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Dynamic differences in dietary polyunsaturated fatty acid metabolism in sputum of COPD patients and controls

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ABSTRACT

Introduction: Disturbances in onset and resolution of inflammation in chronic obstructive pulmonary disease (COPD) are incompletely understood. Dietary polyunsaturated fatty acids (PUFAs) can be converted into lipid mediators here collectively named oxylipins. These include classical eicosanoids, but also pro-resolving mediators. A balanced production of pro-inflammatory and pro-resolving oxylipins is of importance for adequate inflammatory responses and subsequent return to homeostasis.

Objectives: Here we investigated if PUFA metabolism is disturbed in COPD patients.

Methods: Free PUFA and oxylipin levels were measured in induced sputum samples from the Bergen COPD cohort and COPD exacerbation study using liquid chromatography-mass spectrometry. Additionally, effects of whole cigarette smoke on PUFA metabolism in air-liquid interface cultures of primary bronchial epithelial cells were assessed.

Results: Significantly lower levels of free alpha-linolenic acid, linoleic acid and eicosapentaenoic acid (EPA) were detected in sputum from stable COPD patients compared to controls. During acute exacerbation (AE), levels of free arachidonic acid and docosapentaenoic acid were higher than in stable COPD patients. Furthermore, levels of omega-3 EPA- and docosahexaenoic acid-derived oxylipins were lower in sputum from stable COPD patients compared to controls. Cyclooxygenase-2-converted mediators were mostly increased during AE. *In vitro* studies additionally showed that cigarette smoke exposure may also directly contribute to altered epithelial PUFA metabolism, and indirectly by causing airway epithelial remodelling.

Conclusions: Our findings show significant differences in PUFA metabolism in COPD patients compared to controls, further changed during AE. Airway epithelial remodelling may contribute to these changes. These findings provide new insight in impaired inflammatory resolution in COPD.

1. Introduction

Whereas cigarette smoking is considered the primary cause of Chronic Obstructive Pulmonary Disease (COPD) in the Western world, only around 20% of smokers develop COPD [1,2]. After smoking cessation, inflammation in the lungs of COPD patients persists [3], indicating that inflammatory resolution is disturbed in the lungs of these patients. Over the past decades, research has shown that the resolution phase of inflammation is initiated by pro-resolving mediators that predominantly originate from precursors derived from our diet, *e.g.* polyunsaturated fatty acids (PUFAs) [4]. Dietary PUFAs (omega-3 and omega-6) are incorporated in the cell membrane and released upon exposure of the cell to activating/inflammatory triggers. Released PUFAs are subsequently converted *via* enzymatic or non-enzymatic pathways into lipid mediators, here collectively named oxylipins [5]. Oxylipins include all PUFA derived (intermediate-)mediators including the well-known leukotrienes and prostaglandins, but also the more recently discovered pro-resolving mediators such as resolvins, protectins and maresins. The end-stage lipid mediators are converted from PUFAs either directly, or indirectly *via* a multiple-step conversion into biochemical intermediate lipid mediators before reaching their final state. Converting enzymes that are involved include cyclooxygenases (COX), lipoxygenases (LOX) and cytochrome P450 enzymes (CYP). Depending on the PUFA released and the enzymes involved, the produced lipid

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mediators will become for example a leukotriene or prostaglandin (generally omega-6 derived) [6], or a pro-resolving protectin, resolvin or maresin (generally omega-3 derived) [7].

Previous studies have explored associations between dietary intake of PUFA (omega-3 and/or -6) and COPD outcomes. Study results showed associations between high omega-6 fatty acid intake and reduction in lung function [8]. Furthermore, an inverse correlation with high dietary intake of omega-3 fatty acids and the risk to develop COPD was found [9,10]. No correlation was found between fatty acid intake and COPD mortality [11]. In line with these findings, disturbances in fatty acid composition in (erythrocyte) membranes of COPD patients have been reported [12,13]. However, none of these studies were designed to address causal relationships, and direct investigations on dietary fatty acid metabolism in the lungs of COPD patients are limited. Studying PUFA metabolism in COPD might help to understand the impaired resolution of inflammation in the lungs of patients with COPD and identify possible therapeutic targets to promote resolution of inflammation [14].

This study investigates PUFA metabolism in sputum from patients with stable COPD in comparison to smoking controls, and changes herein during acute exacerbations in COPD patients. In addition, alterations in PUFA metabolism were investigated in primary air-liquid interface differentiated bronchial epithelial cell cultures to study direct effects of whole cigarette smoke exposure on PUFA metabolism.

2. Methods

2.1. Study population and sample selection

Induced sputum samples were collected from participants of the Bergen COPD Cohort and COPD Exacerbation studies; information on study design and inclusion criteria was previously published [15–17]. Induced sputum samples from 27 smoking controls (current smokers and ex-smokers; CTRL), 38 COPD patients in a stable phase (STBL), and 37 patients during an exacerbation (AE) were available for the lipid analyses described in this study. Baseline characteristics of included subjects are depicted in Table 1. Controls were age matched to the COPD population and had samples available at one time-point. Except for one, no patients received oral steroids at time of sputum sampling. The full details on sputum induction and processing are previously published [16]. All data presented in this study are available upon request from the corresponding author.

2.2. Cell culture

Primary bronchial epithelial cells (PBEC) were obtained from tumour-free resected lung tissue, collected and stored at the Leiden University Medical Center, Leiden, The Netherlands. PBEC were expanded and cultured at the air-liquid interface (ALI) as described in [18]. A detailed description is enclosed in the online data supplement.

2.3. Whole cigarette smoke exposure

Methods for exposing the cell cultures to whole cigarette smoke have been published previously [18]. Detailed information is enclosed in the online data supplement.

2.4. Liquid chromatography-mass spectrometry sample preparation of induced sputum samples and primary bronchial epithelial cells

Detailed methods regarding sample preparation of induced sputum samples and of primary bronchial epithelial cells are enclosed in the online data supplement.

Table 1
Baseline characteristics of the included subjects.

