



Linoleic acid-derived lipid mediators increase in a female-dominated subphenotype of COPD

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ABSTRACT Chronic obstructive pulmonary disease (COPD) is a leading cause of mortality; however, the role of inflammatory mediators in its pathobiology remains unclear. The aim of this study was to investigate the influence of gender in COPD on lipid mediator levels.

Bronchoalveolar lavage fluid (BALF) and serum were obtained from healthy never-smokers, smokers and COPD patients (Global Initiative for Chronic Obstructive Lung Disease stage I–II/A–B) (n=114). 94 lipid mediators derived from the cytochrome-P450, lipoxygenase, and cyclooxygenase pathways were analysed by liquid chromatography-mass spectrometry.

Multivariate modelling identified a 9-lipid panel in BALF that classified female smokers with COPD from healthy female smokers ($p=6\times 10^{-6}$). No differences were observed for the corresponding male population ($p=1.0$). These findings were replicated in an independent cohort with 92% accuracy ($p=0.005$). The strongest drivers were the cytochrome P450-derived epoxide products of linoleic acid (leukotoxins) and their corresponding soluble epoxide hydrolase (sEH)-derived products (leukotoxin-diols). These species correlated with lung function ($r=0.87$; $p=0.0009$) and mRNA levels of enzymes putatively involved in their biosynthesis ($r=0.96$; $p=0.003$). Leukotoxin levels correlated with goblet cell abundance ($r=0.72$; $p=0.028$).

These findings suggest a mechanism by which goblet cell-associated cytochrome-P450 and sEH activity produce elevated leukotoxin-diol levels, which play a putative role in the clinical manifestations of COPD in a female-dominated disease sub-phenotype.



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Introduction

Chronic obstructive pulmonary disease (COPD) is a leading cause of morbidity and mortality worldwide [1]. It is characterised by persistent and non-reversible airflow limitation associated with airway inflammation and remodelling [2]. The primary risk factor for COPD in western countries is smoking, with 15–44% of the population developing disease [1]. COPD evidences gender dependency with higher mortality in women, even after correction for smoking [3, 4]. Smoking also results in greater impairment in lung function in women [5, 6], especially post-menopause [7].

COPD is a heterogeneous disease with a variety of inflammatory pathways involved in disease pathophysiology [8]. The current study was designed to investigate the potential role of lipid mediators in the heterogeneity of COPD, with particular focus on examining gender associated differences. Female gender has been suggested as a risk factor in susceptibility to the lung-damaging effects of cigarette smoke, and both smokers and nonsmokers with COPD are more likely to be female [4, 6, 9]. Lipid mediators are known to play an important role in the inflammatory cascade in respiratory disease, including COPD (for review, see [10]) [11–13]. The most well studied of these lipid mediators are the eicosanoids, which are synthesised from arachidonic acid *via* three biosynthetic pathways (figure E1 in the supplementary material): cyclooxygenase (COX), lipoxygenase (LOX), cytochrome P450 (CYP), as well as *via* non-enzymatic oxidation. However, there are numerous lipid species synthesised from other polyunsaturated fatty acids that exhibit biological activity. For example, the linoleic acid-derived leukotoxins (epoxy-octadecenoic acids (EpOMEs)) and leukotoxin-diols (dihydroxy-octadecenoic acids (DiHOMEs)) (figure 1) have been reported to play a role in acute respiratory distress syndrome (ARDS) [14–16] and exert toxicity to alveolar epithelial cells [17]. Accordingly, there is interest in further investigating these lipid mediator pathways in the pathobiology of COPD.

Methods

Subjects and study design

This study examined subjects from the Karolinska COSMIC (Clinical & Systems Medicine Investigations of Smoking-related COPD) cohort at the Karolinska University Hospital (ClinicalTrials.gov identifier NCT02627872). The COSMIC study is a three-group cross-sectional study in which each group was stratified by sex with the aim of investigating the differentiation between the genders in early stage COPD and integrating several aspects of COPD and smoking through the use of imaging, transcriptomics, proteomics, metabolomics, and lymphocyte profiling in the context of clinical phenotypes [18–21]. A total of 40 never-smokers, 40 smokers with normal lung function (hereafter referred to as “smokers”), and 38 patients with COPD were recruited with the intent to collect peripheral blood and bronchoalveolar lavage (BAL). Of the 118 recruited individuals, three individuals with COPD and one never-smoker did not undergo BAL because of clinical constraints and were therefore excluded from the current analysis. The study was accordingly performed on 114 subjects from the Karolinska COSMIC cohort (table 1) matched for age and sex from the groups of healthy never-smokers, smokers with normal lung function and COPD patients with mild-to-moderate disease (Global Initiative for Chronic Obstructive Lung Disease (GOLD) stage I–II/A–B; forced expiratory volume in 1 s (FEV₁) 51–97%; FEV₁/forced expiratory volume (FVC) <70) [18, 20].

Study participants were recruited from individuals performing spirometry during “World Spirometry Day”, through advertisements in the daily press and *via* primary care centres. The majority of the individuals with COPD were smokers who were found to have an obstructive spirometry upon screening. Participants had no history of allergy or asthma, did not use inhaled or oral corticosteroids and had no exacerbations for at least 3 months prior to study inclusion. *In vitro* screenings for the presence of specific immunoglobulin (Ig)E antibodies (Phadiatop; ImmunoCAP, Phadia, Uppsala, Sweden) were negative. Reversibility was tested after inhalation of two doses of 0.25 mg terbutaline (Bricanyl Turbuhaler; AstraZeneca, London, UK). Medications (including oral contraceptives, oestrogen replacement and nonsteroidal anti-inflammatory drugs or other potential lipid mediator-modifying drugs) were recorded by means of a questionnaire. Lung function parameters were calculated as post-bronchodilator percentage

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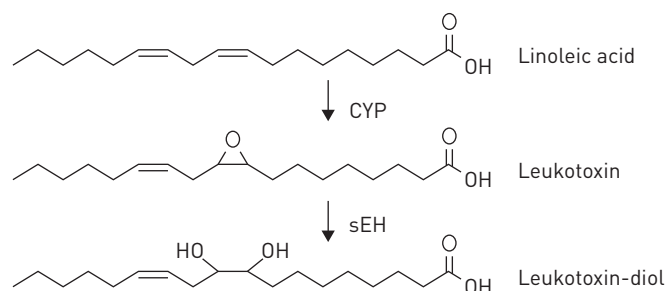


