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Original Contribution

Metabolomic profiling of regulatory lipid mediators in sputum from adult cystic fibrosis patients ☆

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ABSTRACT

Retained respiratory tract (RT) secretions, infection, and exuberant inflammatory responses are core abnormalities in cystic fibrosis (CF) lung disease. Factors contributing to the destructive CF airway inflammatory processes remain incompletely characterized. The pro-oxidative inflammatory CF RT milieu is known to contain enzymatically and nonenzymatically produced regulatory lipid mediators, a panel of structurally defined oxidized metabolites of polyunsaturated fatty acids known to play a role in pathology related to inflammation. Using an extraction protocol that maximizes recoveries of sputum-spiked deuterated standards, coupled with an LC/MS/MS detection system, this study presents a metabolomic method to assess a broad spectrum of regulatory lipid mediators in freshly obtained sputum from CF patients. A broad range of both proinflammatory and anti-inflammatory lipid mediators was detected, including PGE2, PGD2, TXB2, LTB4, 6-*trans*-LTB4, 20-OH-LTB4, 20-COOH-LTB4, 20-HETE, 15-HETE, 11-HETE, 12-HETE, 8-HETE, 9-HETE, 5-HETE, EpETrEs, diols, resolvin E1, 15-deoxy-PGJ2, and LXA4. The vast majority of these oxylipins have not been reported previously in CF RT secretions. Whereas direct associations of individual proinflammatory lipid mediators with compromised lung function (FEV-1) were observed, the relationships were not robust. However, multiple statistical analyses revealed that the regulatory lipid mediators profile taken in aggregate proved to have a stronger association with lung function in relatively stable outpatient adult CF patients. Our data reveal a relative paucity of the anti-inflammatory lipid mediator lipoxin A4 in CF sputum. Patients displaying detectable levels of the anti-inflammatory lipid mediator resolvin E1 demonstrated a better lung function compared to those patients with undetectable levels. Our data suggest that comprehensive metabolomic profiling of regulatory lipid mediators in CF sputum should contribute to a better understanding of the molecular mechanisms underlying CF RT inflammatory pathobiology. Further studies are required to determine the extent to which nutritional or pharmacological interventions alter the regulatory lipid mediators profile of the CF RT and the impact of potential modulations of RT regulatory lipid mediators on the clinical progression of CF lung disease.

Introduction

Cystic fibrosis (CF) is caused by mutations in the gene encoding the CF transmembrane conductance regulator (CFTR). Absent or defective CFTR results in abnormal salt transport and mucus hydration at epithelial surfaces where it is expressed. In the lung, respiratory tract (RT) secretions (mucus) are more viscoid, resulting in compromised mucociliary RT clearance, chronic RT infection, dysregulated and heightened inflammatory responses, and progressive lung tissue destruction [1,2]. Therapeutic interventions include approaches designed to ameliorate the effects of mucus stasis using treatments designed to increase the hydration and removal of RT surface secretions and measures to combat the RT infection and aggressive inflammatory responses [1–8].

Abbreviations: AA, arachidonic acid; CF, cystic fibrosis; CFTR, CF transmembrane conductance regulator; CUDA, 1-cyclohexylureido 3-dodecanoic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; EpETrE, epoxyeicosatrienoic acids; FEV-1, forced expiratory volume in 1 s; HPLC, high-performance liquid chromatography; LC/MS/MS, liquid chromatography/tandem mass spectrometry; LA, linoleic acid; LLE, liquid–liquid extraction; PLS, partial least squares; RT, respiratory tract; sEH, soluble epoxide hydrolase; SPE, solid-phase extraction

☆ Regulatory lipid mediators are a panel of structurally defined oxidized metabolites of polyunsaturated fatty acids. All the regulatory lipid mediators' common names are used in the paper according to LIPIDMAPS nomenclature. The full names are given in Supplementary Table 1.

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Inflammation represents a critical component of the innate host response to infection, and the neutrophil responses generated are critical for killing invading microbes via their generation of antimicrobial products. A hallmark of CF is the early development of persistent and progressively intense neutrophil and inflammatory mediator influxes into the airways [3,6,7,9–12]. The heightened infected/inflammatory CF RT state with its inherent increase in the production of inflammatory mediators, including increased levels of proteolytic and oxidant-generating biomolecule species, is believed to be responsible for the eventual destruction of host RT tissues [6,8,13–15]. More recently, an even more expanded misorchestration of the immune system in CF has emerged [16]. The importance of inflammatory processes is further buttressed by the facts that most known modifiers of the CF gene phenotype that have been reported to date involve inflammatory immune system genes [17–19] and that genomic responses to RT CFTR perturbations are associated with genes regulating inflammation [20]. This is further illustrated by the fact that activation of inflammatory signals, in the apparent absence of any microbes or exogenous stimuli, can damage and destroy RT tissues [21]. Several studies have suggested that once initiated, CF RT inflammatory responses are not well controlled [7,11–13,20–22]. Thus, anti-inflammatory therapies with agents such as steroids and ibuprofen, an inhibitor of arachidonic acid oxygenation pathways, and even inhibitors of NF-κB, a master regulator of inflammation, have been shown to be potentially beneficial, although many have significant side effects [3,5,7,23–28]. More recently, evidence has been presented linking prostaglandin–endoperoxide synthase genes (cyclooxygenase 1 (COX-1) and COX-2) as novel modifiers of disease severity in CF patients [29].

Oxidative lipid products including, notably, the products of unsaturated lipids, are increasingly being recognized as important contributors to chronic inflammatory diseases [24,30–34]. Although most RT therapeutics have been focused on the main routes of the arachidonic acid cyclooxygenase and lipoxygenase pathways [5,11,27–29,35], their P450 and nonenzymatic oxidative products and oxidative products of the other unsaturated lipids (e.g., 20:5 and 22:6) are also responsible for the generation of lipids capable of modifying the intensity and duration of inflammatory processes [24,31,33,36,37], including those of the plant world [38]. Recent advances have facilitated more detailed investigation of profiles of lipid products both in plasma and at inflammatory sites [31,36,39–44]. Further characterization of these profiles is expected to lead to new anti-inflammatory therapeutic approaches directed toward this important class of inflammatory mediators.

