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Rosuvastatin, lycopene and omega-3 fatty acids: A potential treatment for systemic inflammation in COPD; a pilot study

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ABSTRACT

Background/Aims: Chronic Obstructive Pulmonary Disease (COPD) is characterized by airway inflammation, in which contributes to loss of lung function. Systemic inflammation is also a feature of COPD contributing to many associated co-morbidities. Statins, omega-3 fatty acids (docosahexanoic acid, DHA and eicosapentanoic acid, EPA) and lycopene have been shown to decrease systemic inflammation; however their combined effects have not been investigated. This study aims to identify changes in systemic and airway inflammation induced by statins alone or in combination with DHA, EPA and lycopene in COPD.

Methods: COPD patients (n = 11) received rosuvastatin (20 mg/day) for 4 weeks, then a combination of rosuvastatin (20 mg/day), DHA and EPA (1.5 g/day) and lycopene (45 mg/day) for 8 weeks. Blood and sputum were collected and lung function measured by spirometry at baseline, week 4 and 12. Plasma fatty acids were measured using gas chromatography, while plasma carotenoids were analysed using high-performance liquid chromatography. Plasma CRP and IL-6 concentrations were measured using ELISA; and peripheral blood gene expression was measured using the nCounter™ GX Human Inflammation Kit 2.

Results: Following the interventions, clinical characteristics and plasma IL-6 and CRP were unchanged. Sputum neutrophil proportion and absolute count was increased and macrophage proportion decreased by rosuvastatin (P = 0.020 and P = 0.015; respectively). Rosuvastatin increased *LTB4R* and decreased *CXCL10* and *AGER* gene expression in white blood cells. The addition of lycopene and omega-3 fatty acids decreased *LTB4R* and increased *CXCL10* to basal levels, whilst combined use of interventions increased *ALOX15* blood gene expression.

Conclusion: This study shows that rosuvastatin, omega-3 fatty acids and lycopene have some anti-inflammatory effects systemically, but rosuvastatin may increase airway neutrophils, which would be undesirable in COPD patients, warranting further investigation.

1. Introduction

Chronic obstructive pulmonary disease (COPD) is currently suffered by 1.2 million Australians and is expected to rise to 2.6 million by the year 2050 [1]. COPD is primarily caused by smoking and is most prevalent in the elderly population [2]. It is characterized by airway inflammation, resulting in small airway remodelling as well as destruction of lung parenchyma, fibrosis of airways and loss of lung elasticity resulting in irreversible airflow obstruction [3–5]. Systemic inflammation and oxidative stress are also key features of COPD [6,7].

Systemic inflammation involves elevated acute phase proteins, including C-reactive protein (CRP), serum amyloid A (SAA) and surfactant protein (SP)-D [8–10], and inflammatory mediators including interleukin (IL)-6, IL-1β and tumor necrosis factor (TNF)-α [11]. Systemic inflammation is thought to be the cause of many of the co-morbidities that are related to COPD [12], such as cardiovascular disease, diabetes and cachexia [13]. Oxidative stress also occurs in COPD, as reactive oxygen species (ROS) are produced by activated inflammatory cells in both the airways and circulation

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and cause damage to lipids, proteins and DNA [7]. Systemic markers of oxidative stress that are elevated in COPD, include malondialdehyde (MDA), 8-isoprostane, protein carbonyls and oxidised low-density lipoprotein (oxLDL) [14–16]. COPD subjects have also been found to generally have lower circulating levels of antioxidants, which is an indirect marker of oxidative stress [17].

Current therapies for COPD reduce symptoms, decrease frequency and severity of exacerbations as well as increasing exercise capacity [18], yet systemic inflammation and/or oxidative stress persist [19]. Interventions that are prescribed for COPD patients include bronchodilators, corticosteroids, long-term oxygen therapy and pulmonary rehabilitation [18]. Novel therapies targeting systemic inflammation and oxidative stress include 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors or statins, antioxidants and omega-3 fatty acids [20,21]. Previous studies have examined the use of statins, and antioxidants individually in COPD as well as other chronic inflammatory diseases, while omega-3 fatty acids have been investigated for their anti-inflammatory mechanism [20–24].

Statins have anti-inflammatory properties [20,25] and have been shown to reduce markers of systemic inflammation including IL-6, TNF-α and CRP in cardiovascular disease as well as animal models of cigarette smoke exposure [26–28]. Antioxidants such as lycopene, vitamin C and vitamin E have been previously studied in COPD and have been shown to reduce oxidative stress and inflammation [17,21,23,29]. Lycopene has been found to reduce ROS, IL-8 and MMP-9 in THP-1 macrophages exposed to cigarette smoke [30,31] as well as increasing antioxidant enzymes, SOD and catalase, and decreasing inflammatory cytokines IL-6, TNF-α and IL-1β in COPD patients [29]. Omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic (DHA) have been shown to have anti-inflammatory effects through inhibition of the NLRP3 inflammasome [24]. To date there are no studies that have been published on the effects of omega-3 supplementation alone in COPD, however a number of studies have been registered and are currently underway. DHA can decrease inflammatory and oxidative stress markers in mouse models of fatty liver disease and type 2 diabetes [24,32], while the combined use of EPA and DHA in healthy participants decreases inflammatory gene expression in peripheral blood mononuclear cells (PBMCs) [33]. To date, the combined effects of statins, antioxidants and omega-3 fatty acids in COPD have not been investigated. We hypothesised that antioxidants, statins and omega-3 fatty acid supplementation in combination would reduce airway and systemic inflammation in COPD.

2. Materials and methods

2.1. Participants

Thirteen subjects with COPD, aged 35 years and over were recruited. COPD was defined as a doctor's diagnosis as well as having an FEV_1/FVC ratio <2 standard deviations below expected for their age, gender and height. Subjects were ex-smokers with a smoking history of at least 10 pack years and had stopped smoking for at least 12 months prior to commencing the study. Subjects were excluded from the study if they had a respiratory disorder other than COPD, had a moderate exacerbation of COPD in the previous month or a severe exacerbation in the previous 3 months, were receiving long-term oral corticosteroid therapy, were currently taking statins or had any chronic disease other than COPD.

