; $p = 0.015$; Δ, 0.40 ± 0.56; 95% CI, 0.134—0.938) post-EVH on the placebo diet.

The mean pre- and post-EBC Cyst-LT concentration was significantly reduced on the PCSO-524™ diet (pre-EVH: 30.5 ± 10.9 pg/ml; post-EVH: 41.9 + 10.4 pg/ml) compared to the usual (pre-EVH: 41.2 ± 11.2 pg/ml; $p < 0.001$; Δ, 10.7 ± 4.8 pg/ml; 95% CI, 7.5—13.6 pg/ml; post-EVH: 68.9 ± 12.1 pg/ml; $p < 0.001$; Δ, 27.0 ± 12.8 pg/ml; 95% CI, 17.4—34.7 pg/ml) and placebo (pre-EVH: 42.1 ± 2 pg/ml; $p < 0.001$; Δ, 11.6 + 4.5 pg/ml; 95% CI, 7.8 to 13.8; post-EVH: 65.6 ± 15.3 pg/ml; $p = 0.002$; Δ, 23.7 ± 14.9 pg/ml; 95% CI, 11.1—33.9 pg/ml) diet (Fig. 4, Panel B).

Pre- and post-EVH EBC 8-isoprostane concentration was significantly attenuated on the PCSO-524™ diet (pre-EVH: 37.2 ± 14.9 pg/ml; post-EVH: 53.8 ± 13.3 pg/ml) compared to the usual (pre-EVH: 46.4 + 15.1 pg/ml; $p = 0.024$; Δ, 9.2 ± 13.4 pg/ml; 95% CI, 1.6—3.9 pg/ml; post-EVH: 61.1 ± 15.4 pg/ml; $p = 0.043$; Δ, 7.3 ± 13.2 pg/ml; 95% CI, 0.13—17.6 pg/ml) and placebo (pre-EVH: 49.3 ± 15.5 pg/ml; $p = 0.001$; Δ, 12.1 ± 10.4 pg/ml; 95%