	Controls		Stable COPD		p[a]	COPD-AE	
	n	%	n	%		n	%
Sex					0.98		
Women	12	44.4	17	44.7		15	40.5
Men	15	55.6	21	55.3		22	59.5
Body composition[b]					0.48		
Normal	23	85.2	28	73.7		26	70.3
Cachectic	1	3.7	4	10.5		5	13.5
Obese	3	11.1	6	15.8		6	16.2
Smoking status					0.001		
Never-smoker	2	7.4	–	–		–	–
Ex-smoker	3	11	21	55.3		20	54.0
Current-smoker	22	81.5	17	44.7		17	56.0
Charlson Index[c]					< 0.001		
0	20	74.1	–	–		–	–
1	5	18.5	27	71.1		26	70.3
2	2	7.4	8	21.1		8	21.6
3	–		2	5.3		2	5.4
4	–		1	2.6		1	2.7
GOLD stage							
II	–		19	51.2		19	50.0
III	–		14	37.8		15	39.5
IV	–		4	10.8		4	10.5
Using inhaled steroids?					< 0.001		
No	27	100	9	23.7		8	21.6
Yes	–	–	29	76.3		29	78.38
Using LTOT?							
No	27	100	38	100		37	100
Yes	–	–	–	–		–	–

Continuous variables	Controls		Stable COPD		p[a]	COPD-AE	
	Mean	SD	Mean	SD		Mean	SD
Age					0.28		
Years	59.2	5.9	60.8	5.9		61.2	5.9
Smoking load					0.22		
Pack-years	32.8	19.6	38.6	18.4		38.9	18.2
Pre BD spirometry							
FEV_1 (% predicted)	97.6	8.6	46.2	12.8	< 0.001	45.6	12.9
FVC (% predicted)	104.4	10.0	81.8	12.5	< 0.001	81.4	12.5
Post BD spirometry							
FEV_1 (% predicted)	105.9	9.9	49.8	14.8	< 0.001	49.4	14.6
FVC (% predicted)	106.0	10.0	87.3	13.1	< 0.001	86.6	12.4
Arterial blood gases							
pH	7.41	0.021	7.41	0.016	0.10	7.41	0.016
pCO_2 (kPa)	5.27	0.39	5.29	0.43	0.85	5.30	0.42

36 COPD patients are paired (have both stable and exacerbation sample).
[a] Comparing healthy controls to stable COPD.
[b] Body composition based on bioelectrical impedance measurements of fat free mass and fat mass.
[c] All COPD patients will have Charlson 1+ "by default" as COPD = 1 Charlson score point.

2.5. Liquid chromatography-mass spectrometry analysis

PUFA-derived lipid mediators in induced sputum and primary bronchial epithelial cells were measured using RPLC-MS/MS on a Shimadzu LC-system (Shimadzu's-Hertogenbosch, The Netherlands) coupled to a QTrap 6500 (ABSciex, Nieuwerkerk a/d IJssel, The Netherlands). A list of all measured compounds is depicted in Supplementary Table 1 in the online data supplement. Substances were identified according to published protocols and class specific internal standards were used to correct for matrix effects and analyte quantification [19,20]. The full method is described elsewhere [21] and any modifications made in the online data supplement.

2.6. RNA isolation, cDNA synthesis and qPCR

Methods regarding RNA isolation, cDNA synthesis and qPCR analysis are described in the online data supplement.

2.7. Protein concentrations

Detailed information regarding measurements of the ALI-PBEC culture protein concentration is enclosed in the online data supplement.

2.8. Data analysis and statistical analysis

Extracted ion chromatograms of detected analytes were integrated using Multiquant (version 4.1, Sciex) and corrected for their corresponding internal standard. For a subset of analytes which were commercially available at the time of the study, quantitative analysis was carried out using external calibration lines as described in the Supplementary Table 1 in the online data supplement, and in a previous publication [21]. Next, data were corrected for protein levels (PBEC samples only) and either area/ratio or concentration when available was used to depict the data. For the correlation plots, only area/ratios were used in order to enable comparison of all analytes.

Principal component analysis (PCA) was performed with R statistical environment (http://www.r-project.org/, R versions 3.4.3), "Rcpm", "pcaMethods" packages were used. For visualisation of the data correlation structure, the packages "corrr" and "corrplot" were used. Additional lipid mediator data that was collected but not highlighted in the results section can be found in Fig. S1. Univariate statistical analysis was conducted using GraphPad Prism 7 (GraphPad Software Inc., La Jolla, CA, U.S.A.). Data are shown as median with interquartile range unless stated otherwise. The statistical test(s) used are specified in the legend of each figure. Data in Figs. 1–4 were corrected for multiple hypothesis testing using the two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli. A maximum of one outlier per group was removed based on Grubbs testing (alpha = 0.01) performed on the complete data set of that group. Despite a substantial overlap in COPD patients from whom samples were available for both the stable and exacerbation group, we did not perform a paired statistical analysis. The reason for this was that the time between stable sputum collection and exacerbation was highly variable between patients, and the order of sample collection differed: from some patients a sample in the stable phase was collected before the exacerbation sample, whereas in others it was obtained after the exacerbation during which a sample was collected. Significant differences for the parameters in Table 1 were tested using for Sex and Body composition a chi-square test, for Smoking, Charlson Index and Inhaled steroids a Fishers exact test and for all Continuous variables a *t*-test. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Lower levels of free alpha linolenic acid, linoleic acid and eicosapentaenoic acid in sputum from stable COPD patients compared to controls

Free PUFA levels were assessed in sputum from smoking controls and COPD patients in a stable phase or during an acute exacerbation (AE). Significantly lower sputum levels of omega-3 alpha-linolenic acid (ALA) and eicosapentaenoic acid (EPA, both in Fig. 1A) and omega-6 linoleic acid (LA, Fig. 1B) were found in stable COPD patients compared to controls. During exacerbation, slight increases in PUFA levels compared to stable COPD were observed which were significant for omega-3-derived docosapentaenoic acid (DPA, Fig. 1A) and omega-6-derived arachidonic acid (AA, Fig. 1B) and adrenic acid (AdA, Fig. 1B).