CF is known to be associated with abnormalities of lipid absorption and plasma lipid constituents [2,42,45–50]. These abnormalities are primarily secondary to dysfunctional pancreatic and hepatobiliary secretory defects [2]. CF-related lipid abnormalities are also influenced by active inflammatory processes, a measure of oxidative stress (especially in the RT), and more recently described to be influenced by aberrant CFTR modulation of lipid metabolism [51–55]. Thus far studies of amino acid and proteomic profiles of CF [55] and CF RT secretions have been detailed [56–59], but there have been few studies detailing lipidomic profiles of these secretions, which bathe the RT airway surface cells. Given the noninvasive nature of collecting sputum specimens from CF patients and the advent of sophisticated analytical technology for metabolomic analyses [31,36,38–41,60], we reasoned that profiling regulatory lipid mediators (also named "oxylipins" here, to keep the term short in the rest of the paper) in CF sputum would provide a novel means of assessing the possible contributing roles of the spectrum of regulatory lipid mediators to inflammatory processes in the RT of CF patients.

In this study, we have developed and refined an efficient extraction method for sputum oxylipins and characterized the oxylipin profile of freshly obtained sputum from patients attending an adult CF outpatient clinic. We also provide an overview of the substrates and enzymatic pathways involved in generating the identified oxylipins. Finally, we have attempted to provide preliminary correlations between oxylipin profiles and lung function using composite statistical approaches.

Experimental procedures

Patient demographics and sputum collection

Spontaneously expectorated sputum was obtained from 16 patients (10 male, 6 female; age 34 ± 16, range 20–69) attending the University of California at Davis Adult CF Clinic. Fourteen of the patients carried the del F508 mutation (6 were homozygous for del F508), and all had positive sweat chloride tests. The majority of the patients had CF lung disease of moderate severity as assessed by pulmonary function tests (FEV-1 as percentage of predicted; $53 \pm 24\%$ SD, range 27–106%) [61]. Most patients were receiving standard of care CF therapy [62], including antibiotics, aggressive chest physiotherapy including aerosolized bronchodilators, pancreatic enzymes, and vitamin supplements (especially the lipophilic vitamins A, D, E, and K). Eight of the patients were diabetic and none were taking oral corticosteroids. Nine used inhaled preparations that included corticosteroids, several of which were confined to nasal forms alone. As with most CF patient populations, most had low BMI (21.8 ± 2.8 SD, range 16–29). All could produce sputum spontaneously and 13 grew predominantly *Pseudomonas aeruginosa* from their sputa. One of the patients was studied twice over a period of approximately 1 month.

This study was not powered to, nor intended to, relate all of the clinical and therapeutic variables that could potentially affect sputum oxylipin profiles. FEV-1, as a percentage of predicted, was used as a marker of overall CF lung disease severity. Our primary aim was to characterize and quantify the spectrum of oxylipins in the RT of a representative sample of outpatient adults with CF. Sputum samples were coded anonymously and transported on dry ice to the laboratory for analyses. The study protocol was approved by the University of California at Davis Institutional Review Board.

All the sputa were collected directly into a container that already had butylated hydroxytoluene and triphenylphosphine and a broad spectrum COX inhibitor (indomethacin). After the sputum was obtained directly into the container, the specimen was immediately frozen on dry ice. All the samples were then transported on dry ice from the clinic to the lab within 2–6 h and then immediately stored at $-80\ ^{\circ}C$ until analyzed.

Sputum processing

Distilled water was added to the sputum according to the sputum weight (1 ml/g sputum) and vortexed for 10 min to homogenize the sputum. The sputum was then processed by three different extraction protocols (Fig. 1A) as indicated below:

Protocol 1: liquid–liquid extraction (LLE) (CF 1)

The deuterated surrogate solutions (including 500 nmol/L d4-6-keto-PGF1α, d4-TXB2, d4-PGE2, d4-LTB4, d11-14,15-DiHETrE, d6-20-HETE, d4-9-HODE, d8-12-HETE, d8-5-HETE, d4-9(10)-EpOME, and d11-11(12)-EpETrE) were added directly to the sputa. After vigorous vortexing for 10 min, the mixture was extracted three times with ethyl acetate to get optimal extraction

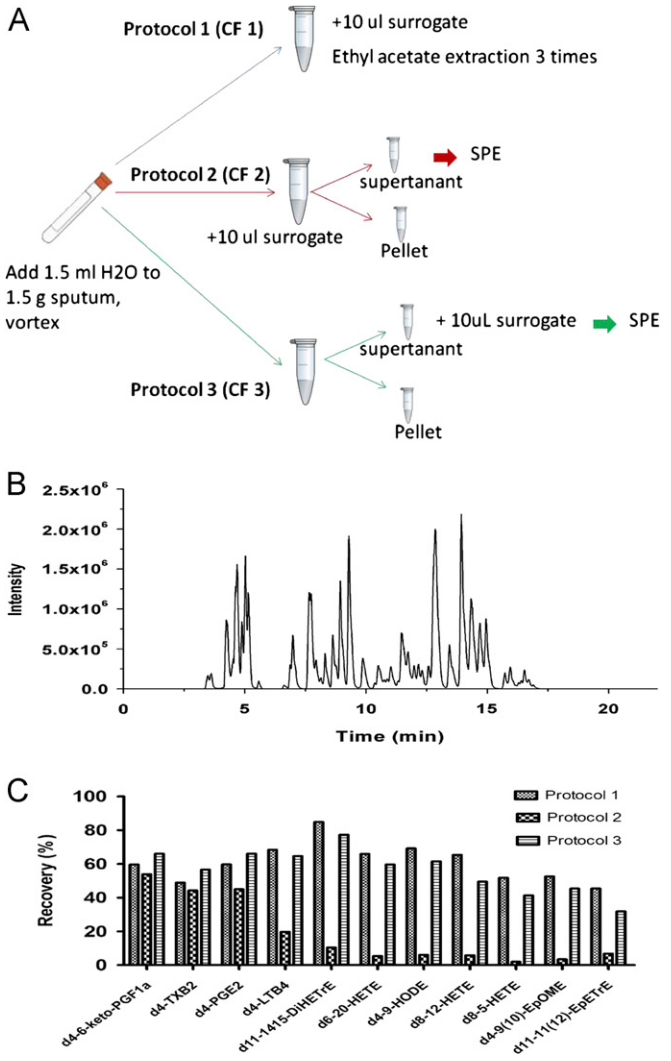

Fig. 1. Schematic illustrations of extraction protocols, mass spectrometric analysis, and extraction recoveries in CF sputum samples. (A) Schematic for the extraction protocols. Protocol CF 1 is liquid–liquid extraction (LLE); protocols CF 2 and CF 3 are the SPE extraction protocols. To assess recovery, the surrogate solutions (including 10 deuterated internal standards) were added to the solution before or after centrifugation, respectively. (B) Representative LC/MS/MS total ion chromatogram of the profile of oxylipins in CF sputum. (C) The average recovery rate comparison among three extraction protocols. The recovery rates of the LLE are from 48 to 85%.

recovery. Extracts from each fraction were combined and evaporated to dryness using a SpeedVac system. The residue from each fraction was then reconstituted with 50 μl of methanol containing 200 nM 1-cyclohexylureido 3-dodecanoic acid (CUDA) as an internal standard. This protocol was used to determine the extraction efficiency of LLE.

