ORIGINAL ARTICLE

Quantitative Lipidomics in Pulmonary Alveolar Proteinosis

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Abstract

Rationale: Pulmonary alveolar proteinosis (PAP) is characterized by filling of the alveolar spaces by lipoprotein-rich material of ill-defined composition, and is caused by molecularly different and often rare diseases that occur from birth to old age.

Objectives: To perform a quantitative lipidomic analysis of lipids and the surfactant proteins A, B, and C in lavage fluids from patients with proteinosis of different causes in comparison with healthy control subjects.

Methods: During the last two decades, we have collected BAL samples from patients with PAP due to autoantibodies against granulocyte–macrophage colony–stimulating factor; genetic mutations in CSF2RA (colony-stimulating factor 2 receptor α-subunit), MARS (methionyl aminoacyl-tRNA synthetase), FARSB (phenylalanine-tRNA synthetase, β-subunit), and NPC2 (Niemann-Pick disease type C2); and secondary to myeloid leukemia. Their lipid composition was quantified.

Measurements and Main Results: Free cholesterol was largely increased by 60-fold and cholesteryl esters were increased by 24-fold. There was an excessive, more than 130-fold increase in ceramide and other sphingolipids. In particular, the long-chain ceramides d18:1/20:0 and d18:1/24:0 were elevated and likely contributed to the proapoptotic environment observed in PAP. Cellular debris lipids such as phosphatidylethanolamine and phosphatidylserine were only moderately increased, by four- to sevenfold. The surfactant lipid class phosphatidylcholine expanded 17-fold, lysophosphatidylcholine expanded 54-fold, and the surfactant proteins A, B, and C expanded 144-, 4-, and 17-fold, respectively. These changes did not differ among the various diseases that cause PAP.

Conclusions: This insight into the alveolar lipidome may provide monitoring tools and lead to new therapeutic strategies for PAP.

Keywords: lipids; pulmonary alveolar proteinosis; BAL

Surfactant is a complex mixture of lipids and specific proteins that line the alveolar space and reduce surface tension, preventing expiratory collapse and warranting gas exchange. Whereas alveolar type II pneumocytes produce and recycle surfactant, macrophages remove surfactant from the alveolar space, maintaining a delicate homeostasis (1, 2). Conditions that result in filling of the alveolar space with abundant surfactant are summarized under the term "pulmonary alveolar proteinosis" (PAP) (3, 4). Many different diseases have been identified that result in such PAP alveolar filling syndromes. Autoimmune PAP, the condition that contributes to approximately 90% of all cases of PAP, is caused by antibodies against granulocyte–macrophage colony–stimulating factor (GM-CSF) (5, 6). Similarly, but more rarely, disruption of

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At a Glance Commentary

Scientific Knowledge on the Subject: Pulmonary alveolar proteinosis is a large group of rare diseases characterized by filling of the alveolar space with lipoprotein-rich material. Comprehensive quantitative analyses of lipid composition may lead to new therapeutic strategies to treat pulmonary alveolar proteinosis.

What This Study Adds to the Field: Here, we report large quantitative changes in lipids related to surfactant, including free cholesterol and cholesteryl esters, but only moderate changes in lipids derived from cellular debris. We noted very large increases in ceramide and other sphingolipids, contributing to a proapoptotic alveolar environment in pulmonary alveolar proteinosis. Therapies targeting such changes may be assessed by monitoring the alveolar lipidome.

alveolar macrophage GM-CSF signaling by mutations in the receptor for GM-CSF leads to PAP (7). Recently, other investigators and we have defined PAP caused by mutations in MARS (methionyl aminoacyl-tRNA synthetase) (8) or FARSB (phenylalanine-tRNA synthetase, β-subunit) (9). Alveolar macrophage surfactant metabolism can also be disturbed in patients with Niemann-Pick disease type C2 (NPC2) from mutations in NPC2 hindering egress of cholesterol from lysosomes and leading to PAP (10, 11). Lastly, disorders that affect the pool of myeloid precursor cells for alveolar macrophages, such as chronic myeloid leukemia (CML) and similar diseases (secondary CML [sec. CML]), can all result in PAP (12, 13). In all of these diseases, the exact pathomechanisms whereby the surfactant steady state is disrupted and leads to PAP remain unknown; however, the recently identified molecular causes of these diseases unambiguously allow a precise definition of the disease that causes PAP.