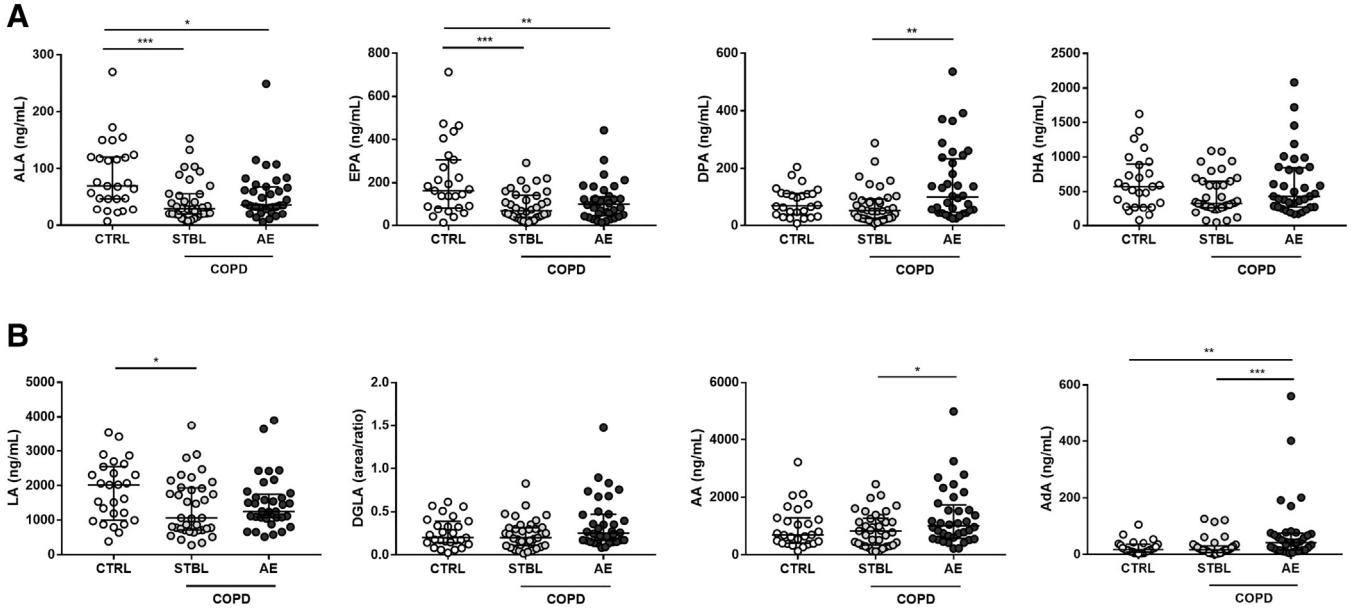

Fig. 1. Free polyunsaturated fatty acid levels in induced sputum from smoking controls and COPD patients in stable phase and during an exacerbation.
LC-MS analysis was performed on induced sputum collected from smoking controls (CTRL, n = 26–27) and COPD patients in a stable phase (STBL, n = 37–38) and during an acute exacerbation (AE, n = 36–37) to assess free polyunsaturated fatty acid (PUFA) levels. A) Levels of free omega-3 PUFA alpha-linolenic acid (ALA)[#], eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA). B) Free omega-6 PUFA levels of linoleic acid (LA), dihomo-gamma-linoleic acid (DGLA), arachidonic acid (AA) and adrenic acid (AdA). Data are depicted as median with interquartile range. Statistical significance was tested using a Kruskal-Wallis one-way analysis of variance test followed by a two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli to correct for multiple comparisons. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ between groups.
[#]Due to technical limitations the signal obtained for the here applied QqQ settings for ALA might result in an approximate 10% overlap with the signal obtained from GLA.

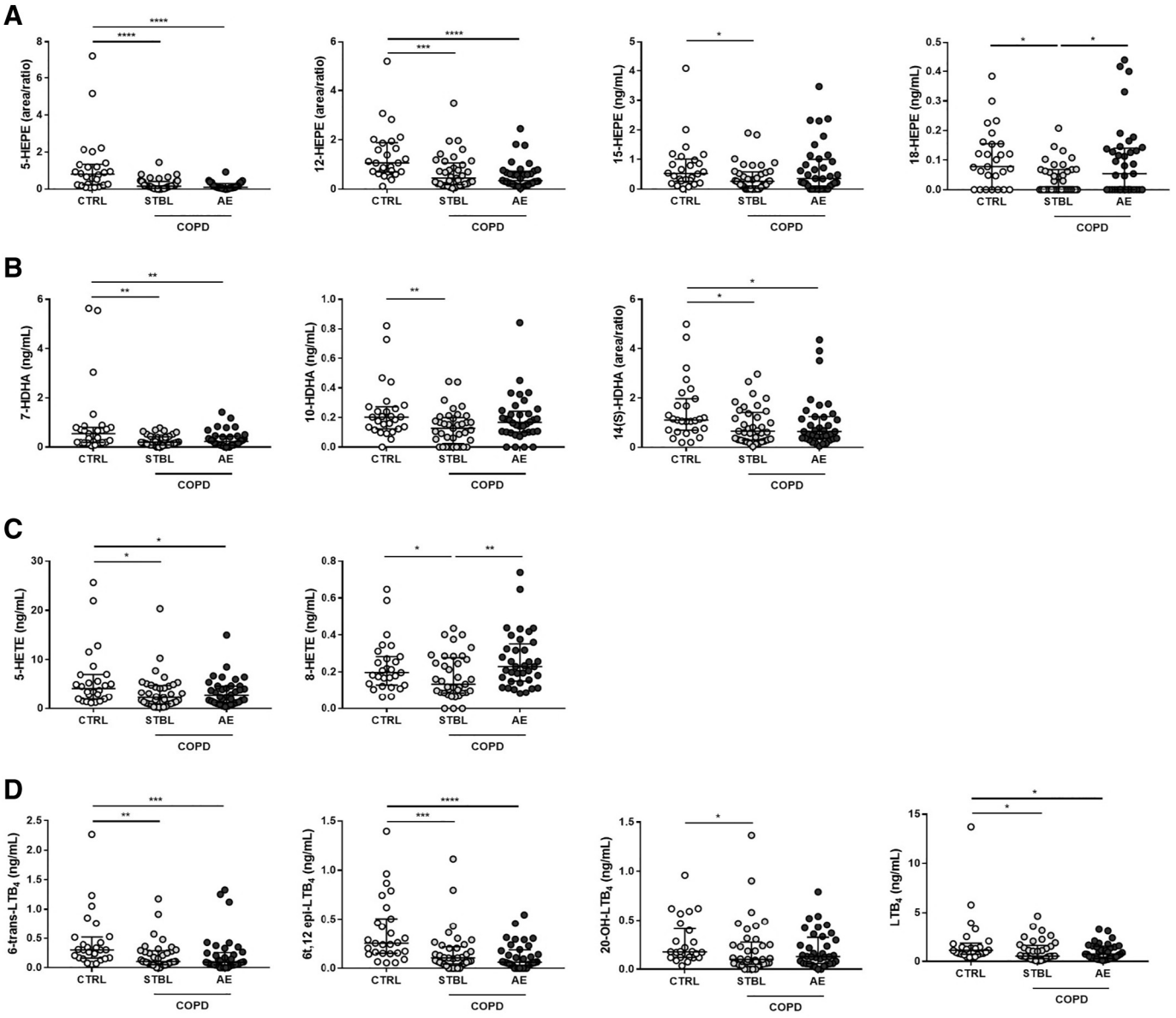

Fig. 2. PUFA-derived lipid mediator levels in induced sputum from smoking controls and COPD patients in a stable phase and during exacerbation.
LC-MS analysis was performed on induced sputum collected from smoking controls (CTRL, n = 26–27) and COPD patients in the stable phase (STBL, n = 37–38) and during acute exacerbation (AE, n = 37) to assess PUFA-derived lipid mediators. Data are depicted as median with interquartile range for A) 5-hydroxyeicosapentaenoic acid (5-HEPE), 12-HEPE, 15-HEPE and 18-HEPE, B) 7-hydroxydocosahexaenoic acid (7-HDHA), 10-HDHA and 14(*S*)-HDHA, C) 5-hydroxyeicosatetraenoic acid (5-HETE) and 8-HETE and D) 6-*trans*-leukotriene B_4, 6t,12epi-LTB_4, 20-OH-LTB_4 and LTB_4. Data are depicted as median with interquartile range. Statistical significance was tested using a Kruskal-Wallis one-way analysis of variance test followed by a two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli to correct for multiple testing. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ between groups.