Protocol 2: solid-phase extraction (SPE) after surrogate solution addition (CF 2)

Surrogate solution (30 μl) was added directly to the sputum. The sputum sample was then centrifuged at 13,200 rpm for 10 min at 4 °C. The soluble supernatant fraction was loaded onto pretreated 60-mg Oasis-HLB cartridges (Waters Corp., Milford, MA, USA) according to methods previously described [60]. The SPE cartridges were then eluted with first 0.5 ml methanol and then 1.5 ml ethyl acetate. The eluents were evaporated to dryness using a SpeedVac system and reconstituted with 50 μl of 200 nM CUDA/methanol solution. This protocol not only provides quantitation based upon the internal standard, but also assesses extraction efficiency. This protocol provides the extraction efficiency of the whole protocol including the SPE step.

Protocol 3: SPE before surrogate solution addition (CF 3)

This sample processing protocol was essentially the same as protocol CF 2; however, 10 μl of surrogate solution was added after all extractions were performed. This protocol provides extraction efficiency just for the SPE step.

Oxylipin profiling by LC/MS/MS

The liquid chromatography system used for analysis was an Agilent 1200 SL liquid chromatography series (Agilent Corp., Palo Alto, CA, USA). The autosampler was kept at 4 °C. Liquid chromatography was performed on an Eclipse Plus C18 2.1 × 150 mm, 1.8-μm column (Agilent Corp.). Mobile phase A was water with 0.1% glacial acetic acid. Mobile phase B consisted of acetonitrile/methanol (84/16) with 0.1% glacial acetic acid. Gradient elution was performed at a flow rate of 250 μl/min. Chromatography was optimized to separate all analytes in 21.5 min. Analytes are then eluted according to their polarity with the most polar analytes, prostaglandins, and leukotrienes, eluting first, followed by the hydroxy and epoxy fatty acids.

The column was connected to a 4000 QTrap tandem mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with an electrospray source (Turbo V). The instrument was operated in negative multiple-reaction monitor mode. HPLC and LC/MS/MS protocols were essentially as previously described [60].

Quality control samples were analyzed at a minimum frequency of 10 h to ensure stability of the analytical calibration throughout a given analysis. Analyst software 1.5.1 was used to quantify oxylipins according to standard curves.

Statistical approaches

A correlation analysis was employed to explore the relationship between the oxylipins and lung function (FEV-1, % of predicted). Prism 4.0 (GraphPad Software, San Diego, CA, USA) was used to perform nonparametric (Spearman) correlation analysis. Partial least squares (PLS) analysis was used as the classification method for modeling the sputum oxylipin profiles. Multivariate analysis was performed using the SIMCA-P 11.5 version (Umetrics AB, Umeå, Sweden). As a powerful multivariate analysis method, PLS can be seen as a particular regression technique for modeling the association between the factors (oxylipin concentrations) of the observations (CF patients) and the responses (lung function) of the observations.

Results

Oxylipin extraction efficiency

To assess the recovery efficiency of the various extraction methods utilized, a surrogate solution, which includes 11 deuterated internal standards (d4-6 keto PGF1α, d4-TXB2, d4-PGE2, d4-LTB4, d11-14,15 DiHETrE, d6-20 HETE, d4-9-HODE, d8-12 HETE, d8-5 HETE, d4-9(10) EpOME, d11-11(12) EpETrE), was added to the samples at different steps during the extraction procedures. Fig. 1A illustrates the three extraction protocols utilized and the stages at which the surrogate solutions were added. Extraction protocol 1 (CF 1) used LLE, whereas extraction protocols 2 and 3 (CF 2 and CF 3) used SPE. The only difference between CF 2 and CF 3 is that the recovery rate of CF 3 reflects only the recovery rate of the SPE, and the recovery rate of CF

Table 1
The measured oxylipin baseline in CF sputum samples[a].

(A)

Pathway:	COX					5-LOX						
Substrate:	AA				DGLA	AA					EPA	
Sample	TXB2	PGF2a	PGE2	11-HETE	PGE1	20-COOH-LTB4	20-OH-LTB4	LTB4	6-*trans*-LTB4	5-oxo-ETE	5-HEPE	LTB5
1	342	535	601	168	79.1	3,760	1380	319	236	7,480	239	99.8
2	88.5	188	103	11.7	30.9	157	103	87.3	31.0	153	12.6	0.00
3	155	69.6	116	84.2	29.7	560	252	253	193	338	24.4	0.00
4	1,340	410	1060	187	70.5	9,740	1500	2630	238	3,440	107	23.0
5	11,800	344	750	207	60.6	1,240	293	201	95.8	747	22.0	ND
6	864	524	745	829	180	2,910	733	979	2730	57.3	267	ND
7	1,080	321	630	208	43.3	899	236	274	53.3	71.3	ND	ND
8	691	372	1220	128	120	726	95.2	170	89.1	553	20.1	ND
9	145	202	191	95.3	29.6	3,500	396	1610	63.9	4,240	119	41.1
10	219	32.9	254	13.0	8.35	1,440	416	644	47.1	1,630	27.1	ND
11	6,840	152	228	22.4	24.9	1,490	156	118	165	1,130	0.00	ND
12	2,330	32.9	85.3	ND	9.07	3,940	514	788	152	1,280	76.9	12.1
13	562	418	536	131	130	5,690	737	5340	1370	23,500	930	108
14	629	786	561	316	49.8	4,240	2240	2810	226	3,990	259	70.7
15	619	154	390	82.2	26.6	3,080	2050	1590	70.9	2,870	46.5	15.9
16	101	133	67.3	817	N.D.	7.42	48.0	280	49.6	2,300	128	ND
17	1,230	699	935	298	67.0	13,700	3230	6750	574	13,700	417	156
Mean	1,710	316	498	212	56.4	3,360	1240	1630	376	3,970	159	31.0
SD	3,050	226	361	249	48.4	3,610	1830	1960	686	6,090	232	48.2

(B)