Data regarding the precise composition of the alveolar surfactant may give clues to the mechanisms involved in the different entities. Such knowledge is also a prerequisite for innovative pharmacological approaches. An example is the recent observation that statins may favorably influence alveolar surfactant lipid cholesterol metabolism (14). Up to now, lipid compositional analyses have focused on the surfactant-related lipids indicating total phospholipids, their class composition, and cholesterol (15–23). Lipidomic data regarding surfactant composition in proteinosis are scarce, as most of the conditions involved are rare, manifest at all ages from birth to old age, and frequently lack precise or genetic definitions.

During the last two decades, we have systematically collected BAL samples from patients with PAP. We batch analyzed the samples and have now generated deep phenotypes of their lipid composition. We expect that the data will stimulate starting points for novel therapeutic interventions.

Methods

Patient Selection

All patients included in this analysis were previously studied in other translational studies to define the molecular basis of their PAP. Fourteen patients (nine males and five females, average age 42 yr [range 32–52 yr]) were positive for GM-CSF autoantibodies and had autoimmune PAP (24), and all other patients were negative for GM-CSF autoantibodies. Three subjects (one male and two females, average age 4.5 yr [1.7–9 yr]) had GM-CSF-Ra mutations (7); 13 subjects (five males and eight females, average age 1.7 yr [range 0.3–7 yr]) had MARS mutations (8); two patients (one male and one female, average age 35 yr [6 yr and 64 yr]) had PAP sec. CML or juvenile myelomonocytic leukemia (12); one patient (male, age 9 yr) had a mutation in FARSB (8); and one patient (female, age 9 yr) had a mutation in NPC2 (11). Aliquots of lavages from 16 healthy subjects (12 males and four females, average age 19 yr [range 1–35 yr]) and 14 subjects with bronchitis (nine males and five females, average age 4.5 yr [range 0.3–16 yr]) were analyzed as described previously (25).

Ethics Statement

The study was approved by the Ethics Committee of Ludwig Maximilians University (EK 026-06). All of the caregivers gave their written informed consent, and the children gave assent.

BAL, Sample Preparation, and Biochemical Analysis

Diagnostic lavages in control subjects were performed with three to four aliquots of 1 ml/kg bodyweight. Recovered fractions 2–4, the alveolar compartment, were pooled, centrifuged at $200 \times g$ for 10 minutes, and analyzed. In patients with PAP, therapeutic whole-lung lavages were done as described previously using large volumes of saline (24, 26, 27). Lavage effluents were collected in large sterile plastic bags or canisters and mixed very thoroughly, and 60 ml aliquots were immediately frozen at −80°C. No inhibitors of lipid oxidation were added. Samples were stored at −80°C for 3 years on average (range 0.1–7.7 yr) before analysis. Long-term stability over such time periods has previously been shown for serum lipids (28), and we did not observe a correlation between storage time and changes in lysophosphatidylcholine (LPC) content (data not shown). After the samples were thawed, lavages were extracted (29) and lipids were quantified by direct flow injection electrospray ionization tandem mass spectrometry in positive ion mode at the University of Regensburg (30, 31). Briefly, after extraction in the presence of not-naturally-occurring lipid species (phosphatidylcholine [PC] 14:0/14:0, PC 22:0/22:0, phosphatidylethanolamine [PE] 14:0/14:0, PE 20:0/20:0 (diphytanoyl), LPC 13:0, LPC 19:0, ceramide [Cer] d18:1/14:0, Cer d18:1/17:0, D7–free cholesterol (FC), cholesteryl ester [CE] 17:0, and CE 22:0) (29) as internal standards, the precursor ion ratio of m/z 184 was used for PC, sphingomyelin (SM), (31) and LPC (32). Neutral loss fragments were used for the following lipid classes: PE 141, phosphatidylserine (PS) 185, and phosphatidylglycerol (PG) 189 (33, 34). PE-based plasmalogens (PE P) were analyzed according to the principles described by Zemski Berry and Murphy (35).