FIGURE 1 Biosynthetic pathway for production of leukotoxin and leukotoxin-diol from linoleic acid. Linoleic acid is converted by cytochrome P450 (CYP) activity to either 9[10]-epoxy-12Z-octadecenoic acid (leukotoxin, coronaric acid, 9[10]-EpOME) or the regioisomer 12[13]-epoxy-12Z-octadecenoic acid (isoleukotoxin, vernolic acid, 12[13]-EpOME), which can be hydrolysed to the corresponding vicinal diols (leukotoxin-diol, 9,10-DiHOME and isoleukotoxin-diol, 12,13-DiHOME, respectively) by soluble epoxide hydrolase (sEH) activity.

predicted using the European Community of Coal and Steel (ECCS) normal values. COPD patients and smokers were matched in terms of smoking history (>10 pack-years) and current smoking habits (>10 cigarettes-day⁻¹ in the past 6 months). The COPD group consisted of both current smokers and ex-smokers (≥ 2 years since smoking cessation). Because the current study focused on mild-to-moderate COPD, there was an overlap between post-bronchodilatory FEV₁ % pred (but not with the FEV₁/FVC ratio) between healthy individuals and participants with COPD; however, in all cases, the current guidelines on COPD were employed when defining study inclusion criteria.

The validation cohort consisted of two gender groups recruited to the same study [22]. Exclusion criteria included a history of asthma or other pulmonary or allergic disease. The participants were clinically stable at the time of the study and were excluded if they had experienced an airway infection during the 14 days prior to study initiation. All medication was withheld 48 h prior to clinical visit and BAL. The female group consisted of seven female current smokers (49–61 years; body-mass index (BMI) 20.5–32.6) with normal lung function and six female current smokers with COPD (48–73 years; BMI 20.0–27.7; GOLD stage II–III; FEV₁ 44–68%; FEV₁/FVC <70) with arterial oxygen saturation (SaO₂) $>90\%$. The male group consisted of nine male current smokers (41–66 years; BMI 21.7–31.4) with normal lung function and five male current smokers with COPD (53–70 years; BMI 17.3–24.6; GOLD stage I–III; FEV₁ 37–99%; FEV₁/FVC <70) with SaO₂ $>90\%$ [22].

Both studies were approved by the Stockholm Regional Ethical Board (COSMIC cohort: No. 2006/959-31/1; validation cohort: No. 2005/733-31/1-4) and participants provided their informed written consent.

Sample collection and preparation

Bronchoalveolar lavage was performed and airway epithelial brushings collected as previously described (see online supplement) [18, 23]. In order to investigate cells involved in first line host defense from both large and small airways, cells from epithelial brushings and bronchoalveolar lavage were chosen. Samples were aliquoted and stored at -80°C until analysis. Blood was drawn between 7–9 AM from fasting individuals by venipuncture and allowed to stand at room temperature for at least 30 min before centrifugation at $1695\times g$ for 10 min at room temperature, and stored at -80°C until use. High-sensitivity C-reactive protein (CRP), platelet and white blood cell counts were measured according to standard methods at the Department of Clinical Chemistry, Karolinska University Hospital. Cytospins prepared from bronchial brushings were fixed with cold acetone, and stained with the alcian blue–periodic acid Schiff (AB-PAS) technique according to standard methods. Differential count of BAL cells was performed by means of Giemsa-Grunwald staining of cytopins.

Lipid mediator analyses

A liquid chromatography-mass spectrometry (LC-MS/MS) method was developed to quantify the reported lipid mediators. The complete method is described in the online supplement, with lipid mediator nomenclature provided in table E1. Briefly, 3.3 mL of BAL fluid (BALF) were mixed with 10 μL of internal standards in concentrations according to table E2 and loaded onto Waters Oasis HLB solid phase extraction (SPE) cartridges (Waters, Milford, MA, USA). SPE cartridges were air-dried, and lipid mediators eluted with organic solvent, evaporated under vacuum and reconstituted in 100 μL of methanol. Following spin filtering, 7.5 μL were injected onto an Acquity ultra performance liquid chromatography with a BEH C18 column (2.1 \times 150 mm, 1.7 μm ; Waters) and analysed on a Waters Xevo TQ-MS in negative mode. The calibration levels and method parameters of all analysed compounds are provided in