Pathway:	12-LOX					15-LOX				CYP 4A/4F
Substrate:	AA		EPA	LA		AA		DGLA	LA	AA
Sample	12-HETE	12-oxo-ETE	12-HEPE	9-HODE	9-oxo-ODE	8-HETE	15-oxo-ETE	15(S)-HETrE	13-HODE	20-HETE
1	371	837	21.7	941	305	92.6	49.5	220	820	488
2	3,070	3,520	209	262	82.6	40.5	76.8	200	912	31.8
3	8,580	11,100	287	3260	3900	258	739	1010	6,700	185
4	10,800	8,430	479	770	350	108	274	448	2,730	195
5	7,010	5,090	141	1030	293	278	507	667	2,320	2250
6	174,000	47,900	4700	3070	2340	1160	419	944	14,400	657
7	10,200	4,450	590	969	357	163	296	227	2,780	531
8	10,100	4,980	314	557	201	41.7	111	224	1,420	411
9	1,280	924	115	746	225	110	97.7	181	2,490	344
10	3,110	4,400	168	196	165	21.3	130	127	397	281
11	15,600	20,600	375	196	36.2	85.5	69.6	96.8	786	471
12	5,510	4,800	329	788	212	54.4	56.4	141	4,460	ND
13	8,540	7,010	282	807	428	219	370	993	2,380	335
14	852	836	23.1	766	403	269	259	420	1,850	667
15	1,820	4,020	15.4	390	164	61.6	215	154	1,520	51.6
16	1,310	777	115	2700	328	1210	670	3360	10,700	845
17	3,860	3,720	174	1790	572	353	406	799	5,490	657
Mean	15,600	7,850	490	1130	610	266	279	601	3,650	494
SD	41,000	11,400	1100	978	994	359	214	783	3,800	514

(C)

Patient	CYP 2C/2J			CYP 2C/2J+sEH			Multienzymes			
	AA	LA		EPA	LA		LA			EPA
	8(9)-EpETrE	12(13)-EpOME	9(10)-EpOME	5,6-DiHETE	12,13-DiHOME	9,10-DiHOME	9,12,13-TriHOME	9,10,13-TriHOME	LXA4	Resolvin E1
1	21.4	54.4	61.6	275	ND	ND	ND	ND	ND	522
2	69.8	49.1	47.2	4.09	ND	ND	ND	ND	ND	ND
3	309	184	81.0	80.6	54.9	24.4	445	103	ND	ND
4	38.9	60.0	16.7	97.5	19.3	29.2	472	48.3	ND	758
5	259	68.3	61.0	27.8	379	299	11,400	622	ND	486
6	4090	474	222	1650	76.4	37.2	3,260	150	ND	495
7	66.3	69.5	107	12.1	59.0	27.7	1,830	215	ND	280
8	84.5	ND	19.9	27.3	579	706	19,700	711	ND	ND
9	11.3	58.3	39.7	80.9	78.7	34.7	1,370	162	ND	ND
10	99.9	8.27	ND	39.0	86.8	7.21	867	95.2	ND	ND
11	163	8.09	ND	33.7	68.9	66.1	2,920	379	ND	106
12	224	47.2	ND	196	29.7	9.55	784	37.7	ND	ND
13	77.9	36.8	ND	1560	67.3	ND	391	57.3	ND	173
14	30.3	ND	61.0	234	74.2	43.0	4,200	113	ND	ND
15	22.4	99.0	41.4	278	12.2	ND	435	149	ND	670
16	6.66	34.3	42.3	135	8.68	2.47	499	97.0	ND	ND
17	251	0.79	85.1	412	77.8	70.8	2,340	209	ND	1040
Mean	343	73.6	52.1	303	52.1	35.7	3,540	332	ND	338
SD	970	113	54.7	504	415	308	7,770	379	ND	358

ND, not detected.
[a] All the concentrations are in pmol/g.

2 actually reflects the real recovery of the whole extraction process. Fig. 1B illustrates an exemplary LC/MS/MS total ion chromatogram of oxylipins included in our profiling method.

A high recovery rate using the CF 3 protocol is expected. CF 3 reflects the recovery rate of the SPE process of the relatively clean matrix–supernatant. The recovery rates of the whole SPE process, the one corresponding to CF 2, were much less than those from CF 3. Extraction recoveries for each of the three methods are illustrated in Fig. 1B. In addition, recovery rates of the deuterated internal standards using CF 2 decreased according to their polarity. The most polar analyte (d4-6 keto PGF1α) has a comparable recovery rate using the CF 3 protocol. The most nonpolar analyte (d11-11(12) EpETrE) was barely recovered from sputum samples using the CF 3 protocol. This suggests that the distribution of these oxylipins is related to their polarity. Given the more lipophilic properties of the insoluble pellet fraction, the oxylipins could be absorbed on some lipid, protein, or DNA fragments. This indicates that the oxylipins predominantly resided in the pellet fraction.

The recovery rates using the CF 1 protocol, LLE of the entire CF sputum sample, showed the most promising results. Most of the deuterated standards have 46–82% recovery rates. Compared to the ~20% recovery rates reported in the literature [63,64], the recovery rate of our current extraction protocol is significantly improved. The reason for the better recovery rates from CF 1 might be that the CF 1 extraction protocol used whole sputum (both supernatant and pellet parts) for the extraction. Therefore, the LLE protocol (CF 1) was utilized in the processing and analysis of all further CF sputum samples in our study.

Oxylipin profiles in adult CF sputum

Table 1 shows the concentrations of oxylipins in 17 distinct CF patient sputum samples using the LLE protocol and LC/MS/MS quantitation. Of the 88 oxylipins that are included in our metabolomic profiling method, 31 oxylipins were detectable in adult CF sputum. Only 9 of them (PGE2, PGF1, PGF2α, thromboxane B2 (TXB2), 6-oxo-PGF1, 8-iso-PGF2α, cys-LTs) have been reported in previous studies [65–67]. To the best of our knowledge, this is the most comprehensive oxylipin profile described in CF RT secretions to date.

Fig. 2A shows the composition of the oxylipins according to the parent fatty acid substrates. By far, the most prominent oxylipins (~75%) are derived from arachidonic acid (AA; C20:4). The arachidonic acid metabolites are typically referred to as eicosanoids, which include many biologically important lipid mediators, such as prostaglandins and leukotrienes. The second largest fatty acid parent molecule is linoleic acid (LA; 18:2). EPA and DHA represent relatively small fractions of the oxylipins detected in this study. Fig. 2B illustrates the breakdown of enzymes involved in the synthesis of the oxylipins that we have quantified in CF sputum. The largest source of oxylipins is the 12-lipoxygenase (12-LOX) pathway (~52%), which produces mainly 12-HETE and 8-HETE, and also 12-oxo-ETE. The second and the third most prevalent enzymes involved in the synthesis of oxylipins in CF sputum are those of the 5-LOX and COX pathways. The major products of the 5-LOX pathway are the leukotrienes, which are potent chemokines. The products of the COX pathway include several prostaglandins and thromboxane. Many of these oxylipins are inflammatory mediators that induce inflammatory cell infiltration, although the full biological activities of many of them remain to be fully characterized, both alone and in combination.

