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Original Article

Lavage lipidomics signatures in children with cystic fibrosis and protracted bacterial bronchitis

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ABSTRACT

Background: Balanced composition of a well-functioning pulmonary surfactant is crucial and essential for normal breathing. Here, we explored whether the composition of lipids recovered by broncho-alveolar lavage (BAL) in children with cystic fibrosis (CF) differ from children with protracted bacterial bronchitis (PBB) and controls. We wanted to differentiate, if alterations are primarily caused by the disease process or secondary due to an increased amount of cell-membrane lipids derived from inflammatory cells.

Methods: Comprehensive lipidomics profiles of BAL fluid from children diagnosed with CF, PBB and controls were generated by electrospray ionization tandem mass spectrometry analysis. BAL cell differential and numbers were examined.

Results: 55 children (37 patients with CF, 8 children with PBB and 10 controls) were included in this study. Results showed comparable total quantities of lipids in all groups. Phospholipids were the major lipid fraction and similar in all groups, whereas the fractions of cholesteryl esters were less and of free cholesterol were increased in children with CF. Among the phospholipids, patients with CF had higher proportion of the non-surfactant membrane-lipids in the classes phosphatidylethanolamine based plasmalogens (PE P), phosphatidylethanolmine (PE) and phosphatidylserine (PS), but a lower proportion of phosphatidylcholine (PC) compared to healthy controls. No such changes were identified in the BAL fluid of children diagnosed with PBB. No differences were observed for the surfactant lipids dipalmitoyl-phosphatidylcholin (PC 32:0) and phosphatidylglycerol (PG).

Conclusions: In CF patients with neutrophilic airway inflammation the lipid composition for surfactant phospholipid components were unchanged, whereas alteration in lipid profile were characteristic for those found in membranes of inflammatory cells. We suspect that the changes in CF were caused by the prolonged inflammation in contrast to a relatively short standing process in PBB.

1. Background

The alveolar spaces of the lungs are covered by a thin layer of surfactant. By mass, the majority of surfactant is composed of lipids which are mainly specific phospholipids (PL), sphingolipids (SL), cholesteryl esters (CE) and free cholesterol (FC) [1,2]. Phophatidylcholines (PC), with dipalmitoyl-phosphatidylcholine (PC 32:0) as the main species, and phosphatidylglycerol are the major contributing surfactant phospholipids [3]. Together with the surfactant specific proteins this highly active compartment is responsible for surface tension reduction allowing appropriate gas exchange and patent small airways [1,4–6]. Another less well characterized function of a preserved lipid composition is the regulation of immune functions including lymphocyte proliferation and immunoglobulin production [4,7,8]. This delicate system may be deranged by several diseases processes [1,9], which affects surfactant composition and their aggregate structure, resulting in an impaired lung function [6,9]. In particular inflammatory airway diseases with chronic eosinophilic inflammation (e.g. asthma) or neutrophilic inflammation (e.g. cystic fibrosis (CF) and protracted bacterial bronchitis (PBB)) have been shown to affect the surfactant system [5,6,10–14].

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CF is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene encoding for a chloride channel [15]. Viscous mucus and defective mucociliary clearance in the airways cause an inflammation dominated by polymorph nuclear cells [1,4,14,16–19]. The inflammation is present early in the course of CF [17,19] and can also be found in patients who have a mild lung disease progress and no symptoms of an active infection [18,20].

PBB is defined by wet cough in children of at least four weeks duration due to failure to clear a bacterial driven respiratory tract infection [21–26]. CF, primary ciliary dyskinesia, airway abnormalities, immunodeficiency or chronic aspiration as cause of the symptoms have to be ruled out. The inflammation is characterized by the presence of polymorph nuclear cells in the lower airways [25]. After prolonged course of antibiotic treatment, the symptoms commonly resolve completely [27].