TABLE 1 Clinical characteristics of study subjects

	Healthy		Smokers		COPD		COPD ExS	
	Male	Female	Male	Female	Male	Female	Male	Female
Group size n	19	20	20	20	14	11	4	6
GOLD stage								
I	NA	NA	NA	NA	7	6	2	4
II	NA	NA	NA	NA	7	5	2	2
A	NA	NA	NA	NA	10	8	3	4
B	NA	NA	NA	NA	4	3	1	2
Age years	58±7	55±7	54±6	54±7	59±6	60±4	62±6	58±6
BMI kg·m⁻²	27±5	26±4	24±4	24±2	24±4	24±4	27±4	28±5
CRP mg·L⁻¹	1.3±0.91	1.9±1.7	4.4±3.2	2.6±1.1	1.2±0.88	1.9±1.9	1.7±2.2	2.7±2.4
Recovery BAL %	62±14	67±8	55±10	63±9	47±12	40±18	42±12	55±10
Smoking history pack-years	0	0	35±15	35±11	44±11	39±9	28±8	28±9
FEV₁ %	118±14	120±12	109±11	109±13	77±9	82±13	76±19	81±11
FEV₁/VC %	79±9	81±6	76±5	76±7	59±9	60±9	54±17	60±7
FEV₁/FVC %	81±5	81±6	78±5	78±5	61±6	60±6	60±12	64±7
Chronic bronchitis (yes/no) n	0	0	7/13	3/17	2/12	5/6	1/3	1/5
Menopause (pre-/post-) n	NA	6/14	NA	8/12	NA	0/11	NA	0/6
Oestrogen therapy (yes/no) n	NA	1/19	NA	4/16	NA	0/11	NA	1/5
BAL cell concentration ×10⁶·L⁻¹	123±55.9	119±44.4	551±267	561±258	528±267	447±244	107±19.9	125±87.2
BAL viable cells %	93.4±3.85	93.2±4.77	91.6±5.58	91.8±3.32	91.4±3.22	92.2±3.97	94.3±2.06	91.4±5.19
BAL macrophages ×10⁶·L⁻¹	105±46.3	102±35.0	529±258	541±248	505±263	427±237	93.0±15.4	106±81.4
BAL lymphocytes ×10⁶·L⁻¹	16.9±20.1	14.3±15.4	15.4±13.7	12.8±9.02	13.0±10.4	13.4±7.21	10.1±7.56	16.9±8.29
BAL neutrophils ×10⁶·L⁻¹	1.57±1.30	2.21±1.84	4.44±3.04	5.33±6.98	4.71±3.23	5.08±4.97	3.45±2.48	2.33±2.01
BAL eosinophils ×10⁶·L⁻¹	1.04±0.940	0.43±0.33	4.88±4.42	3.00±3.67	6.79±14.3	1.70±1.39	0.0±0.0	0.420±0.380
BALF S-Alb g·L⁻¹	40.4±1.95	39.7±2.08	39.6±1.85	38.7±2.24	37.7±1.33	39.0±2.72	37.7±1.71	40.1±1.95
AB-PAS positive cells %	13.8±12.2	15.4±14.9	22.4±16.8	18.8±12.7	23.2±16.1	27.0±16.1	26.3±14.9	35.0±6.3
Blood platelets ×10⁹·L⁻¹	214±33.7	276±36.8	244±46.4	296±65.4	279±65.9	299±55.0	255±54.1	257±64.9
Blood leukocytes ×10⁹·L⁻¹	5.57±1.18	5.44±0.858	7.87±1.38	7.10±1.86	7.31±1.44	8.25±2.56	6.00±1.04	7.80±2.48

Data are presented as mean±sd, unless otherwise stated. Healthy: healthy never-smokers; smokers: healthy smokers; COPD: chronic obstructive pulmonary disease smokers; COPD ExS: chronic obstructive pulmonary disease ex-smokers; GOLD: Global Initiative for Chronic Obstructive Lung Disease; BMI: body mass index; CRP: C-reactive protein; BAL: bronchoalveolar lavage; FEV₁: forced expiratory volume in 1 s; VC: vital capacity; FVC: forced vital capacity; BALF: BAL fluid; S-Alb: serum albumin; AB-PAS: alcian blue-periodic acid-Schiff. NA: not applicable.

table E2 and table E3, respectively. Isoprostanes were screened *via* LC-MS/MS as previously reported [24]. Cysteinyl leukotrienes were measured by immunoassay as described in the online supplement.

mRNA analysis

mRNA was isolated from BAL cells from a subset of the COSMIC cohort based upon sample availability (n=6 female smokers with COPD, n=20 female smokers with normal lung function). The distribution of the female smokers and COPD patients subjected to mRNA analysis was analysed by principal component analysis (PCA) based upon their full clinical profile. The corresponding scores plot demonstrated that the six samples analysed were representative of the population (data not shown). mRNA was subjected to low input quick amplification, and Cy3-CTP single-color labelling (Agilent Technologies, Santa Clara, CA, USA) and hybridised to Agilent human whole-genome arrays containing 41 000 probes corresponding to 19 596 genes. Microarray datasets were normalised using quantile normalisation with limma in Bioconductor (<https://bioconductor.org>). Statistical analyses were performed on probe intensities from a selected subset of 13 probes representing seven genes (EPHX1, EPHX2, CYP2C, CYP1A2, CYP2J, CYP3A4, and CYP2E1) involved in the biosynthesis of the EpOMEs and DiHOMEs.

Statistical analysis

Statistical analyses were performed by comparing smoking and nonsmoking individuals separately in order to limit the confounding effects of smoking. Univariate statistical analysis was performed by non-parametric Mann-Whitney test (p<0.05). False discovery rate estimations were performed by calculating the Storey q-values using the package “qvalue” from Bioconductor in R 3.2.2. Multivariate statistical modelling was performed using SIMCA version 13.0 (Umetrics, Umeå, Sweden) employing a combination of PCA and orthogonal projections to latent structures (OPLS) models. Only variables detected in ≥75% of the subjects in at least one patient group were included. Analytes present at levels