3.2. DHA-, EPA- and AA-derived lipid mediator levels are lower in sputum from stable COPD patients compared to controls

Next, levels of PUFA-derived lipid mediators were assessed. COPD patients displayed significantly lower sputum levels of all measured EPA-derived HEPEs (Fig. 2A), three out of four-measured DHA-derived HDHAs (Fig. 2B), two out of six measured AA-derived HETEs (Fig. 2C) and all measured AA-derived LTB_4's (Fig. 2D) compared to controls. Since the sputum supernatants were cell-free, gene expression levels of the converting enzymes could not be assessed. To still obtain an insight into the activity of the converting pathways, correlations between all lipid mediators were calculated. In sputum from smoking controls no strong correlations were found between PUFA levels and their mediators (Fig. 3A). However, in stable COPD patients PUFA levels correlated strongly to most measured lipid mediators (Fig. 3B), suggesting enhanced activity of several converting pathways (including non-enzymatic) as these correlations were not found in controls. These findings are in concordance with the reduced PUFA levels found in stable COPD patient's sputum and suggest enhanced PUFA conversion in the airways of stable COPD patients compared to smoking controls.

3.3. Fatty acid conversion via cyclooxygenase-2 is increased during acute exacerbation

Next, we compared levels of lipid mediators between sputum collected from COPD patients during an acute exacerbation and during a stable phase, all three measured prostaglandins were significantly increased during AE compared to sputum from stable COPD patients (Fig. 4A). In addition, also thromboxane B_2 (TxB_2), 11-HETE, 9-HODE (all Fig. 4B) and the peroxisome proliferator-activated receptor

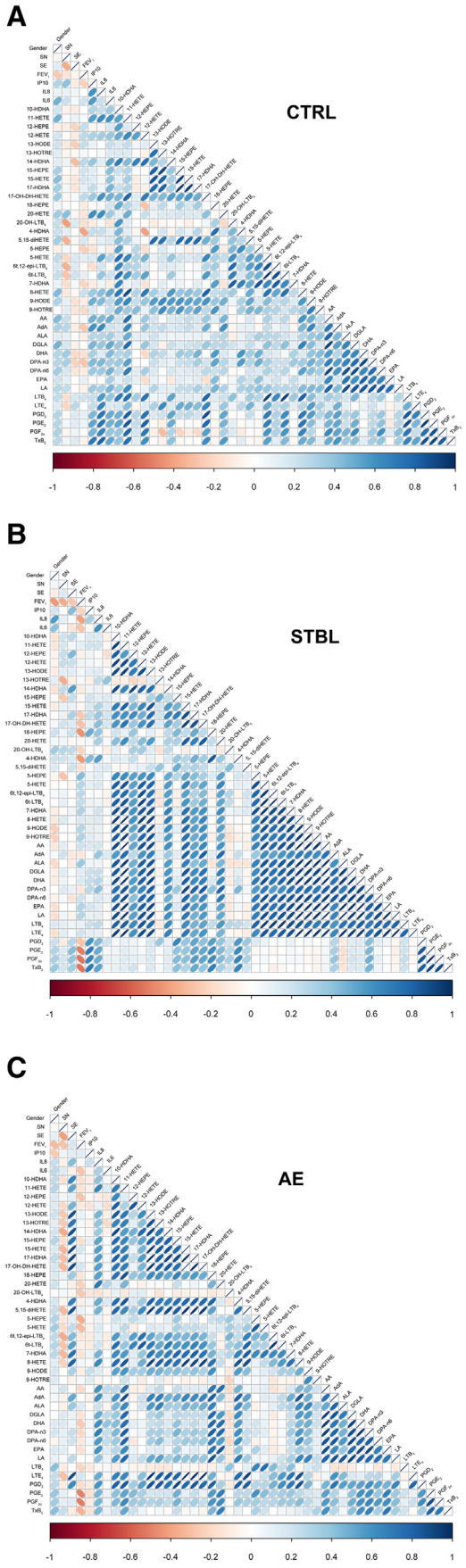

Fig. 3. Correlations between a variety of parameters in induced sputum from smoking controls and COPD patients in a stable phase and during exacerbation. Correlation plots are used to visualize correlations between individual components measured in A) controls (CTRL) or B) COPD patients in a stable phase (STBL) or C) during an acute exacerbation (AE). Negative correlations are depicted in red, and positive correlations in blue. The strength of this correlation is illustrated by the colour key that depicts the correlation coefficient at the bottom of the figures. Correlations are further emphasized by the thickness of the line: a thin line indicates highly correlated, a circle indicates low correlation, empty/white indicates no correlation. All abbreviations depicted in the plot are explained in Supplementary Table 1 in the online data supplement, the order of the included parameters is alphabetical. Gender was included in the calculations as a 0 for women and a 1 for men.

activator 8-HETE [22] (Fig. 2C) were significantly increased during AE. Lastly, 4-HDHA, 9-HOTRE and 18-HEPE were significantly increased during AE (Fig. 4C).

Correlations between all lipid mediators during exacerbation are depicted in Fig. 3C and display several interesting observations: i. PUFA levels did not correlate highly to related mediators, however, COX-2 converted mediators were strongly correlated to each other during AE; furthermore, ii. the levels of several mediators were highly correlated to sputum eosinophil levels. Although sputum eosinophil levels during AE were higher compared to stable (Fig. S2), the number of patients that displayed these elevated levels was limited. It is therefore difficult to assess if the observed correlations are of any biological relevance and these should first be confirmed in a larger cohort.

3.4. Reduced free polyunsaturated fatty acid levels by cigarette smoke-exposed air-liquid interface cultured primary bronchial epithelial cells

Data obtained from the sputum samples gave insight into changes in fatty acid metabolism as a consequence of COPD as these values were compared to those obtained from smoking controls. To investigate whether whole cigarette smoke (CS) exposure directly affects PUFA metabolism of airway epithelial cells, primary bronchial epithelial cells (PBEC) were differentiated at the air-liquid interface (ALI) and exposed to whole cigarette smoke (CS). Significantly increased $cPLA_2$ gene expression was detected at 3 and 24 h after CS exposure (Fig. 5A), indicative of an increased fatty acid release from the cell membrane. Subsequent LC-MS analysis revealed significantly reduced levels of all detected free PUFAs 24 h after CS exposure of the ALI-PBEC cultures (Fig. 5B–F), suggesting an increase in fatty acid conversion.

3.5. Cigarette smoke affects fatty acid metabolism in air-liquid interface cultures of primary bronchial epithelial cells

Next PUFA converting enzymes and lipid mediator release in these CS-exposed cell cultures was assessed. *ALOX15* (15-LOX) expression was significantly reduced 24 h after CS exposure (Fig. 6A), whereas *PTGS2* (COX-2) gene expression was increased 3 h after CS exposure and returned to baseline level 24 h thereafter (Fig. 6A). In line with reduced 15-LOX gene expression, also 15-LOX-mediated PUFA conversion was affected since 15-HETE, 17-HDHA, 15-HEPE and 5,15 diHETE levels were significantly reduced 24 h after CS exposure (Fig. 6B). Unexpectedly, COX-2-converted prostaglandin levels remained similar between AIR and CS, despite increased *PTGS2* gene expression levels (Fig. 6C). This could possibly be explained by the lack of effect of CS exposure on expression of *PTGES* (data not shown), the enzyme that is responsible for the conversion of PGH_2 into PGE_2 following COX-2 conversion, suggesting that PTGES is rate limiting under these conditions.