Several studies have addressed the question of lipid composition of airspace samples in patients with CF [4,6,9,14,28–30], but the results are contradictory. Some studies found concentrations of total phospholipids to be within the normal range [4,14,28], whereas other showed decreased [6] or elevated levels [9]. Differences in disease state and analytical methods may offer potential explanations for these varying results. Current studies also lack disease controls which may help to elucidate whether observed differences are characteristic for the inflammatory response or specific for the disease.

To answer the question, if the distribution of different surfactant lipids were altered due to the underlying disease or a result from debris due to the inflammatory process, we therefore compared lavage lipidomic signatures of patients with CF with neutrophilic inflammation, but preserved lung function, PBB and healthy controls.

2. Methods

2.1. Patient selection

Cystic fibrosis was diagnosed by typical clinical symptoms and a pathological sweat test (chloride >60 mmol/l) and/or the presence of two disease causing mutations. PBB was diagnosed in children undergoing a bronchoscopy for diagnostic purpose after 4 or more weeks of wet cough, showing >5% polymorph mononuclear cells in the alveolar fraction of BAL fluid and after exclusion of CF, primary ciliary dyskinesia, airway abnormalities, immunodeficiency or chronic aspiration. For comparison, data from children without any pulmonary symptoms undergoing an elective operation (i.e. tooth extraction) were included.

All BAL samples were obtained from previous studies within the same time period and stored together. The CF samples were derived from patients participating in the study "broncho-alveolar lavage for the evaluation of anti-inflammatory treatment" (BEAT) [5,6,31–33]. The study population consisted of CF patients with a normal lung function and a clinically stable disease course. All patients had to be free of an acute respiratory exacerbation prior to bronchoscopy for at least 6 weeks. CF patients with anti-inflammatory medicine, allergic bronchopulmonary aspergillosis or severe organ involvements (such as advanced hepatic disease) were excluded. The samples of the patients with PBB and healthy controls were recruited from previous studies on lavage surfactant protein and lipid composition [34,35]. The lavages were centrifuged to isolate and count the cells. The supernatant was immediately frozen in aliquots and stored for later analysis.

2.2. Broncho-alveolar lavages, sample preparation and biochemical analysis

BAL was performed as described with 3 or 4 × 1 ml/kg bodyweight of warmed 0.9% NaCl [31,32] and separately analysed in a standardized manner [36]. The first fraction (bronchial compartment) was kept separate, the other fractions (alveolar compartment) were pooled. The BAL fluids were centrifuged at 200*g* for 10 min, and the supernatant was frozen for lipid analysis. Lavage samples were extracted according to Bligh and Dyer in the presence of not naturally occurring lipid species as internal standards [37]. The following lipid species were added as internal standards: PC 14:0/14:0, PC 22:0/22:0, PE 14:0/14:0, PE 20:0/20:0 (di-phytanoyl), LPC 13:0, LPC 19:0, Cer d18:1/14:0, Cer 17:0, D7-FC, CE 17:0 and CE 22:0. Crude lipid extracts were quantified by direct flow injection electrospray ionization tandem mass spectrometry (ESI-MS/MS) in positive ion mode using the analytical setup and strategy described previously [38,39]. A precursor ion of *m/z* 184 was used for phosphatidylcholine (PC), sphingomyelin (SM) [39] and lysophosphatidylcholine (LPC) [40]. Neutral loss fragments were used for the following lipid classes: Phosphatidylethanolamine (PE) 141, phosphatidylserine (PS) 185, phosphatidylglycerol (PG) 189 [41,42]. PE-based plasmalogens (PE P) were analysed according to the principles described by Zemski-Berry [43]. Sphingosine based ceramides (Cer) and hexosylceramides (HexCer) were analysed using a fragment ion of *m/z* 264 [44]. Free cholesterol (FC) and cholesteryl ester (CE) were quantified using a fragment ion of m/z 369 after selective derivatization of FC [38]. Quantification was achieved using the internal standards (IS) and calibration lines generated by standard addition of a number of naturally occurring species to a pooled lavage sample. Calibration lines were generated for the following naturally occurring species: PC 34:1, 36:2, 38:4, 40:0 and PC O-16:0/20:4; SM d18:1/16:0, 18:1, 18:0; LPC 16:0, 18:1, 18:0; PE 34:1, 36:2, 38:4, 40:6 and PE P-16:0/20:4; Cer d18:1/16:0, 18:0, 20:0, 24:1, 24:0; FC, CE 16:0,18:2,18:1,18:0. Deisotoping and data analysis for all lipid classes was performed by self-programmed Excel Macros as described previously [39].