2.2. Intervention

Subjects participated in an interventional study, of sequential design, with subjects given rosuvastatin (20 mg/day) (AstraZeneca, London, England) for the first 4 weeks of the trial. Following this, subjects were then given a combined intervention for 8 weeks consisting of rosuvastatin (20 mg/day), fish oil (3 g/day; EPA 1055.1 mg, DHA 744.9 mg) (Blackmores, balgowlah, Australia) and lycopene (45 mg/day) (LycoRed, Beer Sheva, Israel) as illustrated in Fig. 1. Subjects completed clinic visits at weeks 0, 4 and 12. At every visit, pulmonary function testing and St George's Respiratory Questionnaire [34] were completed, sputum was induced and blood samples were taken. Unused drugs and supplements were collected at each visit to determine adherence with the intervention. Participants with adherence of >80% were included in the analysis, determined by the pill count back method [35].

2.3. Clinical assessment

Pre and post bronchodilator lung function was assessed using standardised spirometry (KoKo Spirometer, Pulmonary Data Service, Instrumentation Inc., Louisville, USA) according to ATS recommendations [36]. Spirometric classification of severity of COPD was done according to GOLD criteria and based on post bronchodilator FEV_1 as recommended by the GOLD guidelines [37].

2.4. Sputum induction and processing

Sputum induction was performed with hypertonic saline (4.5%), however 0.9% saline was substituted for subjects with FEV_1<40%. Participants inhaled nebulised saline at 30sec, 1 min, 2 min and 3 × 4 min intervals for a maximum time of 15.5 min. If FEV_1 fell by ≥ 15% from baseline, subjects were administered salbutamol and the saline challenge was stopped if FEV_1 did not return to within 10% of baseline. Participants were asked to produce a sputum sample which was processed as previously described [38]. For sputum inflammatory cell counts, selected sputum was dispersed using dithiothreitol (DTT) and a total cell count and cell viability were performed. Cytospins were prepared, stained (May-Grunwald Giemsa) and a differential cell count obtained from 400 non-squamous cells.

2.5. Blood cholesterol analysis

Fasting blood cholesterol measurement was carried out by Hunter Area Pathology Service (HAPS). This included total cholesterol, LDL-cholesterol, HDL-cholesterol and triglycerides.

2.6. Carotenoid assessment

High-performance liquid chromatography was used to measure plasma carotenoid concentrations, including lycopene, α- and β-carotene, lutein/zeaxanthin and β-cryptoxanthin [39] as previously described [40]. Ethanol:ethyl acetate (1:1) containing an internal standard (canthaxanthin) and butylated hydroxytoluene was added to the sample. The solution was centrifuged (3000*g*, 48 °C, 5 min) and the supernatant was collected, this was repeated 3 times adding ethyl acetate twice and then hexane to the pellet. Ultrapure water was then added to the pooled supernatant fluid, and the mixture was then centrifuged. The supernatant was then decanted and the solvents were evaporated with nitrogen. The sample was then reconstituted in dichloromethane:methanol (1:2). Chromatography was performed on a hypersil ODS column with a flow rate of 0.3 mL/min, using mobile phase of acetonitrile:dichloromethane:methanol 0.05% ammonium acetate (85:10:5). Carotenoids were detected at 450 nm using a photodiode array.

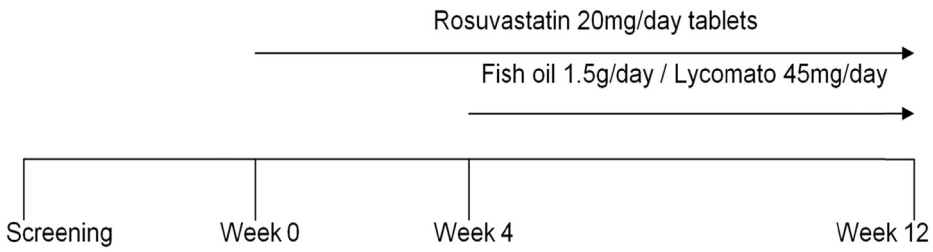

Fig. 1. Study design for the anti-inflammatory intervention. Rosuvastatin (20 mg/day) was given over 12 weeks from week 0 to week 12, while fish oil (1.5 g/day) and lycopene (45 mg/day) were given over 8 weeks from week 4 to week 12 of the study.

2.7. Fatty acid analysis

Total fatty acids were measured by using the method of Lepage and Roy [41] as previously described [42]. A methanol-to-toluene mixture (4:1, by vol), containing C13:0 and C19:0 (0.02 g/L) and BHT (0.12 g/L) was added to plasma. Fatty acids were methylated by adding acetyl chloride drop by drop while mixing by vortex, then heating the sample to 100 °C for 1 h. After cooling, the reaction was stopped by adding 6% K_2CO_3. The sample was centrifuged at 3000*g* for 10 min at 4 °C to facilitate the separation of layers. The upper toluene layer was used for gas chromatography analysis of the fatty acid methyl esters by use of a 30 m × 0.25 mm fused carbon-silica column (DB-225) that was coated with cyanopropylphenyl (J & W Scientific, Folsom, CA). Both the injector and detector port temperatures were set at 250 °C. The oven temperature was initially set at 170 °C for 2 min, was increased 10 °C/min until the temperature of 190 °C was reached and held for 1 min, at which point the temperature was then increased 3 °C/min until the temperature of 220 °C was reached and maintained, giving a total run time of 30 min. A split ratio of 10:1 and an injection volume of 5 mL were used. The chromatograph was equipped with a flame ionization detector, auto-sampler, and auto-detector. Sample fatty acid methyl ester peaks were identified by comparing their retention times with those of a standard mixture of fatty acid methyl esters and were quantified by using a Hewlett Packard 6890 series gas chromatograph with Chemstations software (version A.04.02; Palo Alto, CA) for gas chromatographic analysis.

2.8. Inflammatory mediator analysis

Plasma IL-6 was measured using the Human IL-6 Quantikine HS ELISA Kit (R&D Systems, Minneapolis, Minnesota, USA) as per manufacturer's instructions. Plasma high sensitivity CRP (hsCRP) was measured by Hunter Area Pathology Service from serum mixed with monoclonal antibody-coated polystyrene particles, specific for human CRP (CRP Flex reagent cartridge, Dimension Vista System, Siemens Healthcare Diagnostics Inc. Newark USA).