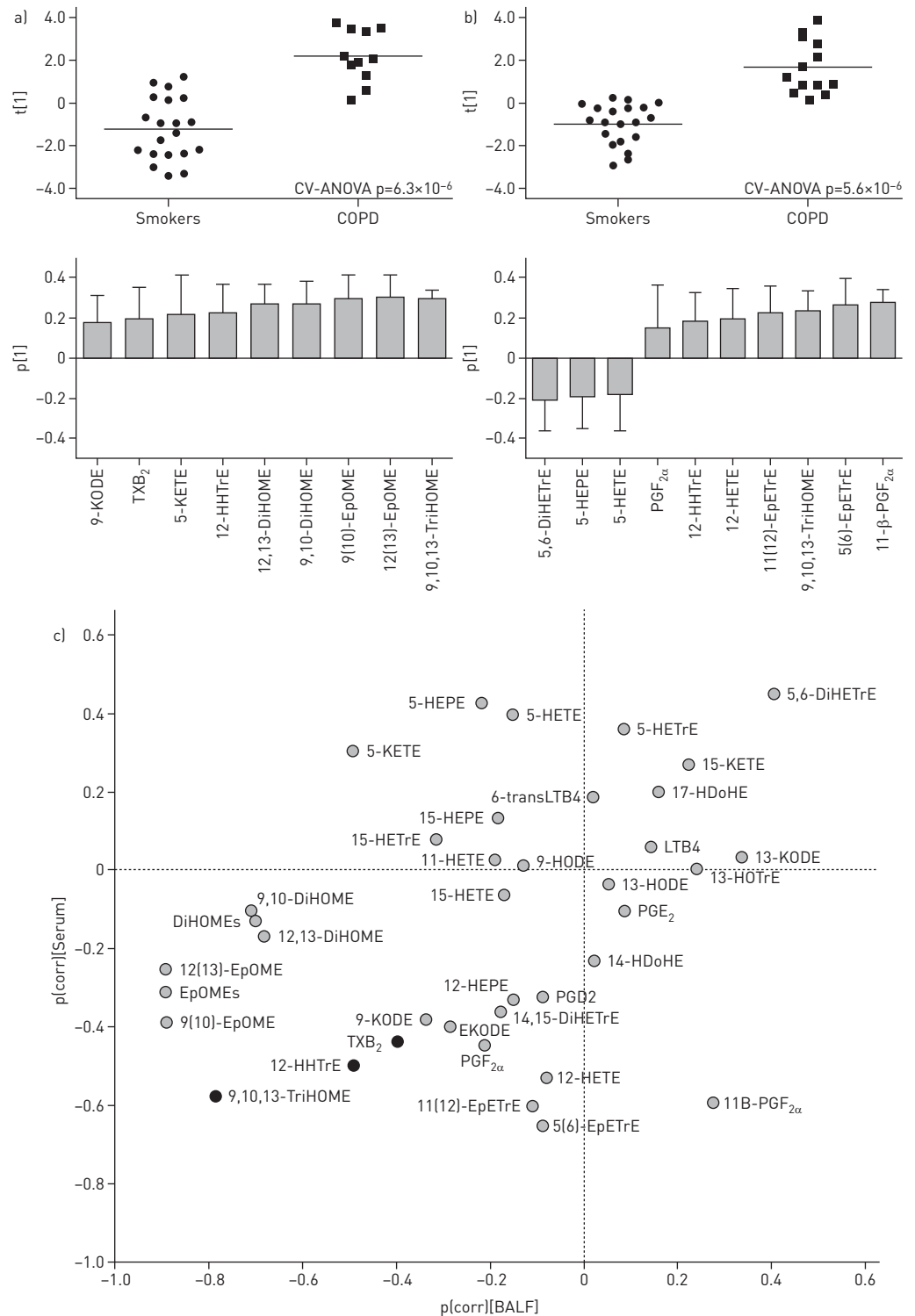


FIGURE 2 Optimised orthogonal projections to latent structures (OPLS) models for female smokers with normal lung function *versus* chronic obstructive pulmonary disease (COPD) patients. a) Bronchoalveolar lavage fluid (BALF) and b) serum. c) Shared and unique structures (SUS) plot for lipid mediators in BALF (x-axis) *versus* serum (y-axis) for females. In the OPLS models (a and b), upper panels display score plots with one predictive component ($t[1]$), and lower panels display loadings ($p[1]$). Smokers with normal lung function (smokers, closed circles), COPD smokers (COPD, closed squares). The corresponding models for the male comparisons were not significant in BALF ($p=1.0$), but did reach significance in serum ($p=0.03$). Lipid mediator nomenclature is provided in table E1 of the supplementary material.

below the limit of detection were set to 25% of limit of detection for multivariate model construction. Data were log transformed, mean centered and scaled to unit variance. Model statistics are reported by the cumulative correlation coefficient (R^2Y), the predictive variance based on seven-fold cross-validation (Q^2), and cross-validated ANOVA p-values for OPLS models. PCA of lipid mediator levels was performed for data quality assessment in BALF and serum (data not shown). Variable selection for OPLS model optimisation was performed iteratively based on the variable influence in projection (VIP) and scaled loadings of the predictive component ($p[corr]$) as previously described [18, 25]. Comparison of two OPLS models was performed using shared and unique structure (SUS) analysis. Correlations between subsets of lipid mediators and clinical variables were evaluated using PLS (SIMCA), reported as the r and p -value of the inner relation.

Results

Cohort description

The clinical characteristics of the subjects are provided in table 1. A total of 94 lipid mediators were screened in BALF using three different analytical methods, with 40 fulfilling the criterion of detection in $\geq 75\%$ of the subjects in at least one group (table E4). In serum, 86 lipid mediators were screened (cysteinyl leukotrienes and isoprostanes were not screened in serum), of which 70 fulfilled the detection criterion (table E5). To minimise the confounding effects of smoking in COPD, the smoking population (smokers with normal lung function (“smoker”) and smoking COPD patients (“COPD”)) was analysed separately from the non-smoking populations (healthy never-smoker subjects (“healthy”) and ex-smoker COPD patients (“COPD ExS”)). No gender differences were observed in the quantified lipid mediators for any of the group comparisons in either BALF or serum ($q > 0.9$, data not shown). The concentrations of BAL immune cells (macrophages, neutrophils, and eosinophils) and blood leukocytes were elevated with smoking (table 1), but not with COPD diagnosis (figure E2). The corresponding decrease in cell counts with smoking cessation in COPD patients was only significant for macrophages and eosinophils (table 1). No gender differences were observed. Blood platelets were higher in females *versus* males in the healthy smoking group, but not with COPD diagnosis (figure E2D).

Multivariate modelling of COPD-related lipid mediators

Supervised multivariate modelling was performed by OPLS analysis of lipid mediator levels in BALF. The initial joint gender model of smokers *versus* COPD resulted in a significant group separation ($R^2Y=0.47$, $Q^2=0.33$, $p=6 \times 10^{-5}$). However, investigating each gender separately revealed that the group separation was driven by the female population. The OPLS model of female Smokers *versus* COPD was optimised *via* variable selection (figure E3) to give a significant robust group separation ($R^2Y=0.59$, $Q^2=0.57$, $p=6 \times 10^{-6}$; figure 2a), while the corresponding analysis on males showed no differences ($p=1.0$). The optimised model consisted of nine lipid mediators: 9,10,13-TriHOME (9,10,13-trihydroxy-11E-octadecenoic acid), 12(13)-EpOME (12[13]epoxy-9Z-octadecenoic acid), 9(10)-EpOME (9[10]-epoxy-12Z-octadecenoic acid), 9,10-DiHOME (9[10]-dihydroxy-12Z-octadecenoic acid), 12,13-DiHOME (12[13]-dihydroxy-12Z-octadecenoic acid), 12-HHTrE (12-hydroxy-5Z,8E,10E-heptadecatrienoic acid), 5-KETE (5-oxo-ETE, 5-oxo-6E,8Z,11Z,14Z-eicosatetraenoic acid), TXB₂ (thromboxane B₂) and 9-KODE (9-oxo-10E,12Z-octadecadienoic acid) (see table E1 for lipid mediator nomenclature). Subjecting the nine-lipid model to permutation tests confirmed that the observed differences did not occur by random (Y-intercept[500 permutations]: $R^2Y=0.05$, $Q^2=-0.24$, figure E4). In addition, no effect of menopause status or oestrogen replacement therapy was observed on the specificity of the nine-lipid panel for female smokers with COPD (figure E5).