Lipid species were annotated according to the published proposal for shorthand notation of lipid structures that are derived from mass spectrometry [45]. The analysed glycerophospholipid species were assigned based on the assumption that only fatty acids with an even number of carbon atoms are present. Sphingomyelin species were assigned based on the assumption of a sphingoid base with 2 hydroxyl groups. The total cell count was measured in a haemocytometer. The differential cell count was assessed by blinded investigators to the different disease groups from May-Grünwald-Giemsa stained cyto-prep slides.

2.3. Calculation and expression of lipid results

Results were presented as total lipids, total phospholipids, cholesteryl ester and free cholesterol. For a better comparison of the different composition of the surfactant lipids, we first analysed for every participant the initial quantities (expressed as nmol/ml) and then normalized the data (expressed as % of analysed lipid class). Species were only included in the analysis, if the species had an abundance of ≥0.5%. To guarantee quality of analysis and to eliminate a sampling error, diluted bronchoalveolar lavages with a total lipid concentration < 15 μmol/l or a phospholipid concentration < 10 μmol/l were excluded from calculations, as most analysed lipid species are below or close to the limit of detection [34]. For PCA analysis data were analysed using R 3.4 with FactoMineR [46] and factoextra [47] for analysis and ggplot2 [48] for plotting.

2.4. Ethics statement

The study was approved by the Ethics Commission at the Ludwig Maximilans University of Munich, Pettenkoferstr. 8, 80,336 München (EK15032011). Informed consent was given by all caregivers and the children gave assent for BAL sample collection [5,35].

2.5. Statistical analysis

Group comparisons were made between CF, controls and patients with PBB. As the data were not normally distributed, the groups were compared by one-way ANOVA (Kruskal-Wallis-Test). For analysis

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Table 1
Amount and cellular components of recovered BAL.

	CF ($n = 37$)	Controls ($n = 10$)	PBB ($n = 8$)	ANOVA p*
Cells/ml *10^4 (1st fraction)	1300 (3–500)#	80 (46.3–205)	65 (20−130)	0.0016
Cells/ml *10^4 (Pool)	400 (61–2150)#	175 (85–210)	215 (525–150)	0.0006
Macrophages	64.2% (35.0–80.4)	83% (76–94)%	41.9 (17.3–53.7)	0.0073
Lymphocytes	6.0% (1.2–10.1)	12% (6–20.2)	11.1% (8.0–14.6)	0.0332
PMN	23.0% (13.0–34.8)	3.4% (0.6–4.2)$	49.5% (24–69)	0.0001
Eosinophil granulocytes	0.2% (0.0–1.0)	0.15% (0–0.55)	1.4% (0.2–6.5)	0.1277

Aberrations: BAL = bronchoalveolar lavage, ml = millilitre, PMN = polymorphonuclear neutrophil. Data are given as median (25. – 75. percentile). [n] is the number of patients with available information.
For the cell-differentiation of the alveolar compartment the result of the pooled fractions are given.
* The groups were compared by one-way ANOVA (Kruskal-Wallis-Test). For FDR correction at a global significance level of 0.05, the new significance cut-off considering multiple comparisons was 0.0237. If p showed significant differences, Dunn's post-hoc analysis was done. Significant difference were found for.
# CF different from controls ($p < .05$) and PBB ($p < .01$).
% Controls different from PBB ($p < .01$).
$ Controls different from CF ($p < .05$) and PBB ($p < .01$).

within the groups we calculated results with the non-parametric Mann-Whiney *U* test. We accounted for multiple testing by false discovery rate (FDR) correction using the Hochberg-Benjamini procedure for *p*-values from 129 tests. For FDR correction at a global significance level of 0.05, the new significance cut-off was 0.0237. If the results showed significant differences the groups were compared separately with Dunn's post-hoc analysis. Data in the tables are expressed as means and 25–75 percentiles. Correlation coefficients were determined according to Spearman. A *p*-value of <0.05 was considered as being significant. All analyses were done with Prism 7 software (San Diego, CA, USA).