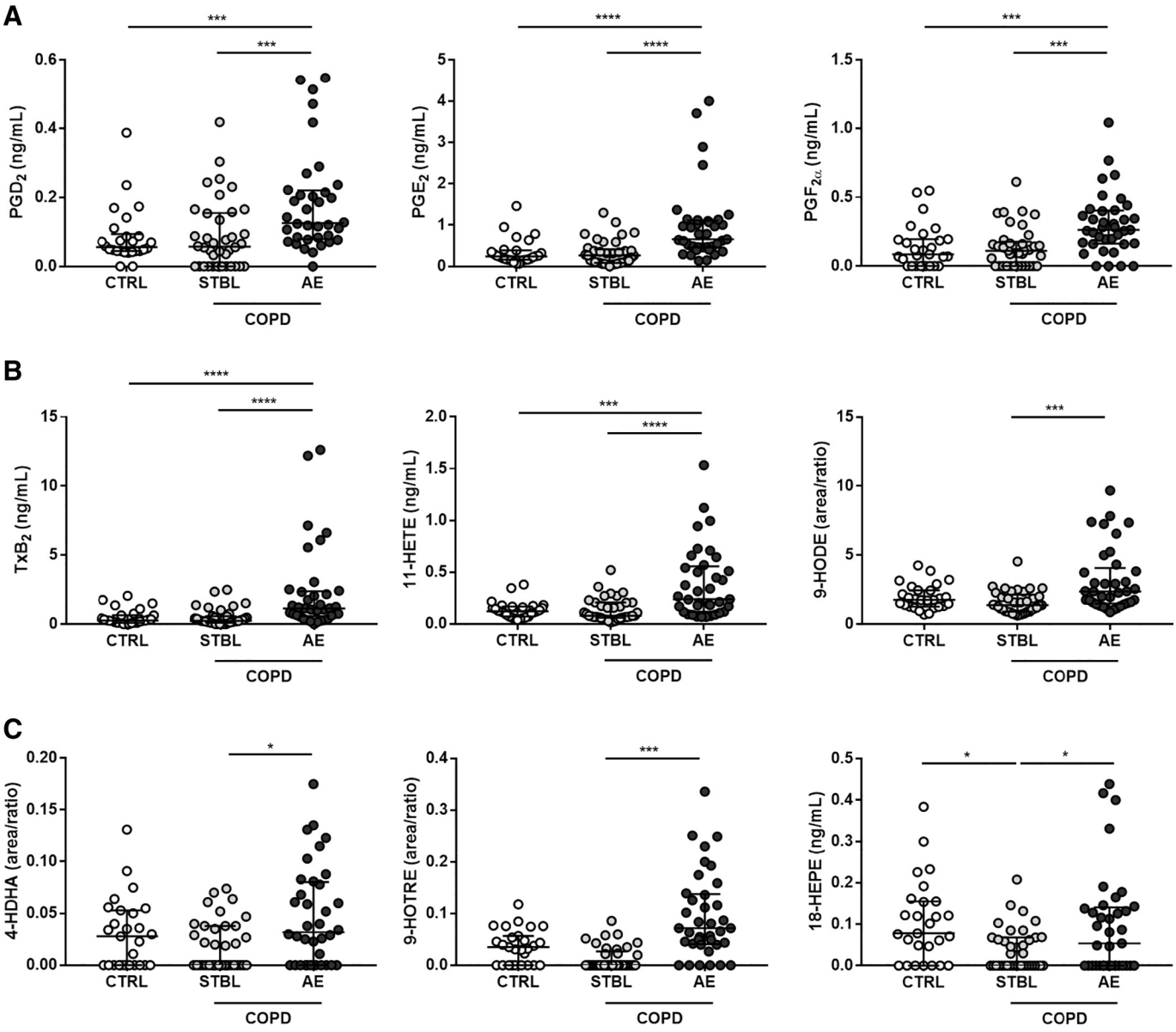

Fig. 4. Changes in lipid mediators during acute exacerbation.
LC-MS analysis was performed on induced sputum from smoking controls (CTRL, n = 26–27) and stable COPD patients (STBL I, n = 37–38) and COPD patients during acute exacerbation (AE, n = 36–37) to assess PUFA-derived lipid mediators. A) Medians with interquartile range are depicted for prostaglandin D_2 (PGD_2), prostaglandin E_2 (PGE_2), prostaglandin $F_2\alpha$ ($PGF_{2\alpha}$), for B) thromboxane B_2 (TXB_2), 11-hydroxyeicosatetraenoic acid (11-HETE) and 9-Hydroxyoctadecadienoic acid (9-HODE) and C) 4-hydroxydocosahexaenoic acid (4-HDHA), 9-hydroxy-10E,12Z,15Z-octadecatrienoic acid (9-HOTRE) and 18-hydroxyeicosapentaenoic acid (18-HEPE). Statistical significance was tested using a Kruskal-Wallis one-way analysis of variance test followed by a two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli to correct for multiple testing. *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$ between groups.

3.6. Differentiation status of the epithelium might contribute to disturbed fatty acid metabolism in COPD patients

To investigate if airway epithelial remodelling resulting from prolonged CS exposure could affect PUFA metabolism, gene expression levels of the converting enzymes were assessed in luminal cell or basal cell enriched fractions of (unexposed) ALI-PBEC cultures in a previously acquired and validated data set [18]. *ALOX15* expression was higher in the luminal cell-enriched fraction compared to the basal cell-enriched fraction, while this was *vice versa* for *PTGS2* expression (Fig. 7A). In an additional existing data set from a study in which we assessed effects of daily exposure (up to 19 days) on differentiating ALI-PBEC cultures to whole CS [18], we measured *ALOX15* and *PTSG2* expression during differentiation in presence or absence of chronic CS exposure. Data showed that chronic CS exposure during differentiation resulted in a markedly decreased expression of *ALOX15* as compared to air-exposed controls where an increase during epithelial differentiation was observed (Fig. 7B). Furthermore, *PTGS2* expression was strongly reduced as a result of epithelial differentiation, but this was not affected by CS exposure (Fig. 7C). As we previously established that chronic CS exposure results in changes in cellular composition of the airway epithelial cell cultures [18], and now also see changes in PUFA converting enzymes, these data indicate that CS-induced remodelling of the airway epithelium may influence the expression of the PUFA-converting enzymes favoring the expression of COX-2.