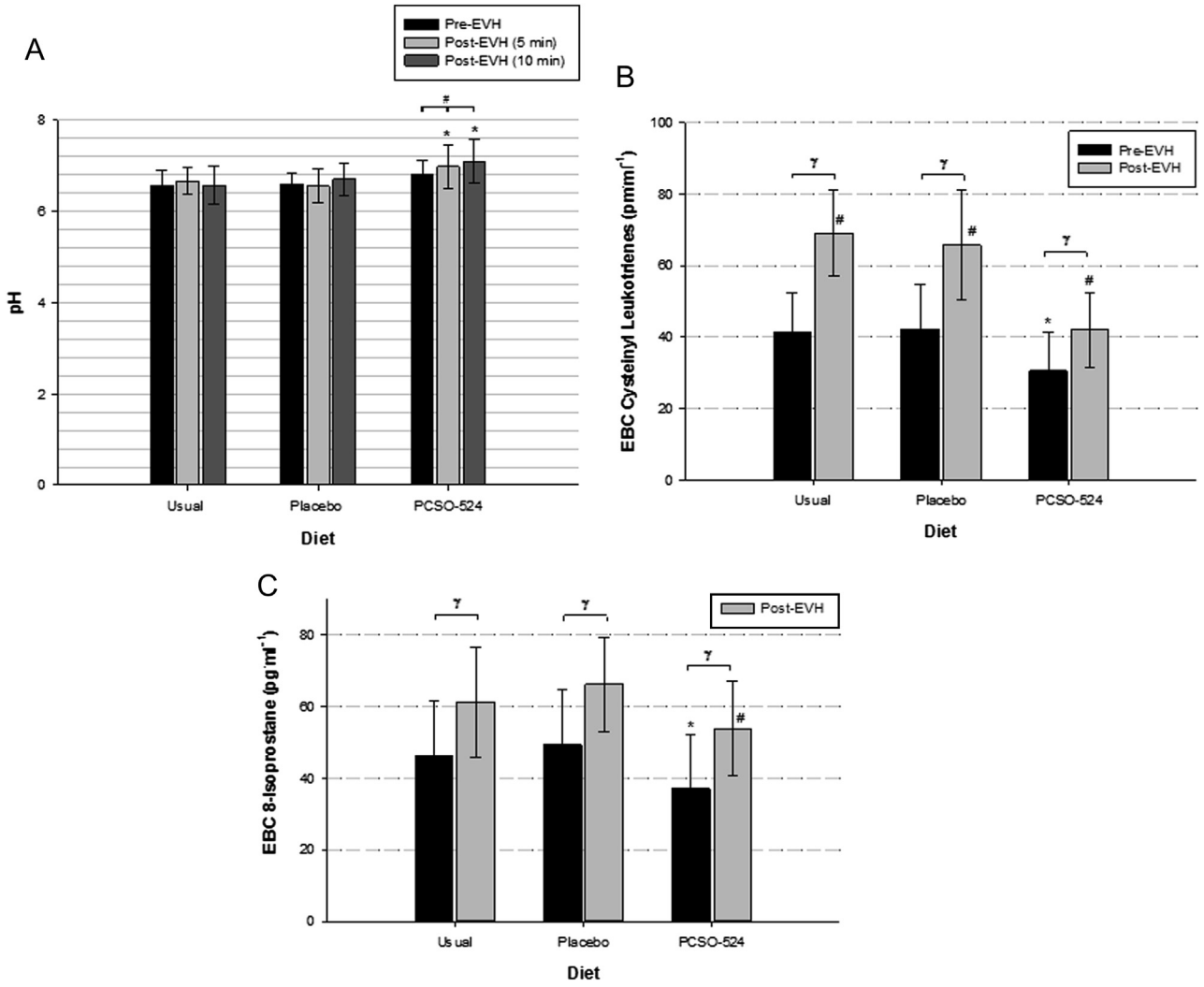

Figure 4 Panel A. Mean exhaled breathe condensate (EBC) pH. # designates a statistical difference ($p < 0.05$) from pre-EVH value within diet. * designates a statistical difference ($p < 0.05$) compared to respective time point between diet. Panel B. Mean exhaled breathe condensate (EBC) cysteinyl-leukotriene concentration (pg mg^{-1}). *, # designates a statistical difference ($p < 0.05$) compared to respective time point between diet. γ designates a significant difference ($p < 0.05$) from pre-EVH value within diet. Panel C. Mean exhaled breathe condensate (EBC) 8-isoprostane concentration (pg mg^{-1}). *, # designates a statistical difference ($p < 0.05$) compared to respective time point between diet. γ designates a significant difference ($p < 0.05$) from pre-EVH value within diet.

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CI, 7.2–21.2 pg/ml; post-EVH: 66.0 + 13.1 pg/ml; $p = 0.040$; Δ, 12.2 ± 12.9 pg/ml; 95% CI, 0.63–20.5 pg/ml) diet (Fig. 4, Panel C).

The mean urinary 9α, 11β PGF_2 levels for pre- and post-EVH was significantly mitigated on the PCSO-524™ diet (pre-EVH: 18.8 ± 8.1 ng/mg creatinine; post-EVH: 31.0 ± 15.4 ng/mg creatinine) compared to the usual (pre-EVH: 23.6 ± 11.9 ng/mg creatinine; $p = 0.036$; Δ, 4.8 + 8.3 ng/mg creatinine; 95% CI, 0.36–9.1 ng/mg creatinine; post-EVH: 36.3 ± 13.6 ng/mg creatinine; $p = 0.041$; Δ, 5.3 ± 11.7 ng/mg creatinine; 95% CI, 0.2–11.5) and placebo (pre-EVH: 26.2 ± 13.8 ng/mg creatinine; $p = 0.008$; Δ, 7.4 ± 9.8 ng/mg creatinine; 95% CI, 2.2–12.5 ng/mg creatinine; post-EVH: 41.2 ± 20.1 ng/mg creatinine; $p = 0.003$; Δ, 10.2 ± 11.6 ng/mg creatinine; 95% CI, 3.9–16.3 ng/mg creatinine) diet (Fig. 5, Panel A).