Sphingosine-based Cer and hexosylceramides (HexCer) were analyzed using a fragment ion of m/z 264 (36). FC and CE were quantified using a fragment ion of m/z 369 after selective derivatization of FC (30). Quantification was achieved using the internal standards 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 O-16:0/20:4; SM d18:1/16:0, 18:1, and

18:0; LPC 16:0, 18:1, and 18:0; PE 34:1, 36:2, 38:4, 40:6; PE P-16:0/20:4; Cer d18:1/16:0, 18:0, 20:0, 24:1, and 24:0; FC, and CE 16:0, 18:2, 18:1, and 18:0. Deisotoping and data analysis for all lipid classes were performed by self-programmed Excel Macros as described previously (31). Lipid species were annotated according to a published proposal for shorthand notation of lipid structures (37). Glycerophospholipid annotation assumed even-numbered carbon chains only. SM species annotation assumed that a sphingoid base with two hydroxyl groups was present. Levels of total protein and surfactant-specific protein A (SP-A), SP-B, and SP-C were determined as described previously (38, 39).

Calculation and Expression of Lipid Results

Data are presented as concentrations of the variable per milliliter of lavage fluid. In addition, lipids are expressed as percentages of total lipids, and the molecular lipid species are expressed as percentages of their respective classes. Species were only taken into consideration for presentation if the species had an abundance of $\geqslant$0.5%.

Statistical Analysis

All results are expressed as means, and the individual data points are given. For ease of communication, we used autoimmune PAP to express the mean fold changes over healthy control subjects for comparison (Table E1 in the online supplement; Figures 1 and E1–E4; individual values in Table E3). Cohort comparisons were made among healthy control subjects, subjects with protracted bacterial bronchitis, subjects with PAP caused by mutations in MARS, and subjects with PAP caused by serum autoantibodies against GM-CSF (autoimmune) using nonparametric, one-way ANOVA (Kruskal-Wallis test) followed by Dunn's multiple-comparisons test. We accounted for multiple testing by false discovery rate (FDR) correction using the Benjamini-Hochberg procedure. The individual *P* values of the 321 ANOVAs run were ranked in ascending order, and the Benjamini-Hochberg critical value was calculated using the spreadsheet at www.biostathandbook.com/benjaminihochberg.xls. With the FDR set at 0.05, the new critical value was 0.0237; that is, all *P* values below that were considered significant and the null hypothesis was rejected. Significant differences between healthy control subjects and the other cohorts are indicated by bars in Figures 1 and E1–E5. The results for PAP due to sec. CML or to mutations in CSF2RA (colony-stimulating factor 2 receptor α-subunit; GM-CSFRa), FARSB, or NPC2 are indicated just for comparison, and were not used for statistical calculations.

Results

The total lipid concentration in the alveolar airspaces was increased 12- to 59-fold in alveolar fluid from subjects with PAP (Figure 1A) compared with healthy subjects. Such a huge expansion of the lipid pool is a hallmark of PAP, although some individual variation within and between the different forms of PAP was noted.

There were substantial changes in all lipid classes. FC was increased 60-fold and cholesteryl esters were increased 24-fold (Figures 1F and 1D, respectively). Total phospholipids were increased 19-fold (Figure 1C). Whereas the major surfactant phospholipid class PC was increased 17-fold, others, including lyso-PC (54-fold), SM (77-fold), and Cer (132-fold), were increased substantially more (Figure 2).

In addition to the increased pool size of the lipid mass, the relative distribution of lipid classes also deviated from that observed in control subjects. This can best be assessed when expressing lipids as a percentage of total lipids or lipid classes (Figures 1E and E2). FC was increased from 10% to 20% of total lipids, whereas CEs remained unchanged at 10%. PC, which normally accounts for more than 60% of all phospholipids in lavage, dropped to 47% in autoimmune PAP (Figures 2 and E4; Table E2).