Oxylipin profiles and pulmonary function

Given that our method allowed for the precise quantitation of oxylipins in adult sputum, we next examined the relationships

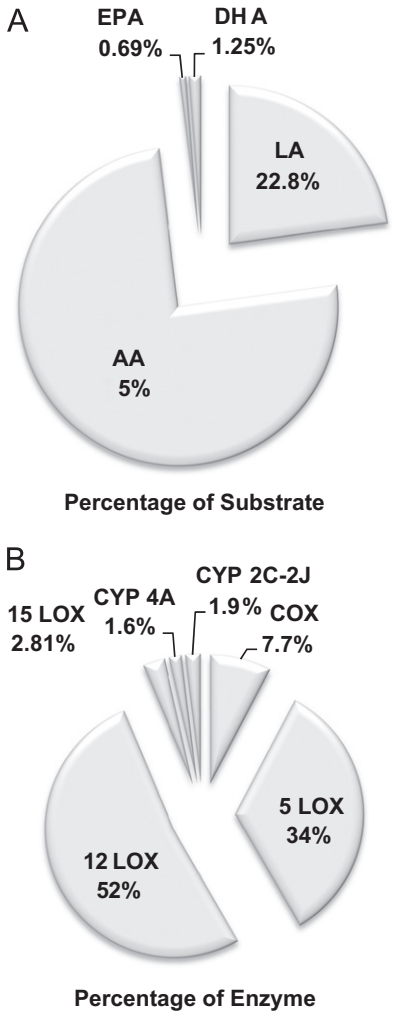

Fig. 2. Distribution of fatty acid precursors and synthetic enzymes of oxylipins profiled in sputum samples from CF patients. (A) Distribution of oxylipins derived from parent fatty acids in CF sputum. LA, C18:2, linoleic acid; AA, C20:4, arachidonic acid; EPA, C20:5, eicosapentaenoic acid; DHA, C22:6, docosahexaenoic acid. (B) Distribution of enzymes responsible for oxylipins detected in CF sputum. COX, cyclooxygenase; LOX, lipoxygenase; CYP, cytochrome P450.

among oxylipin levels and lung function (FEV-1, expressed as % predicted). Spearman correlation analysis was used to correlate exemplary oxylipins with lung function. Fig. 3 illustrates three representative examples. One of the epoxides of linoleic acid, 12(13)-EpOME, was weakly positively correlated with FEV-1 (% of predicted; $r=0.507$, $p<0.05$). 12(13)-EpOME has been regarded as a potentially toxic compound (e.g., leukotoxin); however, previous studies [68] indicate that the toxic effects are actually attributed to its hydrolysis product, the linoleic acid diol (12(13)-DiHOME). Indeed, the epoxide of linoleic acid may actually have anti-inflammatory effects, and high levels of its diol product are clearly proinflammatory. In fact, a correlation between the EpOME/DiHOME ratio and the FEV-1 was observed (data not shown). This implies that DiHOME might also have adverse effects on lung function [68]. A slight negative correlation between FEV-1 and the chemokine LTB4 is shown in Fig. 3B. Additionally, Fig. 3C indicates a minor positive correlation between TXB2 and FEV-1 ($r=0.523$; $p=0.042$). A summary of the synthetic pathways representative of LTB4, 12(13)-DiHOME, and TXB2 is illustrated in Fig. 3D and provides examples of 5-LOX, cytochrome P-450 (CYP 2C/2J), and COX, respectively. As shown in Fig. 4, we observed that CF patients with detectable levels of the anti-inflammatory oxylipin resolvin E1 displayed better lung function than those that did not have detectable levels of this oxylipin ($p=0.059$).

Multivariate metabolomics analysis

As shown in the previous sections, there are at best only weak correlations between individual oxylipins and lung function. As such, we next aimed to define whether oxylipin levels in aggregate had predictive value. To define this, we employed multivariate analysis. The score plot (Fig. 5A) and loading plot (Fig. 5B) of PLS analysis based on the sputum oxylipin profiles are illustrated. The PLS technique is a good way to present the multivariate data because it can reduce the redundancy of the covariance existing in the dataset. In the score plot, each point represents a single sputum sample. The distance between points reflects the similarity of the profiles considering all of the detectable oxylipins in aggregate. From the score plot shown in Fig. 5A, a clear trend can be appreciated in that the lower left points have the lowest lung function and the upper right data points have the best lung function. This suggests that the oxylipin profiles in aggregate presented by a PLS score plot can reflect lung function. The two points labeled in the circle in Fig. 5A are two sputum samples acquired from the same patient at clinic visits a month apart. They are the closet points in the figure and provide some indication of the possible reproducibility of the technique temporally in the same patient. It implies that the point position actually has more information about the patients themselves and might take into account such factors as diet and magnitude of airway inflammatory processes. The loading plot shown in Fig. 5B reflects correlations between specific oxylipins and lung function. Each point in this plot corresponds to a specific oxylipin detected in the total number of patients. The closer the data point lies to FEV-1, the higher the correlation of the oxylipin to FEV-1. From the plot, it can be appreciated, as examples, that leukotrienes (LTB4s) negatively correlate with lung function and 12(13)-EpOME is positively correlated with lung function. Fig. 5C shows clearly the relationships between these oxylipins and lung function. TXB2 correlates with lung function in a highly positive manner, whereas LTB4 and its metabolites negatively correlate with lung function. Moreover, PGE2 also negatively correlates with lung function.

Discussion

This study provides a comprehensive characterization and quantitation of RT oxylipins in adult CF patients. The LC/MS/MS method utilized in this study enabled the detection of 31 diverse oxylipins in CF sputa, which is three times more than the total detected oxylipin numbers from cumulative data from multiple papers in the literature. The representative adult study group showed a wide diversity of bioactive oxylipins, as would be expected from to the intensely bacteria-infested, neutrophil-infiltrated, and inflamed adult CF RT. As has been shown for other biomarkers of CF RT inflammation [12,69], the magnitude of individual proinflammatory bioactive lipids generally display a positive relationship with decrements in pulmonary function (Fig. 3), but that is not always so clear. Interestingly, some oxylipins, such as resolvin E1, show a positive relationship with better lung function (Fig. 5). The novelty of our current metabolomic assessment of the oxylipin profile in CF sputum is that this approach allows for the use of aggregate data to predict lung function. This appears to be superior to assessing lung function/inflammatory state using individual oxylipin metabolites. We discuss the current study from a perspective of technological