In serum, the optimised OPLS model comparing female smokers *versus* COPD yielded a significant group separation ($p=6 \times 10^{-6}$, $R^2Y=0.61$, $Q^2=0.56$; figure 2b). The corresponding male OPLS model also gave a significant group separation ($p=0.03$, data not shown). Both gender models were driven by lower serum-abundances of 5-LOX products and increases in CYP-derived (5[6]-EpETrE, 11[12]-EpETrE) and putative platelet-derived products (12-HETE, 12-HHTrE) in COPD patients (females; figure 2b), and no gender-specific differences were observed by SUS analysis (figure E6).

Univariate analysis of COPD-related lipid mediators

Because the OPLS multivariate model in BALF evidenced strong gender-specificity, it was investigated in greater detail. The members of the 9-lipid panel from figure 2a were examined on an individual basis by univariate statistics (table E6). Correlation analyses were performed between each of the nine lipid mediators with age and pack-years. None of the lipids evidenced a significant correlation, except for 5-KETE, which correlated with pack-years in females ($r=0.42$, $p=0.02$). In both genders, levels of 9,10,13-TriHOME were lower in smokers and then elevated with COPD diagnosis, although the significance of the shifts was greater in females (figure 3a). The 9-KODE levels (figure 3b), 5-KETE levels (figure 3c) as

well as TXB₂ and 12-HHTrE (figure 3d) were significantly greater in female smoking COPD patients compared to non-symptomatic smokers, with no alterations in the corresponding male populations.

The CYP-derived products of linoleic acid evidenced multiple shifts with smoking and COPD (figure 4). Both the EpOMEs (figure 4a) and DiHOMEs (figure 4b) were higher in female COPD patients relative to smokers. Of particular interest is that no differences were observed for the corresponding male populations. For comparison purposes, levels of the CYP-derived products of arachidonic acid, epoxy-eicosatrienoic acids (EpETrEs) and dihydroxy-eicosatrienoic acids (DiHETrEs), were only altered due to smoking, with no alterations between COPD *versus* smokers (figure E7). Accordingly, the gender specificity of the shifts was only associated with the linoleic acid pathway.

The relative level of epoxide formation from linoleic acid was estimated by the sum of EpOMEs and DiHOMEs (figure 4c). The observed trends followed those of EpOMEs: downregulation of linoleic acid epoxide formation in female smokers and higher levels in female COPD patients. In order to estimate the conversion of EpOMEs to DiHOMEs (inferred sEH activity), the ratio DiHOMEs/(EpOMEs+DiHOMEs) in each individual was determined (figure 4d). As expected, the ratio reflected the gender differences observed in the CYP-derived linoleates, although this comparison cannot distinguish increases in substrate availability from differences in the enzymatic activity of sEH.

COPD classification model was validated in an independent cohort

The nine-lipid panel from figure 2a was quantified in BALF from an independent cohort of smokers and COPD patients from both genders. Using the OPLS classification model developed for the initial cohort (figure 2a), the females in the validation cohort were classified with an accuracy of 92.3% ($p=0.005$). Of the 13 females (seven smokers, six COPD), all were correctly classified except for one COPD patient, who was incorrectly classified as a smoker (table E7), resulting in 83.3% sensitivity and 100% specificity. Given that there was no OPLS model for the corresponding male population, the female model from figure 2a was used to perform an equivalent analysis with the males in the validation cohort. The males (nine smokers, five COPD) were classified with an accuracy of 57.1% ($p=0.5$, table E8).