4. Discussion

Traditionally PUFA metabolism has been studied for conversion into eicosanoids such as leukotrienes and prostaglandins. In recent years

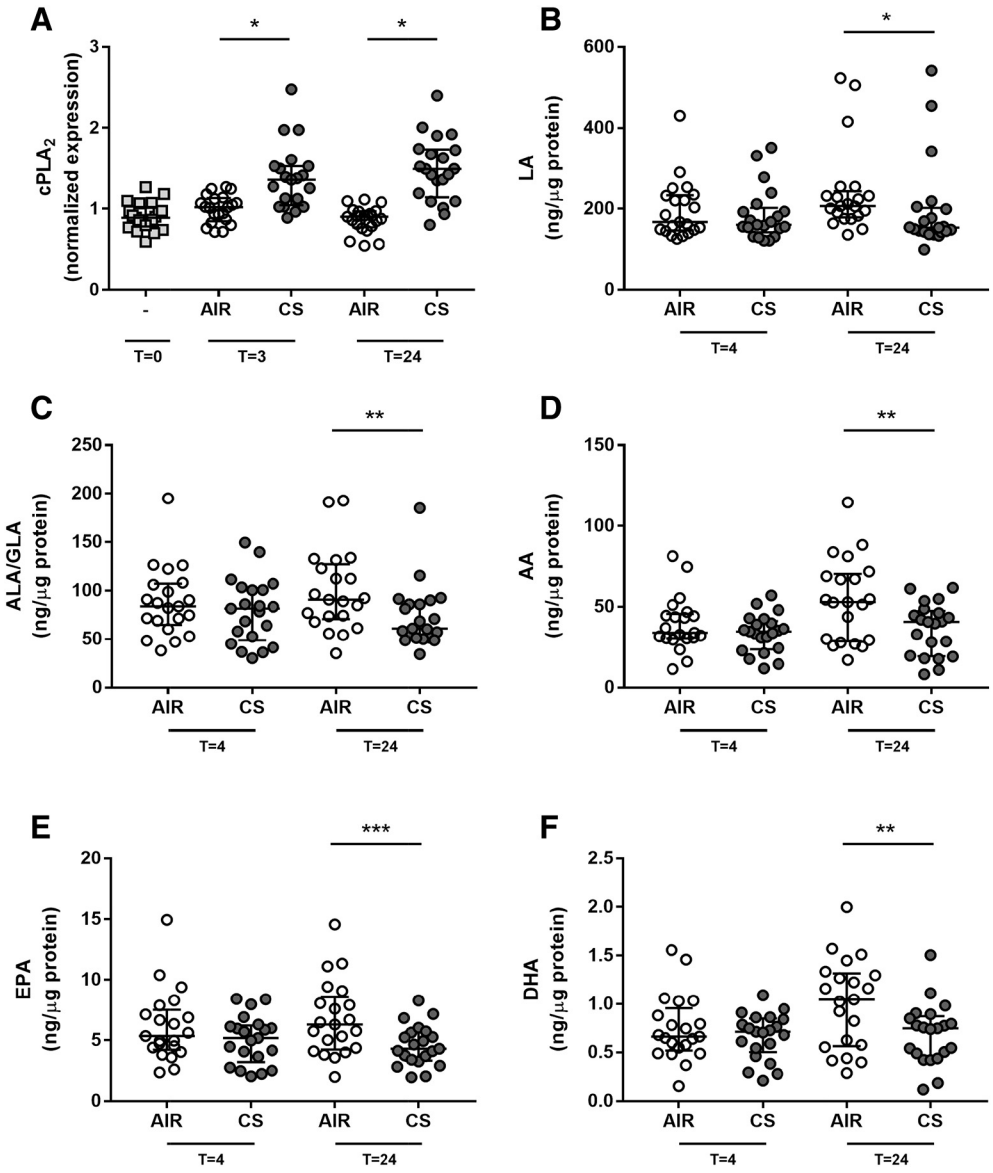

Fig. 5. Whole cigarette smoke exposure of air-liquid interface cultured primary bronchial epithelial cells promotes *cPLA2* gene expression and reduces free polyunsaturated fatty acid levels.
Primary bronchial epithelial cells (PBEC), cultured at the air-liquid interface (ALI) were exposed to whole cigarette smoke, at 3 h and 24 h thereafter, gene expression of cPLA2 was assessed (A), or cultures were harvested for LC-MS analysis to determine levels of linoleic acid (LA), alpha-linolenic acid and gamma linoleic acid (ALA/GLA)[#], arachidonic acid (AA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) (B–F). Data are depicted as median with interquartile range. Data in A are shown as target gene expression normalized for the geometric mean expression of the reference genes ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide (*ATP5B*) and Ribosomal Protein L13a (*RPL13A*); n = 21 different donors. Statistical significance between groups was tested using a non-parametric one-way ANOVA Friedman test with a Dunn's multiple comparisons *post hoc* test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. [#]Due to technical limitations the signal obtained for the here applied QqQ settings (ALA/GLA) shown in panel C accounts for measurement of both ALA and GLA.

important activities of novel PUFA-derived lipid mediators have been discovered that promote resolution of inflammation. Such mediators may be especially important in chronic inflammatory diseases where resolution of inflammation is lacking. These lipid mediators may impact disease pathology and could serve as therapeutic targets to increase resolution of inflammation.

As little is known about PUFA metabolism in COPD, we aimed to investigate PUFA metabolism in stable COPD patients and during acute exacerbations and compare with smokers without COPD. We observed lower levels of several omega-3 and -6 PUFAs in sputum from patients with stable COPD compared to smoking controls. Both EPA and DHA are important sources for pro-resolving mediators, and reduced levels of these PUFAs could therefore limit the availability of these pro-resolving mediators. Unfortunately, the supernatants of sputum we used were cell-free and no plasma or erythrocyte samples were available. We could therefore not assess whether cell membranes in these patients also showed signs of reduction in levels of PUFAs or whether our findings are also reflected on a systemic level. This is relevant, since several studies have reported lower levels of free fatty acids [23] or PUFAs [24] in plasma and of (specific omega-3) PUFAs in erythrocyte cell membranes from patients with COPD compared to controls [13]. Malnutrition was suggested to underlie the observed reduced PUFA levels in one study [23]. However, as our correlation plots from stable COPD patients suggested a general enhancement of PUFA conversion compared to controls, the question arises whether reduced levels of fatty acids solely reflect malnutrition of these patients, or whether enhanced PUFA metabolism in COPD may deplete essential fatty acids also when sufficient amounts are consumed and incorporated into the cellular membranes. Supplementation with omega-3-derived pro-resolving mediators rather than omega-3 fatty acids themselves might therefore be warranted in COPD patients as a possible strategy to promote inflammation resolution. Several studies support this therapeutic strategy as results show beneficial effects of pro-resolving compounds on cellular function and inflammation *in vitro* [25] and in mice models [26–28] using CS or organic dust exposures. A possible explanation for the alteration in PUFA conversion in COPD was provided by our *in vitro* data and those of others [29], suggesting that remodelling of the airway epithelium could underlie the altered conversion of PUFAs in patients with COPD. Remodelling was found to markedly impact the expression of the converting enzymes and possibly also their activity, although the latter has yet to be proven. Since epithelial remodelling is more pronounced in COPD patients than in (ex)smoking controls with a normal lung function, our *in vitro* studies help to explain the observed differences in sputum lipid mediator levels between COPD patients and