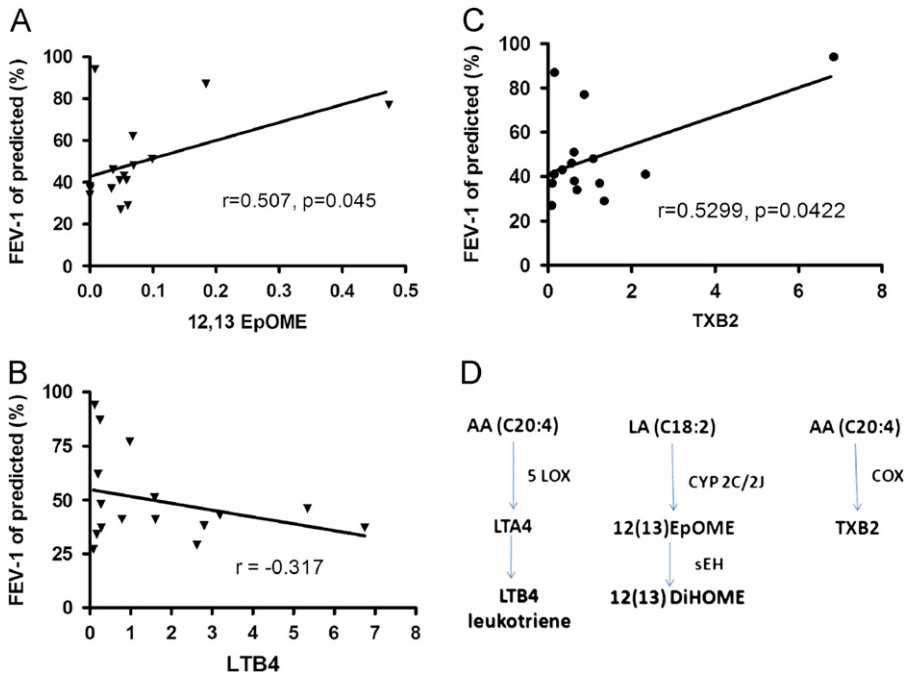

Fig. 3. Positive and negative correlations between lung function (FEV-1, % of predicted) and exemplary oxylipin concentration in CF sputum. (A) Positive correlation between 12,13-EpOME and FEV-1. (B) Negative correlation between LTB4 and FEV-1. (C) Positive correlation between TXB2 and FEV-1. (D) Enzymatic synthetic pathways corresponding to 12,13-EpOME, LTB4, and TXB2.

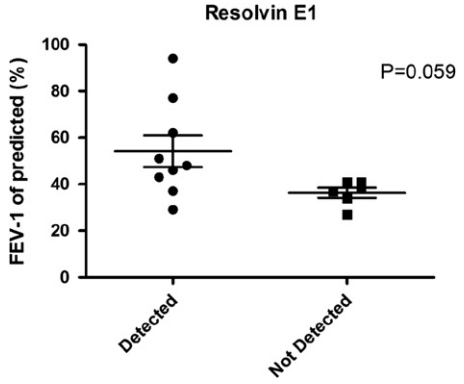

Fig. 4. Detection of the anti-inflammatory lipid mediator resolvin E1 in sputum is associated with better lung function in CF patients. Patients were divided into two groups: those with detectable resolvin E1 and those who had no detectable levels. FEV-1 values were then plotted for the two separate groups. $p=0.059$. The limit of detection for resolvin E1 is 2.4 pmol/g.

consideration, the balance between proinflammatory and anti-inflammatory oxylipins, the role that bacteria (especially *P. aeruginosa*) may play in oxylipin formation and metabolism, clinical relevance and study limitations, and finally, conclusions.

Technical considerations

The oxylipin profiling method reported here is robust, accurate, quantitative, and rapid, all features that allow for rapid transition as a tool to assess the role that oxylipins contribute to overall RT inflammatory processes in patients with CF (and other lung diseases in which airway secretions can be collected). Additional analytes can be added to the basic method as standards become available and knowledge of the role of lipid mediators in pulmonary function expands. A meticulous assessment of extraction recovery of the oxylipins in our protocol, as assessed using a diverse array of deuterated standards, provides confidence in quantitative metabolomic profiling of the measured oxylipins. The current profile of oxylipins detectable by our analytical method exceeds 88. Of these 88, 31 were detected in sputum from adult CF patients. Coupled with multivariate statistical analyses, the current metabolomics method provides a more accurate and comprehensive approach that better assesses lung inflammation and function, compared to using a single biomarker. The oxylipin profiles presented herein by multivariate data analysis method (PLS in this study) were able to reflect lung function very well and differentiate individual patient status (Fig. 5). Whereas our current results suggest the capacity of oxylipin profiling in sputum to assess CF lung health, future studies with a larger, more diverse and detailed patient cohort will be necessary to further validate the overall translational value of this approach for the CF patient.

Table 2 lists the values of selected oxylipins reported by other investigators [65–67]. These investigators used radioimmunoassay (RIA) and ELISA methodologies for their determination. As shown in Table 2, it is readily apparent that the values from our current study are 10–100 times lower than the values obtained using RIA and ELISA. Several possible reasons could contribute to the disparate results of this study compared to the previous reports. For one, the sample groups are very different. The age ranges from the previous studies are of pediatric populations, whereas our study interrogated an older adult cohort with an undoubtedly more advanced disease and a more chronic and intense inflammatory RT milieu. Another possible reason comes from the different natures of these three measurement methods. Both RIA and ELISA are based on the recognition of the metabolites by antibodies. Therefore, the resulting concentrations may represent an overestimation due to detection of multiple highly similar metabolites. For example, the LTB4 concentration quantified by ELISA could account for LTB4 and its bioactive metabolites such as 20-OH LTB4 and 20-COOH LTB4. The advantage of the present LC/MS/MS methodology used in this study is that it is more highly selective.