2.9. Gene expression analysis

RNA was extracted from whole blood using the QIAamp RNA blood mini kit protocol as per manufacturer's instructions (Qiagen, Hilden, Germany). Erythrocyte lysis was performed on EDTA blood and the resulting white blood cell pellet was lysed in buffer RLT (with 0.01% 2-mercaptoethanol) and stored at −80 °C until extraction. The column based RNA extraction was performed using an automated QIAamp RNA blood mini kit protocol (QIAcube, Qiagen, Hilden, Germany) with the addition of an on column DNase digestion. After washing with a reduced volume of RW1 buffer, RNA bound to the QIAamp membrane was treated with DNase I. DNase I was then removed by a second RW1 Buffer wash, washed with RPE Buffer and RNA was eluted. RNA quality and concentration was determined using the Agilent RNA 6000 Nano Assay with the Agilent Bioanalyser 2100. Digital expression of inflammatory genes was measured using the nCounter Human Inflammation Kit Version 2 (NanoString Technologies, Seattle, United States of America), which measures the expression of 255 genes. This consisted of 249 genes which are known to be differentially expressed during inflammation and 6 internal reference genes (CLTC, GAPDH, GUSB, HPRT1, PGK1 and TUBB). Digital gene expression was analysed as per manufacturer's instructions.

2.10. Statistical analysis

Per protocol statistical analyses was performed in STATA IC 11 (STATA corporation, College Station, Texas, United States), using the repeated measures ANOVA with Tukey's post-hoc test (normally distributed data) or Friedman's test with Dunn's post-hoc test (non-parametric data). Gene counts from the nCounter data were first normalised using the geometric means of the housekeeping genes GAPDH, HPRT1 and PGK1 using the nSolver software package provided by NanoString Technologies. The three housekeeping genes were chosen using NormFinder as previously described [43], this was done to determine the most stable housekeeping genes across the samples. Genes that had mean mRNA counts of <20 across all visits were excluded due to low expression levels, genes were also classified as nearing significance if $0.1 > p \geq 0.05$. Parametric data is presented as mean ± standard error of the mean (SEM), while non-parametric data is presented as median (quartile 1, quartile 3). Data was assessed for normality using the D'Agostino-Pearson omibus normality test [44]. Correlations between variables were examined using Pearson's correlation coefficient (normally distributed data) or Spearman's correlation coefficient (non-parametric data). P-values < 0.05 were considered statistically significant.

This was a pilot study aimed at determining the efficacy of these supplements in modifying inflammatory pathways. Hence no primary outcome was nominated. A power calculation using any of the classical markers of systemic inflammation demonstrates the study was underpowered to detect a difference. For example, to detect a 20% change in CRP (0.6 mg/L) with SD = 5 mg/L, we would require $n > 2000$.

2.11. Ethics

Ethics for this study was approved by Hunter New England Health (reference number 11/02/16/3.08). The study was also registered with the Australian New Zealand Clinical Trials Registry (ANZCTR) – Registration number ACTRN12611000165987.

3. Results

13 participants commenced the intervention study and 2 withdrew, hence 11 participants completed the study. One of the withdrawn patients suffered bloating on day 5 of the study and itchiness all over by day 10, while the other withdrew from the study due to muscle aches and pains after visit 2. The clinical characteristics of the 11 subjects who completed the intervention at baseline (visit 1), after statin treatment (visit 2) and after combined statin, lycopene and fish oil treatment (visit 3) are presented in Table 1.

3.1. Clinical characteristics and blood lipids

No clinical characteristics were found to be significantly different between the visits, this included lung function, quality of life, BMI and blood pressure. Total Cholesterol, LDL-Cholesterol and Total Cholesterol/HDL ratio were decreased after statin treatment (Table 1). No additional effect was seen with the nutritional interventions.

3.2. Nutritional biomarkers

The nutritional biomarkers including plasma fatty acids and carotenoids are detailed in Table 2. The supplemented fatty acids, DHA (22:5n-6) and EPA (C20:5n-3) were increased at visit 3 compared to visit 1 and 2. Saturated fatty acids, monounsaturated fatty acids, total polyunsaturated fatty acids and total fatty acids were all found to decrease between visits 1 and 3. Treatment interventions did not significantly change plasma carotenoid levels. However there was a trend for increased lycopene concentration at visit 2 and 3.

3.3. Systemic inflammatory biomarkers

Peripheral blood and induced sputum inflammatory cell counts and plasma IL-6 and CRP levels before and after each intervention are detailed in Table 3. Peripheral blood cell counts remained similar after the interventions, except for red blood cells which decreased at visit 3 following the combined rosuvastatin, lycopene and fish oil intervention (Table 3).

3.4. Airway inflammatory biomarkers

Interestingly, the percentage and absolute counts of sputum neutrophils were increased at visit 2 following rosuvastatin intervention, however this did not reach significance until visit 3 after combined statin, fish oil and lycopene intervention (Table 4, Fig. 2). The percentage of macrophages in sputum were decreased after visit 2 after rosuvastatin intervention but this also did not reach significance until visit 3 following the combined statin, fish oil and lycopene intervention (Table 4).

3.5. Peripheral blood gene expression

Amongst the 255 genes that were measured, there were 5 genes significantly differentially expressed between the visits (Fig. 3), with 15 genes nearing significance (Table 5). *LTB4R* expression significantly increased after statin treatment, however *LTB4R* expression subsequently decreased with the addition of fish oil and lycopene to the treatment regimen. *CXCL10* gene expression significantly decreased after statin treatment, but increased after the addition of fish oil and lycopene (Fig. 3). *AGER* gene expression significantly decreased from visit 1 to visit 2 and *ALOX15* was found to significantly increase from visit 1 to visit 3 (Fig. 3). RIPK2 was also found to be differentially expressed during the study, however

Table 1
Clinical Characteristics and blood lipids at each study visit.