Mean urinary CC16 concentration pre- and post-EVH decreased significantly on the PCSO-524™ diet (pre-EVH: 0.054 ± 0.047 ng/μmol creatinine; post-EVH: 0.69 ± 0.49 ng/μmol creatinine) compared to the usual (pre-EVH: 0.11 ± 0.11 ng/μmol creatinine; $p = 0.048$; Δ, 0.056 + 0.100 ng/μmol creatinine; 95% CI, 0.0005–0.107 ng/μmol creatinine; post-EVH: 1.04 ± 0.45 ng/μmol creatinine; $p = 0.017$; Δ, 0.35 ± 0.51 ng/μmol creatinine; 95% CI, 0.07–0.6) and placebo (pre-EVH: 0.14 ± 0.18 ng/μmol creatinine; $p = 0.048$; Δ, 0.086 ± 0.16 ng/μmol creatinine; 95% CI, 0.0003 to 0.172; post-EVH: 0.98 + 0.44 ng/μmol creatinine; $p = 0.043$; Δ, 0.29 + 0.51 ng/μmol creatinine; 95% CI, 0.011–0.56) diet (Fig. 5, Panel B).

There was no significant difference ($p > 0.05$) between the usual and placebo diets for pre-and post-EVH EBC Cyst-LT and 8-isoprostane, and urinary 9α, 11β PGF_2 and CC16 concentrations.

Nutrient intake and compliance

Subject adherence to the treatment regimens were assured by finding that pill counts at the end of each treatment period reflected that capsules were consumed on a regular basis. Compliance as estimated from return-tablet count was high (median, 99%). Though the usual diet was expected to vary between and among subjects, mean daily nutrient intake of the subject's diets did not differ significantly ($p < 0.05$) between the treatment regimens.

Discussion

This double-blind, randomized, crossover placebo-controlled study has shown that a diet supplemented with PCSO-524™ (Lyprinol®/OmegaXL®) a patented extract of stabilized lipids from the NZGLM, *P. canaliculus*, attenuates airway inflammation, and the bronchoconstrictor response to dry gas hyperpnea, and can reduce bronchodilator use and asthma symptom scores in asthmatic subjects. The PCSO-524™ diet significantly reduced the severity of HIB as measured by AUC_{0-20}, and significantly reduced the maximal fall in post-EVH FEV_1 by approximately 57%. The degree of protection provided by PCSO-524™ on HIB in the present study is similar in magnitude to previous reports showing that 3 weeks of fish oil, rich in n-3 PUFA, reduced the maximum fall in FEV_1 post-exercise by almost 80%[10] and 64%[7] in elite athletes and asthmatic subjects with EIB respectively, and reduced the maximum fall in post-EVH FEV_1 by approximately 49% in asthmatic subjects with HIB.[9]

In the present study the PCSO-524™ diet significantly reduced the pre-EVH F_ENO compared to the placebo and usual diet, suggestive of amelioration in baseline airway inflammation; this finding supports previous observations that n-3 PUFA supplementation can moderate baseline airway inflammation in asthmatics.[9,33] In addition, the present study demonstrated that the PCSO-524™ diet can reduce post-EVH F_ENO values compared to the usual and placebo diets. It has been shown that F_ENO is an indirect marker of asthmatic airway inflammation,[34] and that a relationship exists between F_ENO levels and EIB.[35]

It has been shown that airway pH is a significant determining factor of expired F_ENO and airway inflammation,