3. Results

3.1. Characteristics of the children included

Of the 55 included children 37 patients had CF (median age 10.0 years, 25–75 percentile: 9.4–14.8; 54% male), 8 patients were diagnosed with PBB (median age 2.5 years, 25–75 percentile: 1.1–7.4; 63% male) and 10 were healthy (median age 6.9 years, 25–75 percentile: 2.7–11.0; 80% male). Patients with PBB were younger than patients with CF ($p = .0011$). Body-mass-index (BMI) was comparable between CF (median 16.3 kg/m^2; 25–75 percentile: 15.0–19.75), PBB (median 16.4 kg/m^2; 25–75 percentile: 15.0–19.75 (16.0–17.9) and healthy (median 16.7 kg/m^2; 25–75 percentile: 14.2–21.6). 14% of the patients with CF had a positive culture for *Pseudomonas aeruginosa* and 57% for *Staphylococcus aureus*. Median FEV1 for patients diagnosed with CF was 99.0% (25–75 percentile, 81–110%) of predicted [49]. The individual data is listed in Supplementary Table 2.

3.2. Amount and cellular components of BAL recovered

Patients with CF had increased total cells in both the bronchial and alveolar fraction compared to patients with PBB ($p = .0084$ for the bronchial and $p = .0476$ for the alveolar fraction) and controls ($p = .0324$ for the bronchial and $p = .0014$ for the alveolar fraction) (Table 1). Controls had fewer polymorph mononuclear cells than patients both with CF ($p = .028$) and patients with PBB ($p = .0002$) while no differences existed between PBB and CF. The fraction of macrophages in healthy controls was higher than in patients with PBB ($p = .0057$). No differences between the groups were found for eosinophils. The individual data is listed in Supplementary Table 2.

3.3. Lavage lipidomics

Results of analysed BAL lipidomics profiles of children with CF, PBB and controls are shown in Table 2 (expressed as % of analysed lipid class). Individual results (expressed as % of analysed lipid class and nmol/ml) are listed in Supplementary Tables 3 and 4. Whereas the total quantity of lipids and percentage of phospholipids in all groups were comparable, patients with CF had a higher fraction of free cholesterol than controls ($p = .0026$), but a lower fraction of cholesteryl esters than controls ($p = .0225$) and patients with PBB ($p = .0075$).

Table 2
Concentrations of all lipids, lipid classes, and species of phospholipids and sphingolipids.

	CF ($n = 37$)	Controls (n = 10)	PBB (n = 8)	ANOVA p *
All lipids analysed [nmol/ml]	52.9 (32.5–100.7)	34.9 (27–53)	52.49 (42–102)	0.2565
PL and SL [% of total lipids]	83.6 (77.8–85.4)	84.6 (83.2–88.4)	85.7 (80.2–86.6)	0.0471
CE [% of total lipids]	1.3 (1.0–1.6)#	1.8 (1.4–3.5)	2.1 (1.6–2.6)	0.0026
FC [% of total lipids]	16.0 (13.6–19.6)§	12.3 (10.0–14.6)	12.7 (11.5–17.4)	0.0018
PC [% of all PL and SL]	76.3 (67.6–78.3)#	86.0 (82.4–87.7)	84.9 (80.0–86.5)	<0.0001
PC (32:0) [% of all PC]	44.6 (42.8–46.7)	45.6 (43.1–47.4)	42.2 (35.9–44.8)	0.1610
SM [% of all PL and SL]	1.7 (1.3–2.8)	1.6 (0.9–2.5)	1.8 (1.4–2.8)	0.6687
PE [% of all PL and SL]	2.8 (2.5–3.0)§	2.2 (2.0–2.4)	2.6 (2.4–2.7)	<0.0001
PE P [% of all PL and SL]	9.5 (8.3–15.2)§	2.3 (1.6–3-3)	2.7 (2.5–4.3)	<0.0001
PS [% of all PL and SL]	3.8 (3.3–5.2)	3.3 (2.6–3.6)	3.4 (2.9–4.4)	0.0237
PG [% of all PL and SL]	3.7 (2.8–4.0)	3.8 (3.3–4.2)	3.5 (3.2–4.0)	0.5252
LPC [% of all PL and SL]	1.7 (1.5–2.1)#	0.96 (0.71–1.3)	0.98 (0.92–1.4)	<0.0001
Cer [% of all PL and SL]	0.4 (0.3–0.7)§	0.15 (0.1–0.3)	0.28 (0.21–0.41)	0.0004
HexCer[% of all PL and SL]	0.04 (0.03–0.07)§	0.02 (0.018–0.035)	0.03 (0.023–0.053)	0.0018