n = 11	Visit 1	Visit 2	Visit 3	P-value
BMI[b]	26.5 (24.9, 27.5)	25.84 (24.98, 27.42)	26.05 (24.84, 27.67)	0.6297
Pre FEV_1%[a]	49.6 ± 4.0	48.64 ± 4.49	47.09 ± 4.49	0.2173
Pre FVC%[a]	78.2 ± 3.8	77.27 ± 3.81	75.82 ± 3.30	0.4430
Pre FEV_1/FVC[a]	0.5 ± 0.0	0.47 ± 0.04	0.49 ± 0.03	0.5070
Post FEV_1%[a]	54.6 ± 4.5	54.55 ± 5.05	52.64 ± 5.06	0.2575
Post FVC%[a]	86.5 ± 4.3	84.18 ± 3.84	84.9 ± 4.19	0.4589
Post FEV_1/FVb*	0.53 (0.37, 0.56)	0.51 (0.37, 0.58)	0.53 (0.43, 0.60)	0.3116
Blood Pressure Systolic (mmHg)[a]	134.6 ± 4.9	136.3 ± 6.32	129.2 ± 5.87	0.3515
Blood Pressure Diastolic (mmHg)[a]	83.3 ± 2.1	80.55 ± 1.88	77.73 ± 2.12	0.1118
Pulse (BPM)[a]	78.3 ± 3.9	76.09 ± 3.06	71.18 ± 4.35	0.1751
St. George Respiratory Questionnaire				
Symptoms[a]	43.9 ± 6.8	37.51 ± 7.57	41.43 ± 6.95	0.4893
Activity[a]	51.6 ± 7.3	50.00 ± 8.43	46.57 ± 7.97	0.3076
Impacts[a]	26.3 ± 6.5	23.7 ± 5.66	25.14 ± 6.86	0.5113
Total[a]	36.9 ± 6.3	34.04 ± 5.85	34.42 ± 6.23	0.2213
Blood Cholesterol				
Total Cholesterol (mmol/L)[b]	5.2 (4.4, 5.4)	3.4 (2.7, 3.8)[c]	3.5 (2.7, 4.0)[c]	0.0010
Triglycerides (mmol/L)[a]	1.56 ± 0.33	1.19 ± 0.17	0.99 ± 0.11	0.0510
LDL-Cholesterol (mmol/L)[b]	3.02 (2.19, 3.33)	1.41 (0.89, 1.77)[c]	1.53 (1.06, 1.92)[c]	<0.001
HDL-Cholesterol (mmol/L)[a]	1.37 ± 0.09	1.47 ± 0.12	1.47 ± 0.09	0.3130
Total Chol/HDL Ratio[b]	3.81 (3.06, 4.56)	2.3 (1.99, 2.65)	2.19 (2.09, 2.53)[c]	<0.001

[a] Data is mean ± SEM.
[b] Data is median (Q1, Q3).
[c] $P < 0.05$ vs V1. 'Pre' and 'Post' refer to lung function testing before and after administration of bronchodilator.

Table 2
Changes in nutritional biomarkers before and after intervention.

n = 11	Visit 1	Visit 2	Visit 3	P-value
Plasma Fatty Acids (mg/L)				
C20:5(n-3) Eicosapentaenoic acid [b]	25.48 (17.36, 41.60)	24.70 (14.21, 38.33)	67.50 (43.01, 97.23)[c,d]	<0.0001
C22:6(n-3) Docosahexaenoic acid [a]	61.49 ± 13.25	51.23 ± 9.01	93.10 ± 17.66[c,d]	0.0013
Total short chanin fatty acids [b]	837.2 (609.7, 1330.0)	743.5 (606.8, 1037.0)	691.8 (520.9, 1027.0)[c]	0.0179
Total monounsaturated fatty acids (n-3) [b]	729.7 (496.9, 994.2)	650.8 (510.0, 863.6)	564.0 (402.3, 813.1)[d]	0.0456
Total polyunsaturated fatty acids (n-6) [b]	916.8 (768.8, 1362.0)	749.0 (615.6, 1066.0)[c]	742.7 (653.2, 1187.0)[c]	0.0063
Total Fatty Acids [b]	2461.0 (1875.0, 3904.0)	2155.0 (1798.0, 2841.0)	2031.0 (1579.0, 3028.0)[c]	0.0179
Plasma Carotenoids (mg/L)				
Lutein [b]	0.651 (0.28, 0.84)	0.48 (0.25, 0.85)	0.44 (0.29, 0.87)	0.6854
β-cryptoxanthin [b]	0.07 (0.06, 0.39)	0.11 (0.05, 0.44)	0.13 (0.08, 0.45)	0.6854
Lycopene [b]	0.30 (0.13, 0.54)	0.56 (0.14, 0.77)	0.50 (0.22, 0.96)	0.5690
α-carotene [b]	0.03 (0.01, 0.06)	0.04 (0.01, 0.07)	0.03 (0.0, 0.06)	0.5306
β-carotene [a]	0.25 ± 0.07	0.35 ± 0.09	0.32 ± 0.09	0.2190
Total carotenoids	1.52 ± 0.26	1.73 ± 0.30	1.97 ± 0.48	0.6559

[a] Data is mean ± SEM.
[b] Data is median (Q1, Q3).
[c] $P < 0.05$ vs 1,[d] $P < 0.05$ vs 2.

Table 3
Systemic Inflammation before and after intervention.

n = 11	Visit 1	Visit 2	Visit 3	P-value
IL-6 pg/mL	2.2 (1.62, 3.02)	3.2 (2.3, 5.1)	3.1 (1.6, 4.8)	0.0924
CRP mg/L	3.9 (1.9, 7.9)	3.3 (0.7, 7.6)	3.8 (1.3, 8.9)	0.6862
White Cells (10^9/L)	5.6 (4.4, 6.3)	6.9 (4.3, 9.4)	5.8 (4.7, 6.6)	0.7064
Red Cells (10^{12}/L)	4.6 ± 0.13	4.6 ± 0.12	4.4 ± 0.10[a]	0.0473
Neutrophils (10^9/L)	3.6 (2.5, 4.4)	4.7 (2.5, 5.7)	3.7 (2.6, 4.2)	0.1161
Lymphocytes (10^9/L)	1.6 (1.2, 1.9)	1.7 (1.1, 2.1)	1.7 (1.1, 2.1)	0.6317
Monocytes (10^9/L)	0.5 (0.4, 0.6)	0.6 (0.4, 0.8)	0.6 (0.4, 0.7)	0.1969
Eosinophils (10^9/L)	0.1 (0.1, 0.2)	0.1 (0.1, 0.2)	0.1 (0.1, 0.2)	0.6479

Data is median (Q1, Q3).[a] $P < 0.05$ vs V1.

post-hoc testing could not reveal between which visits.