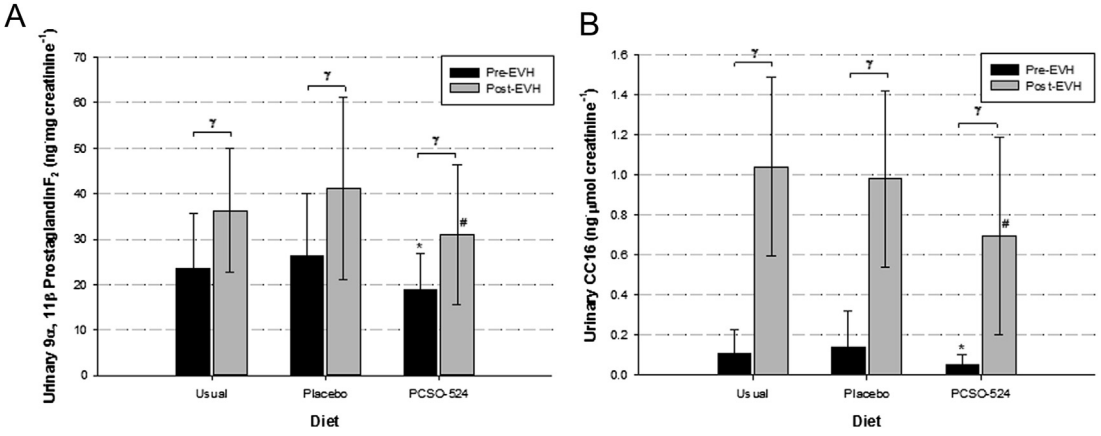

Figure 5 Panel A. Mean urinary 9α, 11β-prostaglandin F_2 concentration (ng mg mmol creatinine^{-1}). *, # designates a statistical difference ($p < 0.05$) compared to respective time point between diet. γ designates a significant difference ($p < 0.05$) from pre-EVH value within diet. Panel B. Mean urinary CC16 concentration (ng μmol creatinine^{-1}). *, # designates a statistical difference ($p < 0.05$) compared to respective time point between diet. γ designates a significant difference ($p < 0.05$) from pre-EVH value within diet.

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and thus there may be a causal relationship between airway acidification and airflow limitation in asthma.[36] Airway pH appears to be lower in asthmatics and correlates positively with sputum eosinophilia, total nitrate/nitrite, and oxidative stress.[37] While airway acidity both accelerates human eosinophil necrosis and can cause the conversion of endogenous nitrate (NO_2^-) to nitric oxide (NO), the acidic airway breath condensate pH in asthma tends to normalize with anti-inflammatory therapy,[36] which supports the findings of the present study, and our previous observation,[9] that a diet supplemented with n-3 PUFA can alkalize airway (EBC) pH in asthmatics.

Our finding that the PCSO-524™ diet significantly decreased EBC Cyst-LT concentration supports previous studies that have shown that n-3 PUFA supplementation can attenuate Cyst-LT levels in urine[10] and induced sputum following exercise,[7] and in EBC following dry gas hyperpnea,[9] compared to a usual (normal) diet in subjects with EIB. Studies have shown a sustained increase in Cyst-LTs and other bronchoconstrictve eicosanoids, such as PGD_2, in the airways after an exercise challenge,[4,7,38] and these markers have been shown to be elevated for up to 6 h after an exercise challenge in asthmatic subjects with EIB.[4] Eosinophils, mast cells and basophils can directly synthesis Cyst-LTs, which can cause tissue edema, stimulate airway secretions, promote cell cycling and proliferation of airway smooth muscle, and may directly increase eosinophilic inflammation.[22] It has been shown that in some patient populations, the amount of eosinophilia in induced sputum is correlated with EIB severity.[39]

The initial rate-limiting step in the formation of Cyst-LTs and other eicosanoids is the release of arachidonic acid from membrane phospholipids regulated by phospholipase (PL) A_2 enzymes.[40] It has been shown that secreted phospholipase A_2 group X ($sPLA_2$-X) is elevated in induced sputum cells of asthmatic patients with EIB,[40] and increased in the BAL fluid of asthmatics in association with lung function and eicosanoid formation.[41] It has been shown that the major source of $sPLA_2$-X is the airway epithelium,[41] and that the effects of exogenous $sPLA_2$-X on human eosinophils can rapidly initiate Cyst-LT formation in eosinophils.[42] These findings[40] suggest that $sPLA_2$-X may serve as a significant regulator of airway eicosanoid formation and that this enzyme is strongly implicated in the pathophysiology of EIB/HIB.