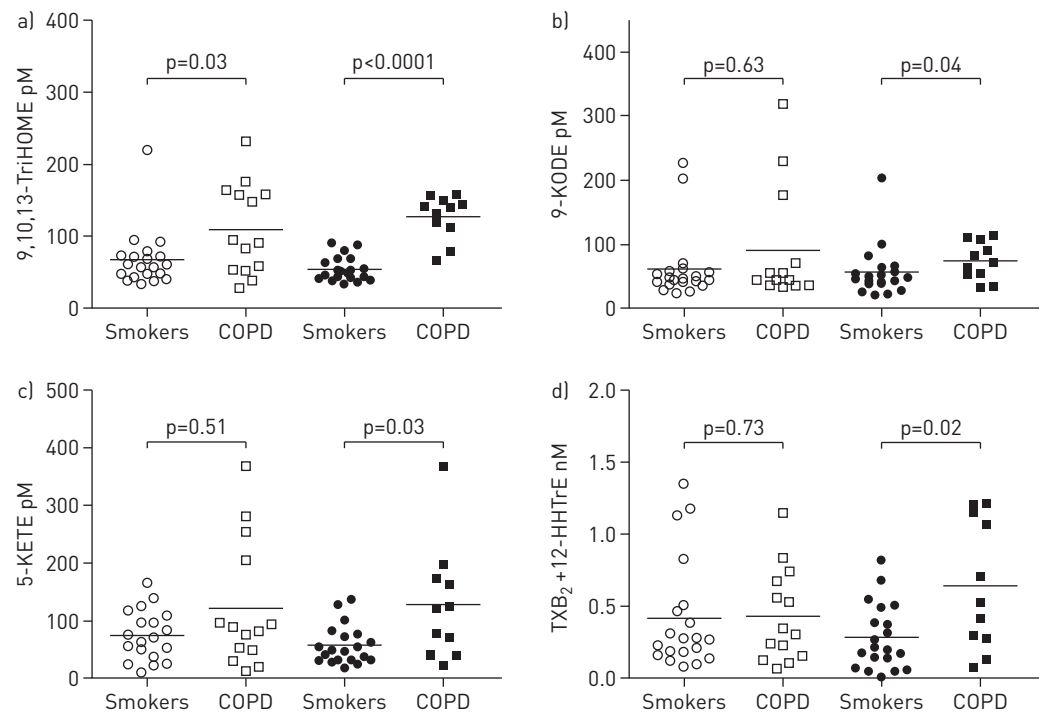


FIGURE 3 Lipid mediator levels in bronchoalveolar lavage fluid [BALF] in relation to sex, smoking status and chronic obstructive pulmonary disease [COPD]. a) 9,10,13-TriHOME, b) 9-KODE. c) 5-KETE and d) sum of TXB₂ and 12-HHTrE [TXA₂ synthase pathway]. Subjects are divided into smokers with normal lung function (smokers, circles) and smokers with COPD (COPD, squares). Open symbols indicate males and closed symbols females. Significance is indicated by the non-parametric Mann-Whitney test. Lipid mediator nomenclature is as follows: 9,10,13-TriHOME [9,10,13-trihydroxy-11E-octadecenoic acid], 9-KODE [9-oxo-10E,12Z-octadecadienoic acid], 5-KETE [5-oxo-ETE, 5-oxo-6E,8Z,11Z,14Z-eicosatetraenoic acid], 12-HHTrE [12-hydroxy-5Z,8E,10E-heptadecatrienoic acid], and TXB₂ (thromboxane B₂).

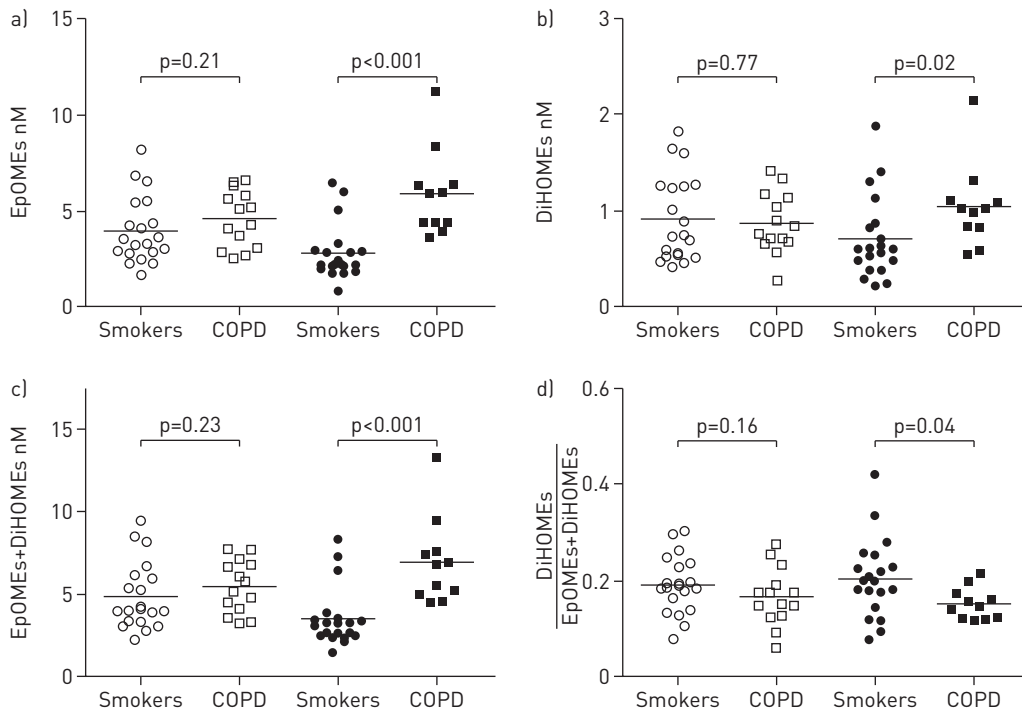


FIGURE 4 Cytochrome P450-derived linoleate mediators in bronchoalveolar lavage fluid [BALF] in relation to gender, smoking status and disease. a) EpOMEs, b) DiHOMEs, c) EpOMEs+DiHOMEs and d) DiHOMEs/(EpOMEs+DiHOMEs). Subjects are divided into smokers with normal lung function (Smokers, circles) and smokers with COPD (COPD, squares). Open symbols indicate males and closed symbols females. Significance is indicated by the non-parametric Mann-Whitney test. Lipid mediator nomenclature is as follows: EpOMEs are 9[10]-EpOME [9[10]-epoxy-12Z-octadecenoic acid] and 12[13]-EpOME [12[13]epoxy-9Z-octadecenoic acid]; and DiHOMEs are 9,10-DiHOME [9[10]-dihydroxy-12Z-octadecenoic acid] and 12,13-DiHOME [12[13]-dihydroxy-12Z-octadecenoic acid].

Linoleic acid-derived species correlate with lung function and mRNA levels in female COPD patients

The EpOME and DiHOME lipid mediators driving the separation between female smokers and COPD patients correlated stronger with lung function (FEV₁ and FEV₁/FVC) in female COPD patients (PLS inner relation $r=0.87$, $p=9 \times 10^{-4}$, figure 5a), than in female smokers (PLS inner relation $r=0.50$, $p=0.02$, data not shown). The EPOME and DiHOME lipid profiles of the female COPD patients also correlated significantly with the individual lung function variables FEV₁ (PLS Inner relation $r=0.69$, $p=0.02$) and FEV₁/FVC (PLS inner relation $r=0.76$, $p=0.01$).

The mRNA for genes putatively associated with the biosynthesis of the EpOMEs and DiHOMEs was identified based upon known biochemical pathways. The gene assignments were as follows: relevant CYPs for EpOMEs based upon the KEGG linoleic acid pathway (hsa00591: CYP1A2, CYP2C, CYP2J, CYP3A4 and CYP2E1), and two epoxide hydrolase (EH) genes for the DiHOMEs (EPHX1, EPHX2). The corresponding correlation between FEV₁ and mRNA levels was stronger for female COPD patients ($r=0.96$, $p=0.003$, figure 5b) than for female smokers ($r=0.66$, $p=0.03$, data not shown). No relationships with lung function or mRNA levels were observed for the corresponding male populations.