Correlation analysis between differentially expressed genes and sputum and blood cell counts revealed that peripheral blood *LTB4R* gene expression had a moderate positive correlation with sputum neutrophils at baseline (Fig. 4). Change in *LTB4R* gene expression had a strong positive correlation with changes in sputum neutrophil count (Fig. 4), and a moderate positive correlation with blood neutrophil counts between visits 1 and 3 (Fig. 4).

Change in peripheral blood *AGER* gene expression had a positive correlation with change in sputum neutrophil count, while change in *AGER* gene expression had a positive correlation with change in

Table 4
Airway Inflammation before and after intervention.

n = 11	Visit 1	Visit 2	Visit 3	P-value
TCC (x10^6/mL)	4.5 (1.98, 13.95)	5.94 (2.25, 12.51)	5.4 (3.42, 13.32)	0.1781
Neutrophils (%)	42.9 (24.3, 81.9)	69.9 (60.8, 82.4)	62.8 (46.3, 82.1)[a]	0.0161
Neutrophils (x10^4 cells/ml)	171.5 (99.8, 327.8)	279.5 (243.3, 329.8)	251.0 (185.3, 328.3)[a]	0.0198
Eosinophils (%)	0.75 (0.69, 3.09)	0.5 (0.5, 1.88)	1.13 (0.44, 3.69)	0.3977
Eosinophils (x10^4 cells/ml)	3.0 (1.75, 5.5)	2.0 (2.0, 7.5)	4.5 (1.75, 14.75)	0.3285
Macrophages (%)	50.5 (14.9, 72.4)	25.5 (15.3, 32.5)	29.25 (11.8, 47.4)[a]	0.0153
Macrophages (x10^4 cells/ml)	174.0 (59.5, 248.0)	102.0 (61.3, 130.0)	117.0 (47.3, 189.8)	0.0542
Lymphocytes (%)	1.63 (0, 3.3)	0.88 (0.25, 4.3)	0.13 (0, 1.19)	0.2906
Lymphocytes (x10^4 cells/ml)	6.5 (0, 11.25)	3.5 (1.0, 8.0)	0.5 (0.0, 4.75)	0.2208

Data is median (Q1, Q3).[a] $P < 0.05$ vs V1.

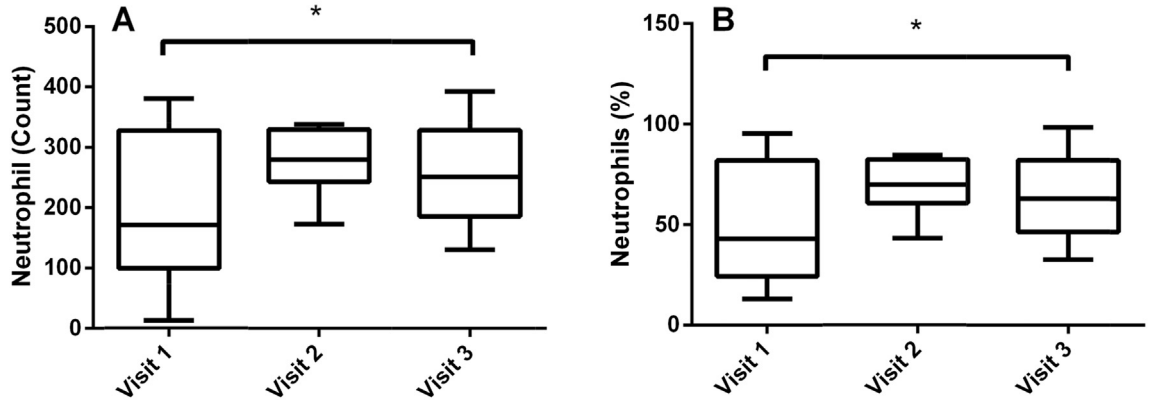

Fig. 2. Neutrophil count and percentage in sputum. Neutrophil counts were performed on sputum samples taken from participants during visits 1, 2 and 3. Graphs show the neutrophil count (A) and percentage of neutrophils (B) in the sputum of the participants over the three visits. Both neutrophil counts and percentage were found to be significantly increased between visits 1 and 3, suggesting that the combined use of statins, fish oil and lycopene increased sputum neutrophils. *$P < 0.005$.