The present study has shown that PCSO-524™ can significantly attenuate the increased urinary levels of CC16 observed prior to, and following, dry gas hyperpnea on the placebo and control diet. CC16 is a low-molecular-weight (16 kDa) protein, secreted in large amounts into the lumen of the respiratory tract by nonciliated bronchiolar Clara cells. Following its passage into the bloodstream across the air-blood barrier, CC16 is rapidly eliminated by glomerular filtration. In humans, CC16 in extrapulmonary fluid has been used as indirect marker of lung epithelial cell damage/dysfunction,[25,30] and it has been suggested that CC16 may play a protective role in response to epithelial stress, such as to protect the airway from dysfunction due to dehydration of the epithelium[43]; most likely via inhibition of PLA_2 and the subsequent suppression of inflammatory eicosanoids.[25] Studies[25,30] have shown that epithelial stress, as shown by increases in urinary CC16 levels, occurs during exercise *in vivo* in elite swimmers, and following an isocapnic hyperpnea challenge in trained and untrained individuals, with and without EIB.

In the present study the PCSO-524™ diet significantly blunted the increase of urinary 9α, 11β-PGF2 prior to and following the dry gas hyperpnea challenge, which confirms previous findings of attenuated urinary 9α, 11β-PGF_2 levels following 3 weeks n-3 PUFA supplementation in elite athletes with EIB[10] and asthmatics with HIB.[9] 9α, 11β-PGF_2, the urinary metabolite of PGD_2, is a sensitive marker of mast cell activation in the airways and a potent bronchoconstrictor,[24] and has been shown to be increased after allergen-induced bronchoconstriction,[44] EIB,[24] mannitol-induced bronchoconstriction[45] and hyperpnea-induced bronchoconstriction.[21]

In the present study EBC 8-isoprostanne levels were reduced prior to, and following, the dry gas hyperpnea challenge on the PCSO-524™ diet compared to the placebo and control diet.

Oxidative damage to lipids (lipid peroxidation) leads to the production of 8-isoprostane, an F_2 isoprostane, formed nonenzymatically by oxidation of arachidonic acid independent of cyclooxygenase (COX) action, and considered a reliable marker of oxidative stress because it is structurally stable and are produced *in vivo*.[46] Levels of 8-isoporstane in EBC are increased in adults[23] and children with asthma,[47] and have been shown to be increased in EBC of asthmatic patients with EIB, as well as correlated with the severity of EIB.[48]

Although previous studies have shown that fish oil supplementation provides a protective effect against EIB[7,10] and HIB[9] as a result of its anti-inflammatory properties, the present study is the first to show that PCSO-524™, a different type of marine oil derived from the shellfish *P. canaliculus* (NZGLM) is similarly effective as fish oil in attenuating airway inflammation and HIB in asthmatic subjects.[9]

While PCSO-524™ has been shown to be effective in treating osteoarthritis, rheumatoid arthritis, and inflammatory bowel disease,[49] only a limited number of studies have examined the efficacy of PCSO-524™ in human asthma[19,50] and animal models of asthma.[20] Emelyanov et al.[19] demonstrated in 46 patients with atopic asthma that PCSO-524™ (Lyprinol®) [two capsules taken twice daily for 8 weeks; each capsule containing 50 mg n-3 PUFA and 100 mg olive oil], compared to placebo, reduced daytime wheeze and exhaled hydrogen peroxide (marker of airway inflammation) and increased morning peak expiratory flow, but did not improve night awakenings or reduce the use of β_2-agonists or forced expiratory volume in 1-sec (FEV_1). Recently, Lello et al.[50] studied the use of PCSO-524™ (Lyprinol®) in 71 children with moderate chronic persistent asthma taking regular inhaled corticosteroids (ICS). These authors[50] found that 16 weeks of PCSO-524™ (Lyprinol®) supplementation improved the percentage of children reporting slight to no problems with their asthma at 3 months of supplementation, and fewer mild and moderate asthma exacerbations overall in the PCSO-524™ (Lyprinol®) group. Wood et al.[20] assessed the effects of 14 days of fish oil and PCSO-524™ (Lyprinol®) supplementation on allergic inflammation and lung function using a mouse model of ovalbumin (OVA)-induced allergic airway disease (AAD).

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The PCSO-524™ (Lyprinol®) diet, but not the fish oil diet, reduced eosinophil influx into the bronchoalveolar lavage fluid, lung tissue and blood, decreased mucus hypersecretion in the lung and attenuated airway hyperresponsiveness (AHR). However, the effects seen on the PCSO-524™ (Lyprinol®) diet were not associated with changes in IgG1 and IgG2a, or the release of the cytokines IL-4, IL-5, IL-13 and IFN-γ.