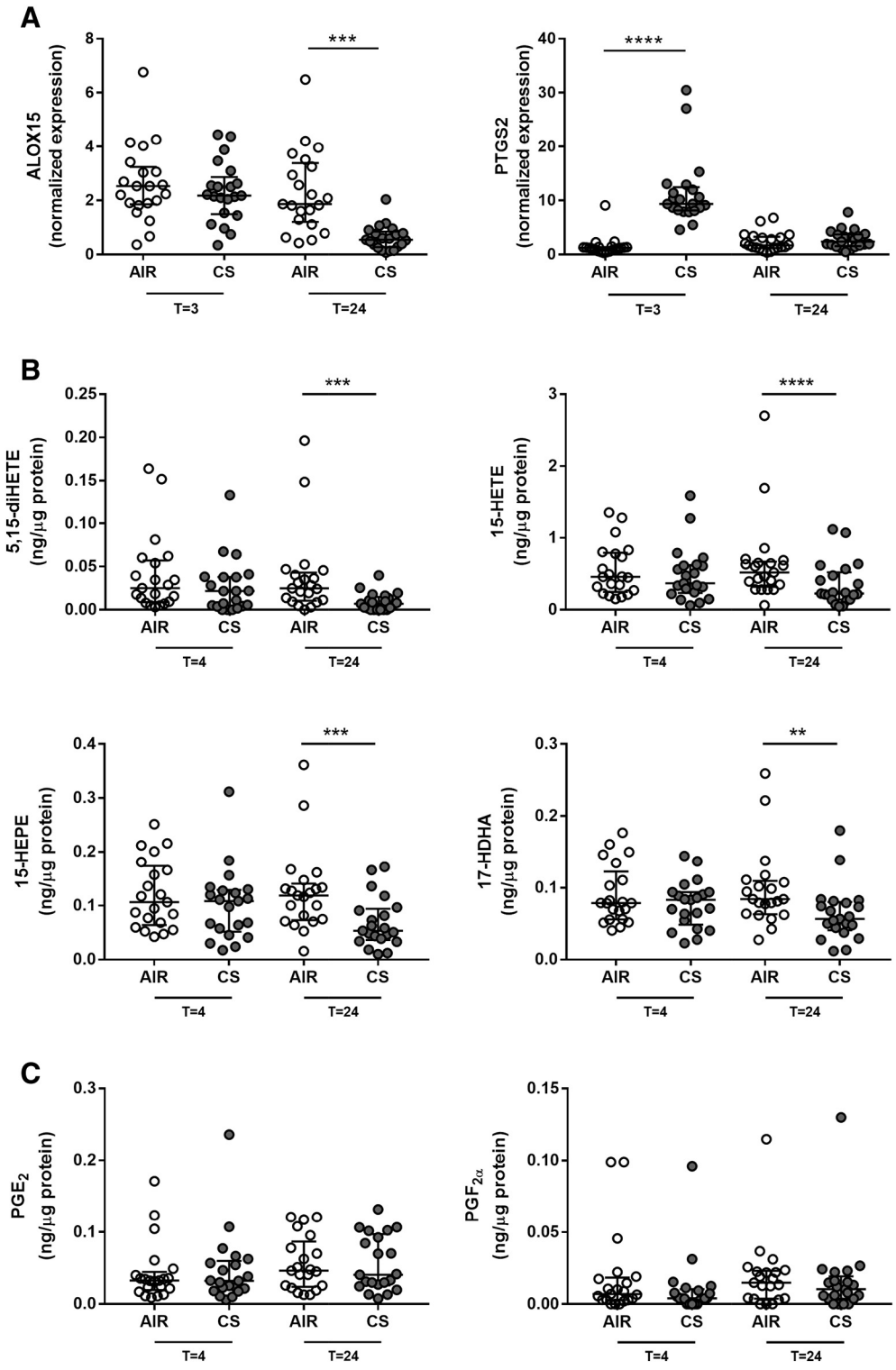

Fig. 6. Whole cigarette smoke exposure of air-liquid interface cultured primary bronchial epithelial cells reduces *ALOX15* (15-LOX) expression and converted mediators. Primary bronchial epithelial cells (PBEC), cultured at the air-liquid interface (ALI) were exposed to whole cigarette smoke (CS), at 3 h and 24 h thereafter, gene expression of *ALOX15* (15-LOX, A) and *PTGS2* (COX-2, B) was assessed. In addition, cultures were harvested for LC-MS analysis to determine levels of 5,15-dihydroxyeicosatetraenoic acid (5,15-diHETE), 15-hydroxyeicosatetraenoic acid (15-HETE), 15-hydroxyeicosapentaenoic acid (15-HEPE) and 17-hydroxydocosahexaenoic acid (17-HDHA), prostaglandin E_2 (PGE_2) and prostaglandin $F_2\alpha$ ($PGF_2\alpha$) (C–F). Data are depicted as median with interquartile range. Data in A and B are shown as target gene expression normalized for the geometric mean expression of the reference genes ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide (*ATP5B*) and Ribosomal Protein L13a (*RPL13A*); n = 21 different donors. Statistical significance between groups was tested using a non-parametric one-way ANOVA Friedman test with a Dunn's multiple comparisons *post hoc* test. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

controls. Additional research is needed to determine the feasibility of a therapeutic strategy targeting this pathway to promote resolution in the lungs of patients with COPD. This is supported by a study in mice, showing beneficial effects of Resolvin D1 on lung inflammation caused by cigarette smoke exposure [28].

This study has several limitations that are worth mentioning: i. no conclusions can be drawn regarding the role of PUFA metabolism in COPD disease severity and progression, since stratification on GOLD stage was not possible because of the small patient numbers in this study, ii. Standardization of sample collection: samples from COPD patients in STBL and AE phases were mostly paired (same donor for both situations), however as these were collected at very different time points between patients and in different order of sampling, paired testing could not be performed; furthermore no long-term follow up samples were available. iii. No end products such as resolvins or maresins could be detected in the sputum samples, however many precursors could be found. One reason for this could be a possible short *in vivo* half-life of these end-products [30,31], or possible binding of these compounds to receptors on cell membranes (*e.g.* for signalling purposes) [32]. Lastly, no dietary information is available of these donors

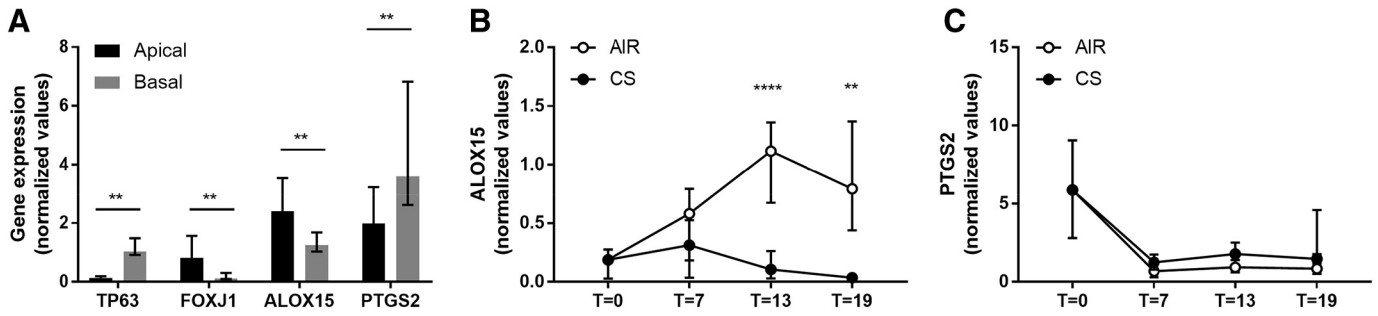

Fig. 7. Impact of repeated cigarette smoke exposure of primary bronchial epithelial cells during epithelial differentiation on *ALOX15* (15-LOX) and *PTGS2* (COX-2) expression.