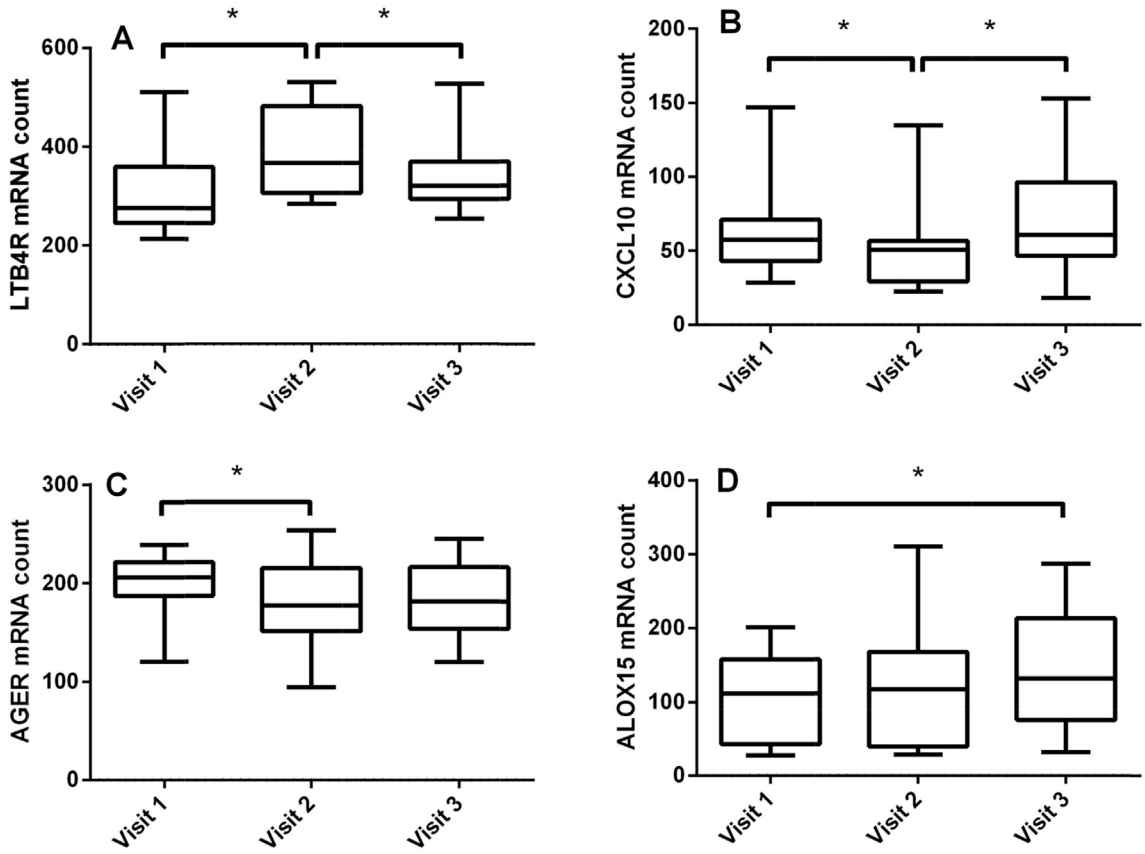

Fig. 3. Genes differently expressed between visits of the intervention study. Graphs showing the mRNA counts of genes found to be changed significantly between visits of the study. The mRNA counts of LTB4R (A), CXCL10 (B), AGER (C), and ALOX15 (D) were determined to be differentially expressed after the treatment interventions. The expression of LTB4R was found to be increased after statin treatment and decreased after the combined use of statins, fish oil and lycopene. CXCL10 expression was decreased by statin treatment and increased after the combined use of statins, fish oil and lycopene. AGER was decreased by the use of statins alone. ALOX15 was found to be increased after the combined use of statins, fish oil and lycopene. *P < 0.005.

Table 5
mRNA counts of genes, before and after intervention, found to be significant or approaching significance.

Gene n = 11	Gene name	Visit 1	Visit 2	Visit 3	P-value	Fold change		
						V1:V2	*V2:V3*	*V1:V3*
LTB4R	*leukotriene B4 receptor*	312.11 ± 22.23	378.38 ± 68.29[a]	329.30 ± 56.75[b]	0.0103	1.25	−1.13	1.09
CXCL10	*Chemokine ligand 10*	68.25 ± 11.72	53.71 ± 27.54[a]	67.67 ± 33.24[b]	0.0176	−1.23	1.30	1.06
AGER	*Advanced glycosylation end product (AGE) receptor.*	201.53 ± 10.62	174.28 ± 51.42[a]	177.11 ± 37.94	0.0216	−1.17	1.05	−1.13
RIPK2	*Receptor-interacting serine-threonine kinase 2.*	340.10 ± 13.11	365.36 ± 45.94	340.43 ± 35.73	0.0377	1.08	−1.07	1.01
ALOX15	*Arachidonate 15-lipoxygenase.*	109.19 ± 20.73	120.51 ± 94.59	146.25 ± 89.90[a]	0.0493	1.06	1.44	1.53
TGFB1	*Transforming growth factor beta 1*	1380.28 ± 34.52	1386.87 ± 115.95	1453.53 ± 97.48	0.0559	1.01	1.05	1.06
PTGDR2	*Prostaglandin D2 receptor 2*	164.08 ± 23.24	155.06 ± 97.72	198.54 ± 96.90	0.0618	−1.08	1.51	1.32
NFE2L2	*Nuclear factor erythroid 2-like 2*	627.90 ± 25.73	699.56 ± 57.08	663.57 ± 65.71	0.0629	1.13	−1.05	1.08
HIF1A	*Hypoxia inducible factor 1, alpha subunit*	2112.10 ± 139.69	2355.18 ± 372.34	2237.20 ± 315.75	0.0639	1.14	−1.04	1.08
TCF4	*Transcription factor 4*	93.85 ± 10.00	81.33 ± 33.48	98.59 ± 40.87	0.0703	−1.14	1.31	1.07
MAP3K7	*Mitogen-activated protein kinase kinase kinase 7*	400.95 ± 13.86	385.08 ± 53.87	416.28 ± 35.53	0.0761	−1.04	1.10	1.04
CCL3	*Chemokine Ligand 3*	214.08 ± 43.46	177.00 ± 108.36	157.82 ± 97.86	0.0828	1.25	1.24	−1.23
HMGB2	*High mobility group box 2*	764.96 ± 36.99	861.08 ± 112.21	800.25 ± 117.19	0.0929	1.14	−1.06	1.06
IFNG	*Interferon Gamma*	56.09 ± 6.07	48.33 ± 13.42	61.23 ± 21.55	0.0929	−1.07	1.31	1.13

Data is mean ± SEM, [a] P < 0.05 Vs 1, [b] P < 0.05 Vs 2; P-value is difference in mRNA counts between visits.

blood neutrophils between visits 1 and 3 (Fig. 4).

Change in *ALOX15* gene expression had a positive correlation with change in blood neutrophils between those visits (Fig. 4).

4. Discussion

This study examined the effect of a novel anti-inflammatory intervention on systemic inflammation in COPD. During the study total cholesterol, LDL-cholesterol and the total cholesterol/HDL ratio decreased with rosuvastatin treatment as well as combined rosuvastatin, lycopene and fish oil intervention. These results are expected with the use of statins and confirm that participants complied with the treatment [45]. Markers of systemic inflammation (plasma IL-6 and CRP) however, were found to be unchanged throughout the course of the study. Previous studies have found that statins have decreased IL-6 and CRP in both rats exposed to