Of interest are changes in the fatty acid composition of the various lipid classes, as they may point toward disturbed underlying molecular pathways. Changes in the specific molecular species of CE in PAP were not as pronounced as in other lipid classes, i.e., by 1.1-fold (range 0.7–25). In particular, the major classes did not change much: CE 18:1 dropped from 35% to 26%, and CE 16:0 dropped from 28% to 24% (Figures 2 and E4; Table E2).

Whereas a relative increase in dipalmitoyl-PC (PC 32:0), the major surfactant phospholipid in PAP (Figure E4), suggested its unimpaired provision, a diversity of additional observations extensively characterized the pulmonary surfactant system in PAP. Increases in the relative masses of lyso-PC 16:0 pointed toward a high activity of phospholipase A2, generating this molecular species. The relative contribution of PG 32:0 to total PG increased from 2% to 8%, whereas no big changes were observed for phosphatidylinositol molecular species.

PE, PL P, and PS were only increased to a small extent and the changes in their fatty acid compositions were also not large. Whereas PE 32:0 accounted for 14% of all PE in healthy control subjects, this increased to 24% in subjects with PAP. PS 36:1 increased from 14% in control subjects to 24% in subjects with PAP.

Not only were the absolute concentrations of SM, Cer, and HexCer increased in PAP by 77-fold, 132-fold, and 140-fold, respectively, but their relative contribution to total lipids expanded by 4-fold, 10-fold, and 142-fold, respectively (Figures E1 and E2; Tables E1 and E2). On the level of molecular species, Cer d18:1/16:0, Cer d18:1/20:0, and Cer d18:1/24:1 increased by 106-fold, 152-fold, and 174-fold, respectively. These large changes were associated with a shift of Cer d18:1/16:0 from 60% in control subjects to 40% in subjects with PAP, and of Cer d18:1/24:1 from 10% to 20%.

Our comprehensive assessment of the alveolar surfactant system included a determination of the levels of total protein and SP-A, SP-B, and SP-C, which were increased by approximately 10- to 100-fold (Figure E5).

PAP can be distinguished by the underlying molecular cause; however, there were no differences in composition between autoimmune PAP and PAP due to mutations in the MARS gene. Similarly, the other rare PAP forms, although not available in sufficient number, were within this range.

Discussion

Here, we quantified broadly the PAP alveolar lipidome in relation to that of healthy control subjects. We noted huge increases in lipid pool sizes that substantially varied with the lipid classes considered and were not greatly dependent on the disease-causing molecular mechanisms of PAP. Up to now, lipid compositional analyses have focused on the surfactant-related lipids indicating total phospholipids, their class composition, and cholesterol (14–20).