PCSO-524™ is a mixture of the five main lipid classes including sterol esters, triglycerides, free fatty acids, sterols and polar lipids.[51] PCSO-524™ contains approximately 13% EPA, 21% DHA, 30% cholesterol and up to 91 fatty acid components such as 5,9,12,15-octodecatretraenoic acid, 5,9,12,16-nondecatertraenoic acid, 7,11,14,17-eicosatetra enoic acid, 5,9,12,15,18-heneicsapententaenoic acid, myristic acid, palmitic acid, palmitoleic acid, stearic acid and oleic acid.[51] PCSO-524™ has been shown to reduce the pro-inflammatory LTB_4 in human monocytes,[17] inhibit the formation of 5-HETE (products from the lipoxygenase pathway,[52] attenuate the formation of LTB_4 and 5-HETE from human neutrophils,[16] directly inhibit the COX-1 and COX-2 enzymes,[53] and to inhibit IL-1, IL-2, IL-6, TNF-α, IFN-γ synthesis from several LPS-stimulated isolated cell preparations.[54]

In the present study the attenuation of airway inflammation and subsequent improvement in pulmonary function cannot be explained entirely by the EPA and DHA content of PCSO-524™, since the amount of EPA and DHA consumed daily was only 72 mg and 48 mg respectively, which is substantially lower than our previous studies examining the effect of fish oil on EIB/HIB (3.2 g EPA/day and 2.0—2.2 g DHA/day).[7,9,10] Interestingly, Wood et al.[20] have shown that PCSO-524™, but not fish oil, provides a protective effect against eosinophilic inflammation, mucus production, TH2 cytokine responses in the lungs and airways, and airway hyperresponsiveness, in a murine model of AAD. In addition, Whitehouse et al.[52] showed that the lipid-rich oil of PCSO-524™ showed potent anti-inflammatory activity in rat paw edema assays at a concentration two orders of magnitude lower than fish oil containing abundant EPA, while Tenikoff et al.[55] has shown that PCSO-524™ is more effective than fish oil in reducing symptoms of experimentally-induced inflammatory bowel disease, which suggests that the potent anti-inflammatory effect of PCSO-524™ may not be due solely to the EPA and DHA content, since in these two studies the n-3 PUFA content of PCSO-524™ compared to fish oil is significantly lower. Therefore, it is possible that additional constituents of PCSO-524™, which may act synergistically with the n-3 PUFAs, may also be partially responsible for its anti-inflammatory effects. PCSO-524™ is rich in anti-inflammatory polyphenols (oleuropein and hydroxtyrosol) and oleic acid (18:1n-9), which are postulated to reduce risk factors for heart disease, lower cancer mortality, and reduce inflammation.[55] It is also possible that the anti-oxidant component (D-α-tocopherol) of PCSO-524™ may be required to protect cell membranes from an increased susceptibility of oxidation which occurs with incorporation of n-3 PUFAs.[56] Interestingly, Wakimoto et al.[57] have shown that furan fatty acids, which are a minor component of PCSO-524™, exhibit more potent anti-inflammatory activity than EPA in a rat model of adjuvant-induced arthritis, and may explain, at least in part, why in the present study PCSO-524™ was effective in attenuating airway inflammation, given the very low dose of EPA and DHA.

In conclusion, the present study has shown that a lipid extract of NZGLM (PCSO-524™) attenuates airway inflammation and provides protection against HIB in asthmatic subjects. This study supports data from previous studies[19,20] that PCSO-524™ may have beneficial effects in HIB/asthma, by serving as a pro-resolving agonist and/or inflammatory antagonist. Further studies are required to determine the minimum effective dose needed to attenuate HIB/asthma, and to determine the bioactive components of PCSO-524™ responsible for its anti-inflammatory effect in asthmatic airways.

Authorship

All authors fulfilled conditions of authorship: (1) substantial contributions to conception and design of the study, acquisition of data, or analysis and interpretation of data; (2) drafting the manuscript or revising it critically for important intellectual content; and (3) final approval for the version to be published.

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Conflict of interest

The authors declare no conflicts of interest.

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