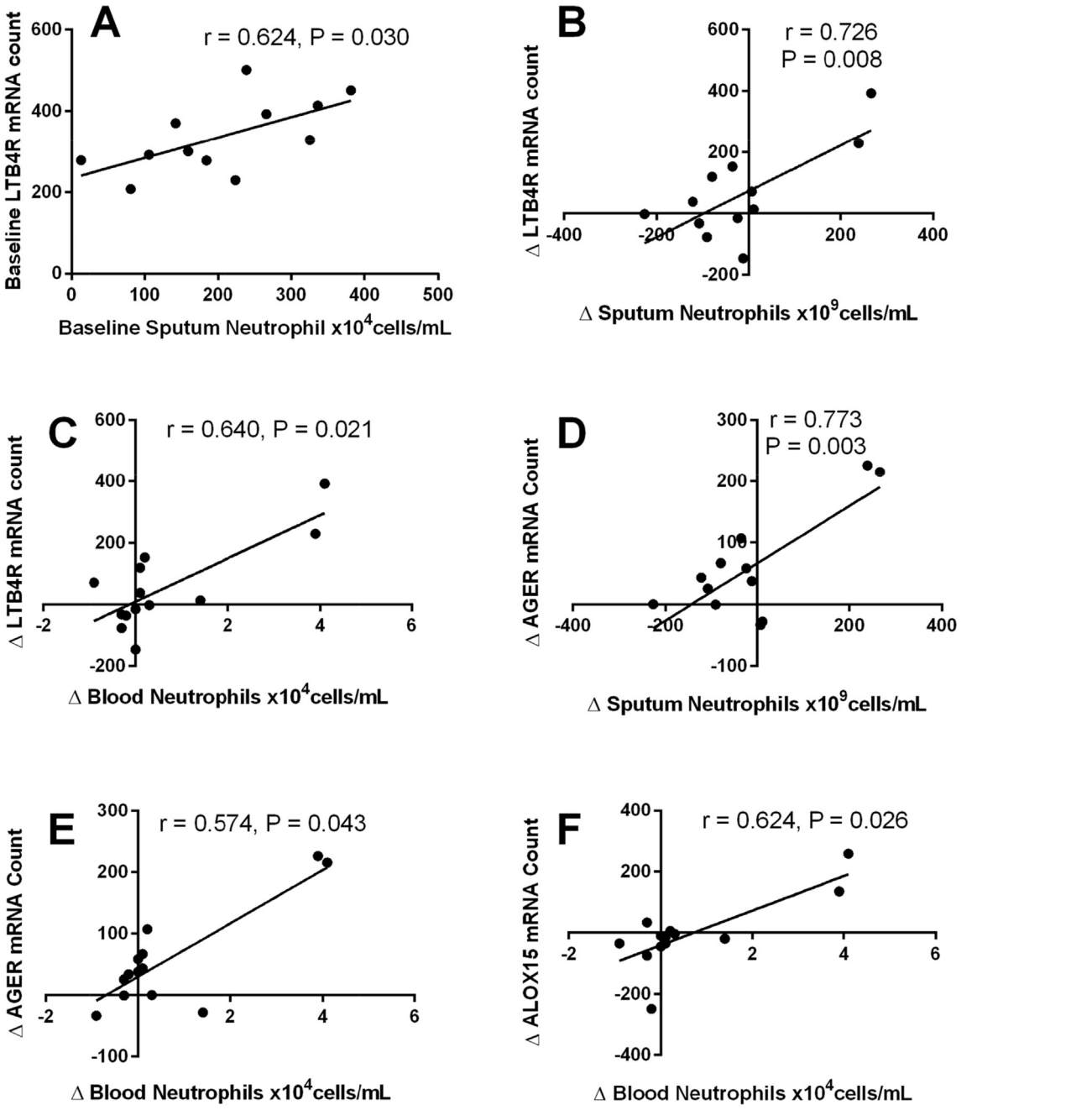

Fig. 4. Graphs of significant correlations between Sputum and blood neutrophil and genes significantly changed by the anti-inflammatory interventions. *LTB4R* gene expression was positively correlated to sputum neutrophil counts at baseline (A), while change in *LTB4R* expression between visits 1 and 3 of the study was positively correlated with changes in sputum (B) and blood (C) neutrophils between visits 1 and 3. Change in *AGER* gene expression was positively correlated with change in sputum (D) and blood (E) neutrophil count between visits 1 and 3, change in *ALOX15* gene expression was also positively correlated to change in blood (F) neutrophils between visits 1 and 3. The correlations of A and B were calculated using Pearson's correlation, while C–F were calculated using Spearman's correlation for nonparametric data.

tobacco smoke and in humans with Cardiovascular disease (CVD) [27,28,46]. Previous studies have also reported that lycopene supplementation (20 mg/day, 4 months) reduced plasma IL-6 in COPD [29] and lycopene (15 mg/day for 8 weeks) reduced plasma CRP in healthy men [47]. These previous reports do not align with the results of this study, which suggests that neither statins, nor the combined use of statins, fish oil and lycopene lower systemic inflammation. While the current study may have been too short in duration or underpowered to detect a change in classical markers of systemic inflammation, our data provides no convincing evidence of a systemic anti-inflammatory effect.

In the current study sputum neutrophils were increased, and sputum macrophages decreased after the combined rosuvastatin, lycopene and fish oil treatment. The increase in sputum neutrophils was seen with 4 weeks of rosuvastatin alone, but did not reach statistical significance until the end of the combined intervention. A previous study on patients with atopic asthma also found that giving atorvastatin (40 mg/day) along with inhaled corticosteroids increased neutrophils and decreased macrophages in sputum when compared to the placebo group [48]. Other *in vitro* studies however, have found that the use of pravastatin, simvastatin and atorvastatin on neutrophils isolated from blood impairs their ability to migrate

[49]. Lycopene has previously been found to reduce sputum neutrophils in non-eosinophilic asthma [50], and neutrophils found in bronchoalveolar lavage fluid of mice with ovalbumin induced asthma [51]. Similarly, a number of previous studies have found that the use of fish oil attenuates neutrophil function, including chemotaxis [52,53]. Hence it appears likely that the increased neutrophils observed after intervention in our study are due to the rosuvastatin treatment. The clinical importance of this observation requires further investigation, as an ongoing increase in neutrophilic inflammation in the airways of COPD patients may have adverse consequences in those taking rosuvastatin.