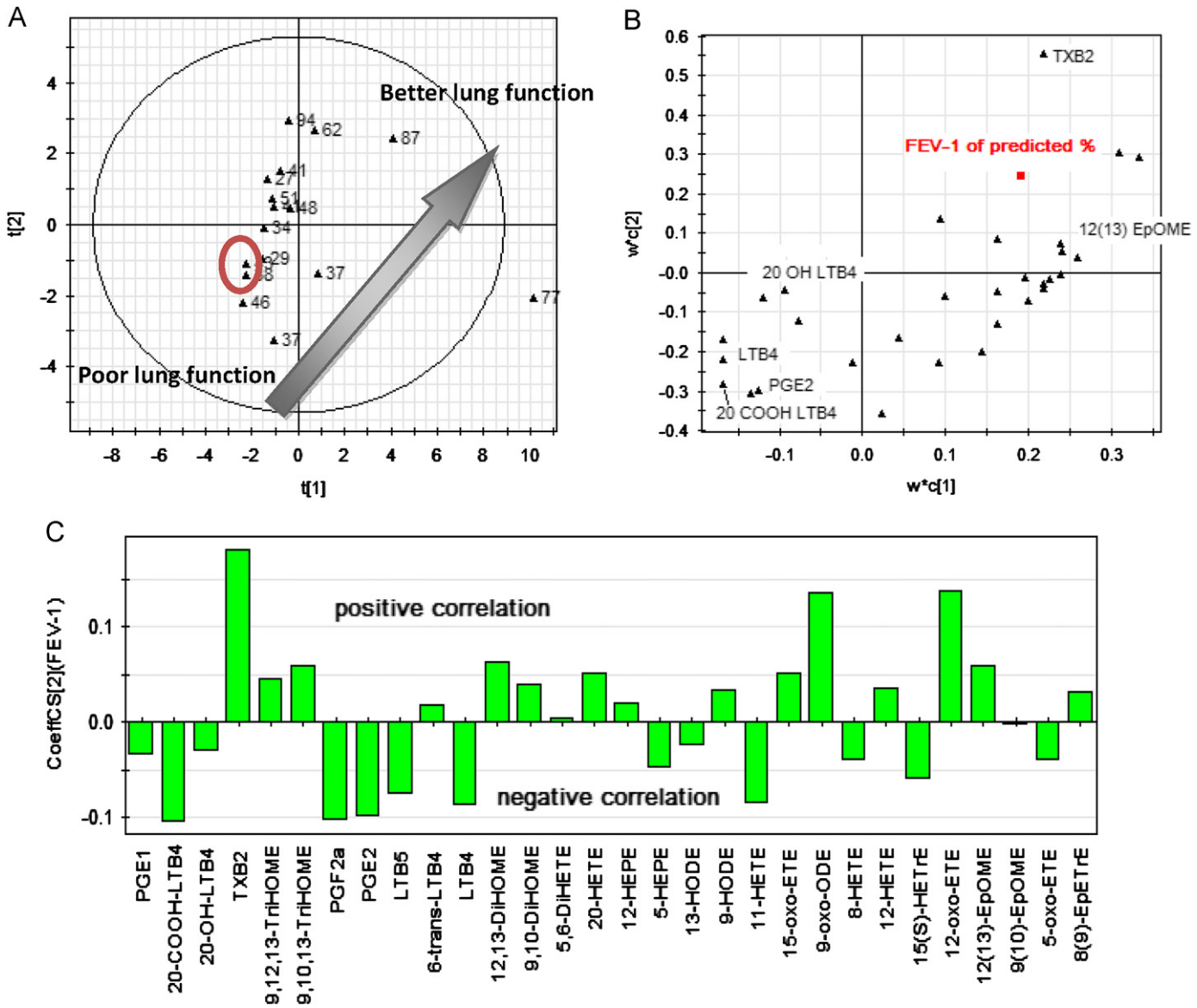

Fig. 5. Based on the cystic fibrosis patients' sputum oxylipin profiles, (A) the score plot of the PLS (partial least squares) analysis shows the clear trend matching the lung function–FEV-1 % of predicted; (B) the loading plot of the PLS analysis indicates the important metabolites responding to the aggregation; (C) the coefficient plot of PLS indicates the positive/negative relationship between the analytes and the lung function–FEV-1 % of predicted. As a powerful multivariate analysis method, PLS can be seen as a particular regression technique for modeling the association between the factors (oxylipin concentrations) of the observations (cystic fibrosis patients here) and the responses (lung function) of the observations. (A) The score plot. Each point corresponds to a patient's sample. The label is the value of the FEV-1. (B) The loading plot. Each point in the plot corresponds to a variable (oxylipin or the FEV-1). Shorter distance means similar contribution to the score plot distribution. (C) The coefficiency plot summarizes the relationship between the analytes and the lung function. These are directly analogous to, but not identical to, coefficients obtained from multiple regression.

Table 2
Comparison of our oxylipin profiling method with that quantified by RIA and ELISA methods.

	PGE2	PGF2α	TXB2	LTB4
LC/MS/MS[a]	0.5	0.32	1.7	1.6
RIA [65]	45	10	12	20
ELISA [66]	6.6			

All values are reported as nmol/g.

[a] Our method.

Possible microbiota contribution to RT oxylipin profile

We and others have made the presumption that oxylipins detected in CF sputum arise from the host cells and tissues/lipid substrates. The presence of large numbers of bacteria (particularly *P. aeruginosa*) begs the question as to the possible involvement of microorganisms in the synthesis and metabolism of the analyzed RT oxylipins. Previous studies have revealed that *P. aeruginosa* harbor primarily shorter chain and mostly saturated fatty acids [70], thus they are not a source of the parent fatty acids (i.e., AA, LA, EPA, DHA) of the oxylipins we have detected in this study. However, recent studies have interestingly demonstrated that *P. aeruginosa* express a secreted cytotoxin (ExoU) with enzymatic phospholipase activity that is capable of liberating free unsaturated fatty acids (LA and AA) from host cells [71–74], including neutrophils [75], which are highly abundant in the CF airway. Moreover, *P. aeruginosa* express a number of fatty acid-metabolizing enzymes including dioxygenases, hydroperoxide isomerases, and arachidonate 15-lipoxygenase (contributes to the formation of 15-HETE) [76,77] that may directly contribute to the oxygenation of fatty acids in the CF airway [76,78] and contribute to the virulence and airway persistence of the organism [79]. One

report has revealed that *P. aeruginosa* may be a source of secreted inhibitors of 5-lipoxygenase [80]. Moreover, a recent study has identified an epoxide hydrolase produced by *P. aeruginosa* [81] that could potentially metabolize epoxide-based oxylipins to the corresponding diols, which compromise the functions of these anti-inflammatory oxylipins in the CF RT. Therefore, it is reasonable to presume that *P. aeruginosa* may use their own molecular machinery to modulate the profiles of oxylipins in the CF airway to benefit their own survival. Not surprisingly, we find no reports in the literature on the effects of oxylipins on the biology of *P. aeruginosa.* Future studies in this area seem warranted and may prove fruitful in further defining the mechanisms by which *P. aeruginosa* interact with the host in the CF airway.