Linoleic acid-derived species correlate with goblet cell abundance in female COPD patients

To obtain insight into the cellular origin of the nine-lipid panel, the abundance of the BAL immune cells (macrophages, lymphocytes, neutrophils, eosinophils) as well as structural cells from bronchial brushings (goblet cells) and blood platelets were correlated with the lipids on a pathway-specific basis. In the female COPD group, 9,10,13-TriHOME levels correlated with neutrophil abundance ($r=0.77$, $p=0.005$; figure 5c), while the remaining five linoleates (DiHOMEs, EpOMEs and 9-KODE) displayed a weaker correlation with eosinophil abundance ($r=0.67$, $p=0.02$). No significant correlations were found for the corresponding male groups (figure E8). Both female and male COPD patients exhibited a weak correlation between blood platelet levels and the nine-lipid panel ($r=0.64$, $p=0.047$; $r=0.66$, $p=0.03$, respectively; data not shown). No correlations were observed for blood platelets and any of the individual compounds from the nine-lipid panel. All nine lipids correlated with goblet cells in female COPD patients ($r=0.84$, $p=0.005$), with the correlation driven primarily by the EpOMEs ($r=0.72$, $p=0.03$; figure 5d). The corresponding correlation in

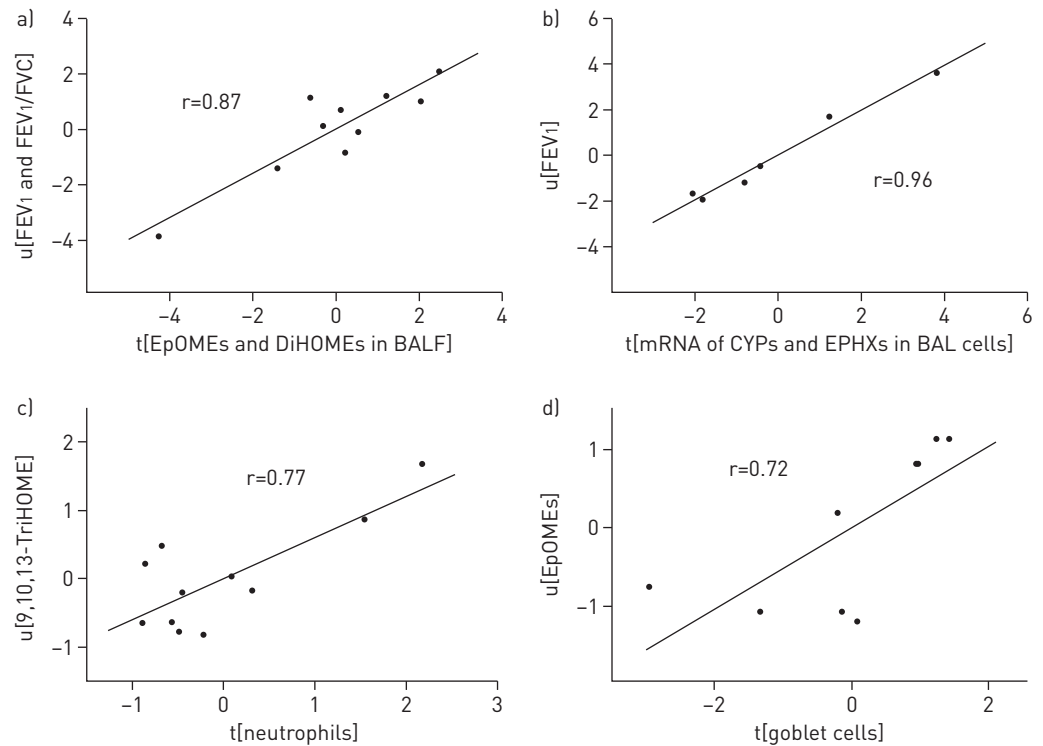


FIGURE 5 Correlation between lipid mediators in bronchoalveolar lavage fluid (BALF) with lung function, mRNA, neutrophils and goblet cells. a) The correlation between lung function parameters (forced expiratory volume in 1 s (FEV₁) and FEV₁/forced expiratory volume (FVC)) versus the concentrations of EpOMEs and DiHOMEs in female COPD smokers (PLS inner relation, $r=0.87$, $p=0.0009$, $n=10$). b) The correlation of FEV₁ versus mRNA related to the EpOME and DiHOME lipid mediators (EPHX1, EPHX2, CYP1A2, CYP2C, CYP2J, CYP3A4 and CYP2E1) of a subset of the female COPD smokers group (PLS inner relation, $r=0.96$, $p=0.003$, $n=6$). c) The correlation between neutrophil abundance in BALF and 9,10,13-TriHOME (PLS inner relation, $r=0.77$, $p=0.005$, $n=11$). d) The correlation between goblet cell abundance versus the 2 EpOME lipid mediators (leukotoxin and isoleukotoxin; PLS inner relation, $r=0.72$, $p=0.03$, $n=9$). Lipid mediator nomenclature is as follows: EpOMEs=9[10]-EpOME [9[10]-epoxy-12Z-octadecenoic acid] and 12[13]-EpOME [12[13]epoxy-9Z-octadecenoic acid]; DiHOMEs=9,10-DiHOME [9[10]-dihydroxy-12Z-octadecenoic acid] and 12,13-DiHOME [12[13]-dihydroxy-12Z-octadecenoic acid]; 9,10,13-TriHOME [9,10,13-trihydroxy-11E-octadecenoic acid].

male COPD patients was weaker for the nine-lipid panel ($r=0.65$, $p=0.02$; data not shown) and non-significant for the EpOMEs ($r=0.46$, $p=0.14$, figure E8C).

Discussion

COPD is a heterogeneous inflammatory disease that is a leading cause of morbidity and mortality worldwide. This heterogeneity may manifest in patient sub-phenotypes involving different molecular pathways in onset and development of the disease. Results from the current study identified increases in lipid mediators from the linoleic acid-derived CYP pathway, as well as arachidonic acid-derived products of thromboxane synthase and 5-LOX in smokers with early-stage COPD compared with smokers with normal lung function. These differences were driven by the female population, with a nine-lipid panel classifying female smokers with COPD from female smokers with normal lung function ($p=6.3 \times 10^{-6}$; figure 2a). While the classification model for women was robust, and confirmed with 92.3% accuracy in an independent validation cohort, no differences were observed in the corresponding male populations ($p=1.0$). In contrast, no gender-based differences were observed for the corresponding smokers with normal lung function or healthy never-smokers groups, indicating that the observed gender-related differences were associated with COPD disease phenotype rather than generic gender-differences. As such, the putative pattern identified in this study is applicable to a female-dominated disease sub-phenotype, thereby corresponding to our previously reported alterations in the BAL cell proteome of the same cohort [18].