5 genes were significantly changed between the three visits of the intervention study. These genes included *LTB4R*, *CXCL10*, *AGER*, *RIPK2* and *ALOX15*. *LTB4R* is the receptor for leukotriene B4, and acts as a neutrophil chemoattractant [54]. During the study *LTB4R* was increased by statins, however the combined use of statins, fish oil and antioxidants lowered *LTB4R* back to basal levels. No previous studies have looked at *LTB4R* expression with statin treatments, but a previous study has shown high doses of atorvastatin had no effect on LTB_4 protein levels [55], while another study found that atorvastatin inhibited the 5-lipoxygenase pathway involved in LTB_4 production [56]. Fish oil has been shown to reduce production of LTB_4, via substitution of DHA and EPA for arachidonic acid in cell membranes, which results in production of LTB_5 instead of LTB_4 [57,58]. Our study suggests that rosuvastatin increases the expression of LTB_4 receptor, thus stimulating an increase in neutrophils, while the addition of fish oil may have subsequently led to a decrease in LTB_4 gene expression, back to baseline levels.

CXCL10, also known as IP-10, is a chemoattractant for activated T-lymphocytes and is produced by neutrophils [59]. *CXCL10* gene expression was decreased with statin treatment, however this returned to basal levels with fish oil and lycopene supplementation. Previous studies have seen that the use of fluvastatin and atorvastatin reduces CXCL10 levels systemically, which supports the results seen in the current study [60,61]. However, it is unknown why addition of lycopene and fish oil reverses this effect, as these nutrients have previously been shown to also reduce CXCL10 [62,63].

Advanced glycation end products (AGE) are produced when reduced sugars react with the amino groups of proteins forming reversible Schiff bases and then amadori products, undergoing complex reactions to become cross-linked fluorescent protein derivatives [64]. AGE receptor (AGER, or RAGE) has been shown to interact with AGEs [65]. AGER is involved in the activation of a number of inflammatory genes, such as NF-κB regulated pathways, as well as being important in a number of chronic inflammatory diseases [66]. *AGER* was found to be significantly decreased after treatment with rosuvastatin. Previous studies have shown that pravastatin and simvastatin can reduce AGER [64,65], as well as reducing NF-κB signalling suggesting a potential anti-inflammatory mechanism of statins [67,68].

Receptor-interacting serine/threonine protein-kinase 2 (RIPK2) is a caspase-recruitment domain (CARD) containing kinase [69], and has previously been implicated in NF-κB signalling and inducing cell death [70]. The results of this study show that statins may increase *RIPK2*, while lycopene and fish oil may decrease its expression. No previous studies have found an association with RIPK2 and the use of statins, lycopene or fish oil. RIPK2 may be involved in the innate immune response of neutrophils. Decreased RIPK2 expression has been shown to result in decreased neutrophil chemokine and cytokine expression [71]. This may explain the differential expression of RIPK2 in this study, and its possible relationship to the increase in airway neutrophils.

ALOX15 encodes the enzyme arachidonate 15-lipoxygenase (15-LO), which metabolizes arachidonic acid to yield peroxidised products that are then reduced, or converted enzymatically, to form 12-hydroxyeicosatetraeonic acid (12-HETE) [72]. 12-HETE is known to be a potent chemoattractant for neutrophils [72]. *ALOX15* expression was increased after the statins, lycopene and fish oil intervention. No previous studies have looked at *ALOX15* expression and the use of these interventions, however one study has seen that DHA may be a substrate for 15-LO forming neuroprotectin D1 (NPD1) [73], which exerts a negative feedback system to control oxidative stress as well as up regulating Bcl-2 anti-apoptotic proteins and inhibition of the production of pro-inflammatory eicosanoids such as prostaglandin [73]. The increase in ALOX15 may also have contributed to the increase in neutrophils seen between visit 1 and 3, as a previous study found that knocking out *ALOX15* reduced neutrophil recruitment in an acute lung injury model in mice [72].

Several correlations were seen between the changes in the expression of genes *LTB4R* and *ALOX15* and the changes in the proportion or count of neutrophils in the airways and blood between baseline and after rosuvastin/combined treatment. These results suggest that the changes in these genes seen in the study may be related to the increase in neutrophils seen between visit 1 and 2/3. This is expected, as both *LTB4R* and *ALOX15* have been previously shown to play an important role in the recruitment of neutrophils [54,72]. As the primary change that we observed in inflammation in this study was an increase in sputum neutrophils, this suggests that the changes in expression of these genes dominated the inflammatory changes in this study.

There are several limitations to this study. In particular, the intervention study was a pilot study with only 11 participants. While we have seen some important effects on neutrophilic airway inflammation and associated gene changes, a larger study cohort is required to determine whether these effects are clinically important, particularly in light of previous studies which have found that the use of simvastatin, lovastatin, atorvastatin and fluvastatin have been associated with reduced decline in lung function in patients with COPD, ex-smokers and current smokers [74,75].

Another limitation of the study is the sequential study design, which does not allow us to determine whether the effects seen between visits 1 and 3 and visit 2 and 3 are from the combined use of rosuvastatin, lycopene and fish oil or whether they are an effect of long term statin use or just an effect of lycopene and fish oil supplementation. Another potential limitation of this study was that patients ranged from mild to severe COPD; this may play a role in the changes seen as the effect of these treatments may vary with disease severity. During the study, plasma DHA and EPA were increased after supplementation, at visit 3 compared to visit 1 and 2. However, plasma lycopene was not significantly changed between any of the visits of the study. It is unknown why this occurred and suggests that either the participants were not adherent, or the bioavailability of the supplement was poor. However, the latter is unlikely, as the bioavailability of the supplement used has been previously confirmed [76,77]. The use of a 0.05 significance level for the gene analysis may also be seen as a potential limitation for this study, as there were a large number of genes assessed for expression. A more conservative p-value could have been used, however this would have reduced the power of the study to detect any changes in gene expression following the interventions due to our small sample size.

In summary, this study has shown that rosuvastatin, fish oil and lycopene may have some anti-inflammatory effects systemically, but rosuvastatin may also have pro-inflammatory effects, leading to increased airway neutrophilic airway inflammation. Further investigation of the effects of rosuvastatin on clinical outcomes in COPD is needed. Investigation of alternative forms of statins is also warranted, as other statin drugs may not have the same undesirable effect on airway inflammation observed with rosuvastatin.

There still remains a need for alternative anti-inflammatory treatments in COPD, hence additional studies of potential treatments is needed.

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