Balance between proinflammatory and anti-inflammatory oxylipins in the CF RT

Although it seems that proinflammatory oxylipins predominate in the CF RT and have garnered the attention of most investigators, considerable interest has recently been placed on identifying, quantifying, and establishing the functions of anti-inflammatory oxylipins [36,82]. Examples of classes of such anti-inflammatory lipid mediators include resolvins, protectins, EpETrEs and lipoxins, three of which have been quantified in this study. Resolvin E1 is an anti-inflammatory and proresolving mediator derived from ω-3 fatty acids. Herein, we observed that patients with detectable sputum levels of resolvin E1 display better lung function than those patients who have no detectable levels of this anti-inflammatory mediator. Although our data are reflective of a rather small cohort, they suggest that increasing the production of resolvin E1 in the airways, either by providing its parent fatty acid (EPA) in the diet or by directly instilling resolvin E1 into the lung as an aerosol, may prove beneficial to the CF patient. Of interest, resolvin E1 administration has already recently been shown to ameliorate toxic and septic RT inflammation and to improve survival in a mouse sepsis model [83,84] and to counteract some of the proinflammatory circuits mediated by LTB4 [84]. However, it should also be recognized that ω-3 fatty acids (e.g., DHA and EPA) may affect antimicrobial resistance via their modulation of host immune responses [85].

Another class of anti-inflammatory lipid mediators quantified is represented by the epoxy fatty acids, particularly EpETrEs. Enhancing EpETrE levels in the CF airway via preventing their metabolism to diols (DiHETrE) by inhibitors of soluble epoxide hydrolase (sEH) may provide a particularly novel and perhaps effective therapeutic approach for treating CF. Also, EpETrEs or the corresponding ω-3 fatty acid epoxides could also conceivably be given by inhalation. It is also worth pointing out that *P. aeruginosa* have recently been shown to express an enzyme with sEH activity, again suggesting that *P. aeruginosa* may play an active role in oxylipin metabolism [81]. One study has suggested that there is a seemingly pathophysiologically important defect in lipoxin A4 (LXA4)-mediated anti-inflammatory activity in the CF lung [86]. However, another study using a larger and more heterogeneous CF population failed to show defects in LXA4 in CF RT secretions compared to a group of subjects with chronic bronchitis [87]. Because our current LC/MS/MS technique was less sensitive than the mentioned studies that used an ELISA for quantitating LXA4, we were unable to detect LXA4 in any of the CF patient sputa analyzed. Thus, our observations may not allow us to comment on the possibility that modulation of LXA4 synthesis could be therapeutically useful for maintenance care of CF patients. Taken together, it seems clear that the balance of proinflammatory and anti-inflammatory lipid mediators may be an important consideration in inflammatory lung diseases, including CF, and that strategies aimed to promote the synthesis or persistence of anti-inflammatory lipid mediators should be further investigated [36].

Clinical relevance and study limitations

The clinical implications of this study are multifaceted. Aggressive inflammatory processes represent a dominant feature of the RT deterioration in CF [2] and thus have been viewed as a desirable target for therapeutic intervention [5,6,9,12]. Bioactive oxylipins, including metabolites of arachidonic and linoleic acids, represent known contributors to pulmonary functions [36,37,50]. Yet, to date (including this study), only partial characterization of oxylipins in the available CF airway secretions is available. Interestingly, these profiles have perhaps been better profiled in blood and urine samples and in bronchoalveolar fluids [42,88], compared to the most relevant RT inflammatory milieu, bathing airway epithelial cells.

The present studies demonstrate that oxylipin profiles can be potentially used to evaluate the effects of interventions with both nutritional and pharmacological strategies. In addition, these oxylipin profiles can also provide a predictive basis for designing effective interventions. For example, dietary 20:5 and 22:6 lipid administration [54,89–91], antioxidant micronutrients [92], and statins [93,94] are known to affect these pathways, along with a multitude of pharmaceuticals, yet their overall impact on RT oxylipin bioactivities has been incompletely characterized. More rigorous quantitative techniques for direct analysis of oxylipin profiles in RT secretions should lead to further understanding of the overall CF inflammatory airway pathobiology and lead to further developments of measures to target these pathways from both diagnostic and therapeutic perspectives.

Although we define a greater spectrum of oxylipins than previously reported in the CF airway, not all oxylipins were profiled. This study focused on most, but not all, of the arachidonic acid pathways. Further studies are needed to more fully characterize the important 5-lipoxygenase cysteinyl leukotrienes, other 20:5 and 22:6 fatty acid metabolites, and oxysterols.

Because of the limited number of patients in our study, we did not attempt to further characterize the myriad of nutritional, infectious, and therapeutic factors that could potentially have affected the CF oxylipin profile beyond the associations we attempted to make with FEV-1, a physiological parameter often used as a reflection of CF disease severity [95]. Further studies are necessary to better understand such related factors as: (1) the dynamic nature of RT oxylipin metabolites; (2) the relative contributions of phagocytes, epithelial cells, and even bacteria to the oxidative metabolism of polyunsaturated fatty acids (PUFAs) in CF airways; (3) the potential modification of significant variability of diet and therapeutic drugs; and (4) oxylipin outcome contributions to CF pathobiology and disease natural history. Longitudinal studies and correlation of oxylipin profiles with other CF RT biomarkers of inflammation will be particularly insightful in this regard, as will clarification of precise cellular locations of the enzymes responsible for the generation and metabolism of oxylipins in airway CF fluids.

It is noteworthy that oxylipins are probably sensitive to the oxidative milieu, including atmospheric oxygen tension. Thus, precautions need to be taken to limit the artifacts formed by non-enzymatically generated pathways. Although we collected freshly obtained sputum into an antioxidant cocktail, we did not use the more rigorous technique of performing all sputum processing and workup procedures in an oxygen-free environment.

On the other hand, nonenzymatic free radical-derived species usually do not have much specificity. Thus, should workup artifact oxidations have occurred to a major degree, we should have determined comparable quantities of various isomers such

as 5-HETE, 8-HETE, 12-HETE, 15-HETE, 9-HETE, and 11-HETE. Our results, which showed large differences in these isomers, probably indicate that nonenzymatic free radical mechanisms were unlikely to be a major concern in our study. An interesting fact is that airway secretions in CF in vivo interface directly with airway surfaces for long periods of time exposed to room air. Should "artifactual" generation of oxidized "oxylipins" be occurring during workup they would also be likely to be formed in vivo on the surface of respiratory tract epithelial cells.

Conclusions

Studies of CF RT oxylipins, together with their metabolic profiles, as demonstrated in this paper, create a unique set of biomarkers for further characterization, biologic impact, and exploitation. Sputum from CF patients is readily available and its constituency appears to exhibit some corrections with lung pathophysiology, including pulmonary function, the CF RT microbiology, and other biomarkers of RT and systemic inflammatory processes. The integrative impact of nutritional, antioxidant, anti-inflammatory, and therapeutic measures that modulate PUFA oxidative metabolism can now be evaluated using metabolomic oxylipin profiling. Such data should provide unique opportunities for more detailed assessment of the contribution of oxylipins to CF disease processes and rationally guide anti-inflammatory therapeutic measures in CF.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.freeradbiomed.2012.05.001.

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