Aberrations: PL = Phospholipids, SL = Sphingolipids, CE = Cholesteryl esters, FC = Free cholesterol, PC = Phosphatidylcholine, SM = Sphingomyelin, PE = Phosphatidylethanolmine, PE P = Phosphatidylethanolmine based plasmalogens, PS = Phosphatidylserine, PG = Phosphatidylglycerol, LPC = Lysophasphatidylcholine, Cer = Ceramide, HexCer = Hexosylceramide. Data are given as median (25. – 75. percentile).
* The groups were compared by one-way ANOVA (Kruskal-Wallis-Test). For FDR correction at a global significance level of 0.05, the new significance cut-off considering multiple comparisons was 0.0237. If p showed significant differences, Dunn's post-hoc analysis was done. Significant difference were found for.
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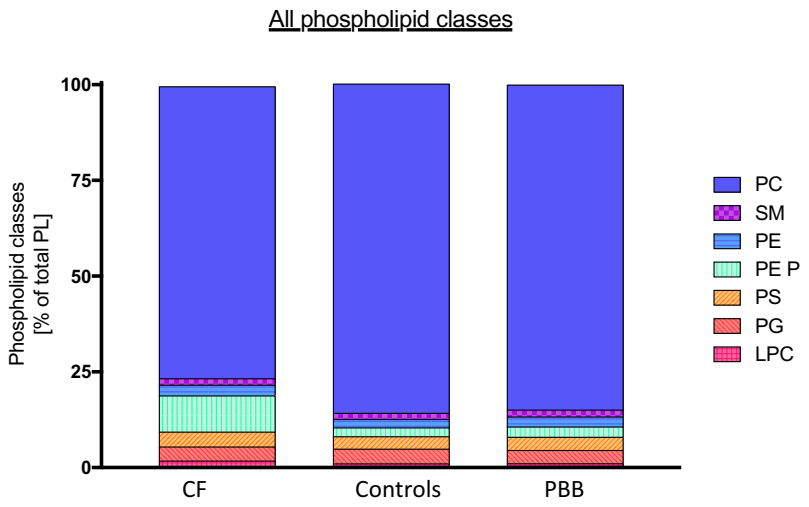

Fig. 1. Composition of the analysed phospholipid species PE, PE P, LPC in the airspace fluid recovered by broncho-alveolar lavage in patients with cystic fibrosis, protracted bacterial bronchitis and healthy controls. Data are indicated as means for the different groups.

The quantitatively largest changes were observed among the analysed phospholipid species. Patients with CF had higher percentage of the non-surfactant lipid classes PE P than both controls ($p < .0001$) and patients with PBB ($p = .002$). Similar changes were observed for PE ($p < .0001$) and PS ($p = .0307$) (Fig. 1). This was at the expense of PC, the most abundant phospholipid in all groups, which was reduced in children with CF compared to both controls ($p < .0001$) and children with PBB ($p = .0012$). No differences were found for the major surfactant phospholipid PC 32:0 which was the most abundant of the analysed lipid species of PC. Patients with CF had a decreased SM-Cer ratio (median 3.835) compared to patients with PBB (median 6.229; $p = .0040$) and healthy controls (median 9.824; $p < .0001$), whereas no differences were found between PBB and healthy controls. (See Fig. 2.)