The strongest drivers of the gender-specificity were the linoleates in BALF (the EpOMEs (leukotoxin and isoleukotoxin) and DiHOMEs (leukotoxin diol and isoleukotoxin diol) as well as 9,10,13-TriHOME). The EpOMEs are epoxides that can be produced enzymatically from linoleic acid *via* CYP activity, which are further converted to the corresponding 1,2-diols, DiHOMEs, *via* sEH [26] (figure 1). EpOMEs and DiHOMEs have been studied in models of respiratory diseases [16, 27, 28], especially in rodent models of

ARDS [14, 29], with the DiHOMEs demonstrated to exert cytotoxicity [17, 30]. The DiHOMEs were reported to decrease net ion flux in alveolar epithelial monolayers and increase intercellular junction permeability [17, 30], both of which are known contributing factors to chronic bronchitis [31]. Chronic bronchitis has, with some contradictions [32], been reported to be a more prevalent clinical manifestation among female COPD patients by many investigators [4, 33, 34], including twin studies [35]. BALF EpOME levels in female COPD patients correlated with goblet cell abundance, which have been reported to increase with smoking [36] and possess CYP activity [37]. Accordingly, these findings suggest a mechanism, by which goblet cell hyperplasia in female smokers leads to elevated production of EpOMEs and the commensurate DiHOMEs, which potentiates the onset of chronic bronchitis and COPD. These findings are of particular clinical relevance due to the interest in targeting the sEH pathway for COPD treatment. For example, GlaxoSmithKline has conducted two Phase I clinical trials to evaluate the use of sEH inhibitors for the treatment of COPD (NCT01762774 and NCT02006537).

Little is known about the 9,10,13-TriHOME, with its route of formation and mechanism of action unclear. TriHOMEs have been reported to exert prostaglandin E₂-like activity of relaxing vascular smooth muscle cells *in vitro* [38] and to be altered in asthma [39]. Levels of the 9,10,13-TriHOME correlated with neutrophils (figure 5c), which possess 9,10,13-TriHOME synthesis activity [40]. Accordingly, the 9,10,13-TriHOME is likely formed in the lung *via* a neutrophil LOX-dependent process, followed by sEH activity. The biosynthetic route and function of the 9-KODE are equally unclear, although the linoleic acid oxidative metabolites including the monohydroxy- and keto-octadecadienoic acid are implicated in a variety of pathologies [41, 42] as well as reported to exert peroxisome proliferator-activated receptor- γ agonist activity [43].

Other primary differences between female smokers and COPD patients include production of the arachidonic acid-derived TXB₂, 12-HHT and 5-KETE. The thromboxane synthase products 12-HHT and TXB₂ are classic platelet-derived lipids [44], thereby suggesting a role of platelet activation in COPD. These two lipids as well as 9,10,13-TriHOME were the only species to correlate between female BALF and serum models (SUS plot, figure 2c), indicating a systemic relationship for these three mediators. Furthermore, the BALF levels of the full nine-lipid panel correlated with blood platelets for smoking COPD patients in both sexes. Platelet-neutrophil transcellular interactions have been recently shown to regulate lung inflammation *via* the production of lipid mediators [45], further suggesting that platelets may play a role in the inflammatory-derived pathobiology of lung disease. 5-KETE is a potent chemoattractant for eosinophils [46] that can be synthesised by airway epithelial cells [47], neutrophils [48] and blood platelets [49] in response to oxidative stress. Levels of 5-KETE have been postulated to prolong pulmonary inflammation and potentially contribute to conditions such as severe asthma [46].

Mechanistically, it is unclear what is driving the observed gender-specific shift. Linoleic acid is the major dietary fat and cannot be *de novo* synthesised by mammals [50]. Its metabolites are known to be biologically active, with the DiHOMEs generally considered to be pro-inflammatory and the EpOMEs anti-inflammatory [51]. A meta-analysis found no gender differences in the levels of linoleic acid in plasma lipids [52], suggesting that the observed differences are not associated with gender-related dietary intake. Higher concentrations of EpOMEs and the corresponding DiHOMEs in BALF of females with COPD suggest a gender-specific upregulation of the CYP-pathway, which is related to smoking-driven goblet cell hyperplasia. Gender specificity in the expression of CYP enzymes has been extensively reported in rodent tissues [53], although less is known about the patterns in humans, especially the lung [54]. Accordingly, it would be of interest to investigate the gender-dependent expression and activity of CYP enzymes in goblet cells and their role in the onset and progression of COPD.

These findings also support recent reports of platelet activation in COPD. Platelets have been proposed as key mediators in inflammatory lung disease [55], playing a critical role in the recruitment of neutrophils, eosinophils and lymphocytes [56]. The production of platelet-activation factor (PAF) has been reported to increase in response to cigarette smoke [57]. Patients with stable COPD have increased circulating platelet-monocyte aggregates, and PAF increases during an acute exacerbation [58]. Anti-platelet therapy (*i.e.* clopidogrel) [59] as well as statin therapy [60] (which reduces platelet activation) have been suggested in the treatment of COPD. Given that several components of the nine-lipid panel identified in this study can be linked to platelet activation, these findings further support the theory of platelet involvement in the pathobiology of COPD.

Conclusions

Female gender has been suggested as a risk factor in susceptibility to the lung-damaging effects of cigarette smoke, and both smokers and nonsmokers with COPD are more likely to be female. In the current study, a nine-lipid panel evidenced specificity in COPD. The identified lipid mediators included compounds previously suggested to be of importance in disease including 5-KETE and thromboxane synthase

products (TXB₂ and 12-HHT), as well as more novel lipids produced *via* the CYP pathway from linoleic acid. These results suggest a gender-based phenotypic difference in the production of lipid mediators associated with inflammatory disease. The female-specific correlation between goblet cells and EpOME levels in BALF in combination with their purported role in decreased ion flux in airway epithelial monolayers and increased intercellular junction permeability suggests a mechanism by which increased production of EpOMEs could lead to more frequent clinical manifestations of chronic bronchitis in the female COPD population. In combination with the reported association between linoleic acid-derived mediators with multiple organ failure and ARDS, it is possible that this pathway plays an important role in the aetiology of certain COPD sub-phenotypes.

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