Fully differentiated (unexposed) PBEC cultured for ~3 weeks at the air-liquid interface (ALI) were split to obtain a luminal cell-enriched fraction and a basal cell-enriched fraction. A) Enrichment was confirmed using the basal cell gene expression marker *TP63* and the luminal (ciliated cell) gene marker *FOXJ1*. In addition, *ALOX15* and *PTGS2* expression was assessed. B–C) Primary bronchial epithelial cells (PBEC) were air exposed (T = 0) after which the cultures were once daily exposed to whole cigarette smoke (CS) or air as a control (AIR); this routine was repeated for 19 days in total and cells were harvested at the indicated time points for qPCR analysis of *ALOX15* (15-LOX, B) and *PTGS2* (COX-2, C) gene expression. Data in A are shown as target gene expression normalized for the geometric mean expression of the reference genes ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide (*ATP5B*) and Ribosomal Protein L13a (*RPL13A*). Data in B and C are shown as target gene expression normalized for the geometric mean expression of the reference genes *ATP5B*, *RPL13A* and β2-microglobulin (*B2M*) (n = 8 different donors for A–C, data are depicted as median with interquartile range). Statistical significance between groups was determined using a Wilcoxon matched-pairs signed rank test (A) and a two-way ANOVA and Sidak's *post-hoc* test (B and C). $^{**}p < 0.01$, $^{****}p < 0.0001$ between groups.

during the time of the study and we therefore cannot discriminate if the observed findings were partly a result of changes in diet in this patient group, an intrinsic change in the metabolism, or both. Despite these limitations, the results show clear differences between groups, further supported by unsupervised multivariate analysis. A score plot of a principal component analysis (PCA) model (Fig. S3) illustrates a degree of the groups clustering; with the first two components covering > 50% of the total variance trend for the group relate clustering is evident. Off note, only one COPD patient used oral steroids at time of sputum sampling, and therefore this is unlikely to have affected the data.

It is interesting to note that during AE, the pattern of produced lipid mediators was strongly skewed in presence of high sputum eosinophil numbers. In contrast to neutrophils, eosinophils express the 15-LOX converting enzyme [33] and we indeed found that high eosinophil levels strongly correlated with the presence of 15-LOX-converted mediators. In addition, high sputum eosinophil levels correlated with high LTE_4 levels, an eicosanoid produced by eosinophils. LTE_4 is a known bronchoconstrictor [34], that also activates Th2 cells, induces IL-8 gene expression by Th2 cells and promotes neutrophil activation (*e.g.* migration and survival) *in vitro* [35]. As not all, but only a small subgroup of patients displayed high sputum eosinophil levels (Fig. S2), it is tempting to speculate that patients with high levels of eosinophils and the correlated mediators represent a specific subgroup of COPD patients such as for example ACOS patients (Asthma COPD Overlap Syndrome) who are characterized by Th2 inflammation, including increased levels of eosinophils. Our observations suggest a molecular basis for the use of cysteinyl-leukotriene receptor (CysLT) antagonists such as montelukast in COPD, which have shown possible beneficial effects on the therapeutic control of COPD [36]. Possibly COPD subgroups with high LTE_4 levels during AE may benefit from a personalized therapeutic approach using Cys-LT receptor antagonists and/or COX-2 inhibitors.

During AE, we furthermore noticed increased levels of COX-2-converted prostaglandins and thromboxane B2 that showed a negative correlation with FEV_1, particularly in the case of PGE_2 and $PGF_{2\alpha}$. An inverse correlation between PGE_2 and FEV_1 in induced sputum from COPD patients was also reported by others [37,38] and suggested to be mediated by MMP-2 [37]. Our *in vitro* data show how both acute cigarette smoke exposure and remodelling of the airway epithelium as a result of this exposure could favour expression of COX-2 at a cost of expression of LOX enzymes, therefore we hypothesize that the production of prostaglandins could also be related to the level of epithelial remodelling. Unexpectedly, in contrast to the previously mentioned study [38], we did not find increased LTB_4 levels in sputum from patients with COPD, rather a reduction. This could be explained by the fact that our controls are smoking controls and possibly these already have elevated levels in their sputum. It could also be a discrepancy between exhaled breath condensate used in the referred study and sputum in our study as this has been shown to not give similar results with regard to LTB_4 levels [39].

In summary, we show that patients with COPD have disturbed PUFA metabolism in stable state and during AE with indications of compromised production of precursors for pro-resolving mediators. We furthermore show *in vitro* that both acute exposure of the airway epithelium as well as smoke-induced remodelling of the airway epithelium could contribute to changes in PUFA metabolism.

Further investigations are needed to understand how changes in PUFA conversion and derived lipid mediator levels modulate airway epithelial functioning and differentiation. This knowledge is essential to delineate the potential of PUFA metabolism as therapeutic target to promote resolution of chronic lung inflammation in COPD patients. Furthermore, our findings propose that molecular investigations such as those in the presented study may contribute to the development of personalized therapies for specific groups of COPD patients.

Author contributions

Conception and design: AD, MH, TE, PH, MG; Sample preparation: AD, MH, LP, MA, PB, TE; analysis and interpretation: AD, MH, OM, TE, PH, MG; drafting the manuscript for important intellectual content: AD, TE, PH, MG. All authors have read the manuscript, provided input and agree with its submission.

Conflict of interest

AD reports grants from EU Marie Curie Intra-European Fellowship and a grant from ZonMW enabling technologies hotels during the conduct of the study; LP reports grants from Chiesi outside the submitted work; PB reports grants from GlaxoSmithKline during the conduct of the study; personal fees from AstraZeneca, personal fees from Pfizer, personal fees from Mundipharma, personal fees from Boehringer-Ingelheim, personal fees from Advisory board AstraZeneca, personal fees from Advisory board Boehringer-Ingeheim, personal fees from Advisory board GlaxoSmithKline outside the submitted work; TE reports personal speaker fees from AstraZeneca, personal speaker fees from Boehringer Ingelheim, outside the submitted work. PH reports grants from ZonMW enabling technologies hotels grant (grant

#435002003) during the conduct of the study and grants from Boehringer Ingelheim and grants from Galapagos N.V. outside the submitted work; MH, OM, MA and MG have nothing to disclose.

Transparency document

The Transparency document associated with this article can be found, in online version.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbalip.2018.11.012.

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