No differences of lipid classes were found within the CF population subdivided into two distinct groups with and without a positive culture of *Pseudomonas aeruginosa*.

Higher percentage of polymorphonuclear neutrophils correlated with higher fraction of PE ($r = 0.3572$; $p = .0413$), Cer ($r = 0.3868$; $p = .0262$) HexCer ($r = 0.3599$; $p = .0397$), but a lower fraction of PG ($r = -0.3974$; $p = .0220$). Higher percentage of lymphocytes correlated with higher fractions of PC ($r = 0.3827$; $p = .0336$), but lower fractions of PE P ($r = -0.3403$; $p = .0210$) and PS ($r = -0.3752$; $p = .0379$). Higher percentage of macrophages correlated with a lower fraction of PE ($r = -3.798$; $p = .0293$). See also Supplementary Table 4.

4. Discussion

We analysed the lipid profile of BAL fluid recovered from patients diagnosed with CF and compared the results with patients with PBB and controls. Whereas the total concentration of all lipids and the fraction of phospholipids, which represented >80% of all lipids, were similar between all analysed groups, we found alterations in the composition of cholesteryl esters, free cholesterol and the species of phospholipids (PC, SM, PE, PE P, PS, PG, LPC) and sphingolipids (Cer and HexCer).

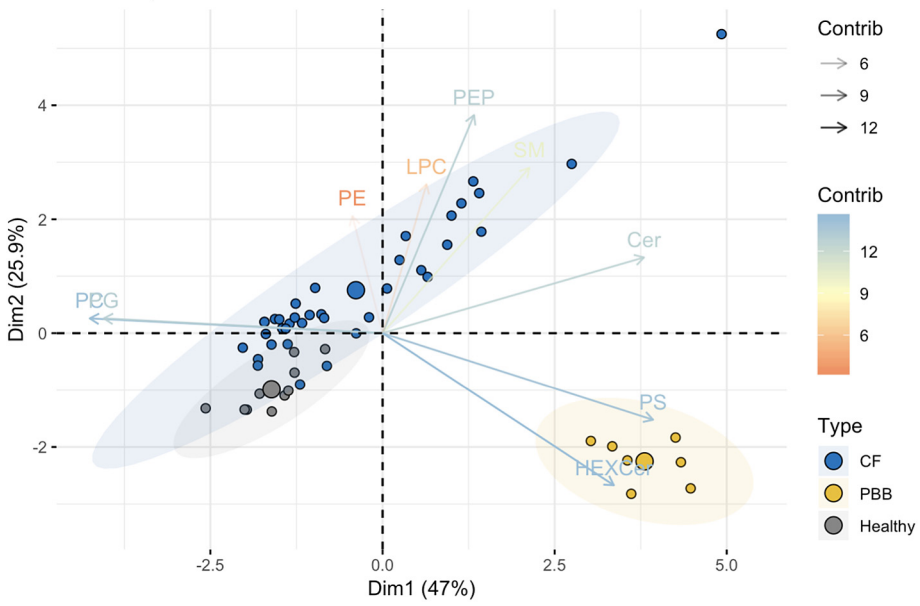

Fig. 2. Principal component analysis using dimensions 1 and 2 demonstrates separation of PBB patients by evaluation of HEXCer and PS content. Healthy donors and samples from CF patients overlap partially though CF lipid content appears separable by expression of Cer, PE P, LPC and SM. Small dots = samples, large dot = centroid of the group, ellipses = 0.05 confidence region for localisation of samples, arrows = eigenvectors contributing to localization of samples.

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A major finding was that patients diagnosed with CF had an increased proportion of FC and the analysed phospholipid species PE, PE P, LPC with corresponding decreased content of PC (Fig. 1). Such changes are consistent with the accumulation of cell membrane-derived lipids [4,9,50,51]. FC, PE, PE P, Cer and HexCer are the determinate of the membrane organization of PMN [52]. This is in line with the marked high fraction of PE P found in patients diagnosed with CF that correlates with the increased amount of PNM in the alveolar fraction of these patients [52]. We suspect that the high fraction of LPC is associated with the increased activity of Phospholipase A2 (PA 2), generating LPC from PC, found in CF patients [53].

The reduced SM-Cer ratio of patients with CF compared to patients with PBB and healthy controls supports the hypothesis of a modified activity of sphingomyelinase in patients with CF.

Also, in accordance with these findings are the deviations found in the above mentioned phospholipid species of patients with CF (Supplementary Table 1). The increased proportion of PC O-34:1, PE 36:1, PE P-16:0/ 18:1, PE P-18:0/20:4 and Cer d18:1/16:0 correspond to a high fraction of those lipids in the membrane of PMN [52]. Decreased fraction of PC 36:4 is caused by cleaved arachidonic acid generating eicosanoids as inflammatory mediators [54–56].

In line with previously published data, the fraction of the most active and surface-tension reducing surfactant component PC 32:0 and also of PG were unchanged [4,5,9,28]. Interestingly, in our cohort of CF patients we found that CE were decreased compared to Controls and PBB, and FC were increased compared to healthy controls, but not patients with PBB. CE are an effector of innate immunity, alter the susceptibility of the lungs to bacterial colonization and exert antimicrobial activity against *P. aeruginosa* [4,57]. Increased CE was found in lavages from CF children [29,58] and chronic smoker [59], but normal CE and FC levels in BAL fluid from children with chronic bronchitis [57]. Increased CE in BAL fluid is linked to elevated levels of CE in plasma of chronically infected CF patients [60] and reflects the inflammatory process [58]. Nonetheless, elevated FC represents a distinct mechanism of surfactant dysfunction [1,8,34,61] and may lead to impairment in surface activity and contribute to surfactant dysfunction [30]. We interpret decreased CE and elevated FC as signs for an apparent, but mild inflammation within our cohort of CF patients.

However, we did not find such changes in BAL fluid of children diagnosed with PBB. This is likely due to the fact, that PBB is a relatively short standing process of only several weeks, compared to the chronic inflammatory infiltrates of CF airspaces from early infancy onward [17–19]. This notation is also supported by the higher absolute cell count in the patients with CF (see Table 1).

In our point of view there are basically two possible explanations for our findings. In CF changes are caused by (1) the prolonged chronic inflammation or (2) the neutrophilic inflammation is not the same in CF and PBB. We suspect the first conclusion to be more likely. We suppose that the higher proportion of cell membrane lipids is caused by the higher amount of cells, in particular the increased amount of PMN. And thus, the higher fractions of PMN correlates with a higher fraction of cell-membrane lipids derived from these cells. Such processes may only play a transient, but major role in children with PBB, where abnormal neutrophilic airway inflammation may be reversed with antibiotic treatment [62].

A limitation of this study is that we did not analyze the surfactant lipid phosphatidylinositol (PI), which accounts 1.6% of all lipids [63], the membrane lipids diphosphatidylglycerol, Cardiolipin, (CL) and phosphatidic acid. An analysis of free fatty acids would have been interesting, as this could have supported the above-mentioned hypothesis of an increased PLA2 activity in CF patients.

This study underscores the susceptibility of the lipid composition to influences from airspace inflammatory processes. Lipidomic analyses of this inflammatory process of the lower airways may help to differentiate the type and extent of inflammatory processes. With a better understanding of the underlying pathophysiological processes causing changes in the lipid composition of the airspaces may help to identify deranged processes. The increased proportion of cell-membrane derived lipids found in the composition of alveolar lipids of patients with CF support the hypothesis of a secondary effect in the CF patients caused by an inflammatory process. Such a process is less pronounced in patients diagnosed with PBB, who lack similar alterations of lipid profile.

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