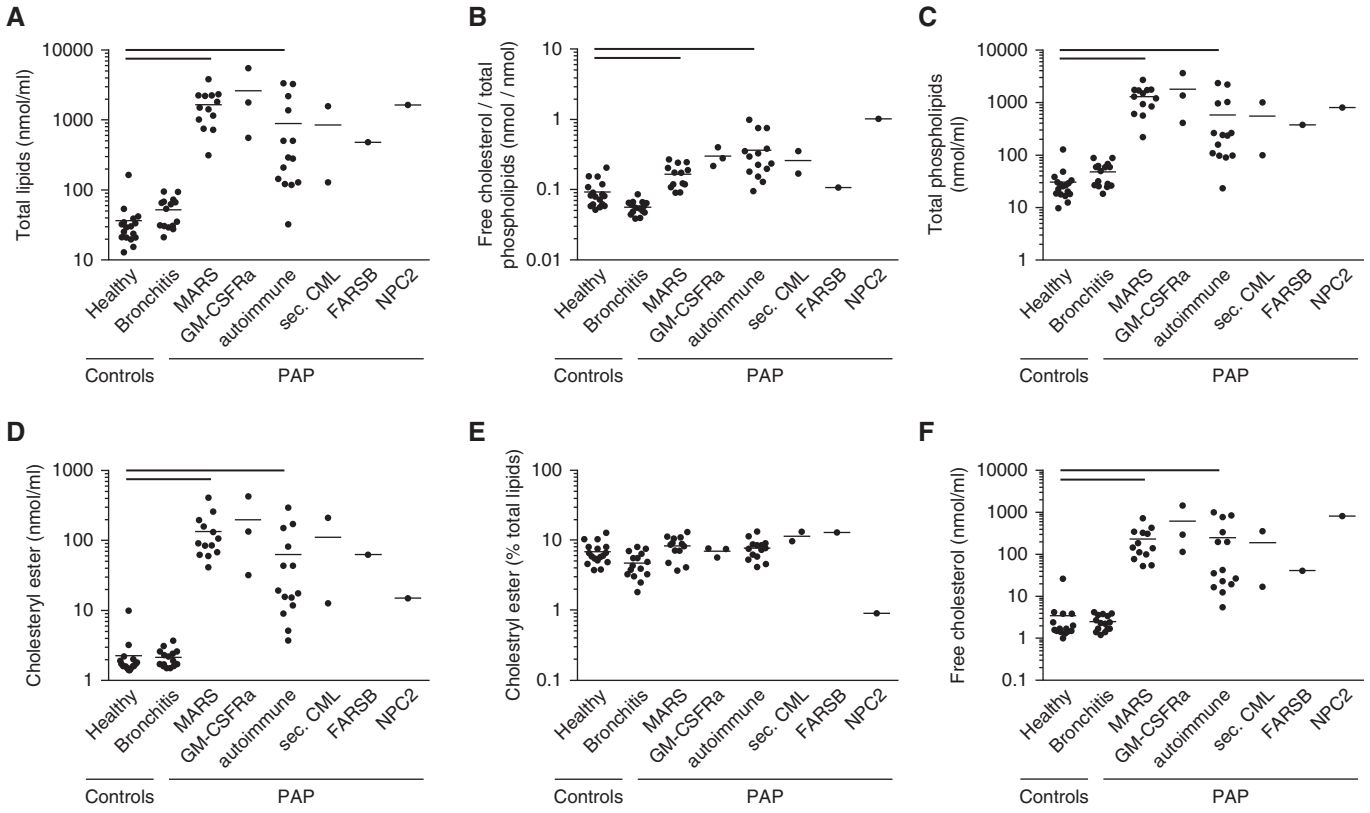

Figure 1. Concentration of lipids (*A–D* and *F*) and percentage of cholesteryl esters (*E*) in BAL recovered from patients with different forms of pulmonary alveolar proteinosis (PAP) and control subjects. PAP was caused by mutations in MARS (methionyl aminoacyl-tRNA synthetase), mutations in CSF2RA (GM-CSFRa), serum autoantibodies against granulocyte–macrophage colony–stimulating factor (GM-CSF; autoimmune), secondary to chronic myeloid leukemia (sec. CML), or mutations in FARSB (phenylalanine-tRNA synthetase, β-subunit) or NPC2 (Niemann-Pick disease type C2). As healthy controls, lavages from patients without lung disease were used, and, as disease controls, lavages from patients with protracted bacterial bronchitis were used. Individual data points and means are given. Differences among the four major cohorts (healthy control subjects, subjects with bronchitis, subjects with PAP caused by mutations in MARS, and subjects with PAP caused by serum autoantibodies against GM-CSF [autoimmune]) were calculated by nonparametric, one-way ANOVA (Kruskal-Wallis test) followed by Dunn's multiple-comparisons test. The Benjamini-Hochberg procedure was used for false discovery rate correction. Significant differences between healthy control subjects and the other cohorts are indicated by bars.

Predominantly at the expense of PC, the most abundant lipid component, the relative contribution of FC increased 2.9-fold, the proportion of cholesteryl esters was unchanged, that of cellular membrane lipids decreased, and that of the apoptosis-related SMs and Cer increased 4- to 10-fold in all forms of PAP. Such changes in the composition of the lipids point to potentially druggable pathways, including the increase in FC, which was recently targeted therapeutically by statins in autoimmune PAP and cell models of *Csf2rb* deficiency (14).

McCarthy and colleagues suggested that statin therapy may reduce the synthesis of new cholesterol by alveolar macrophages and thus lead to a reduction of their CE content via export as high-density lipoprotein into the blood (14). This hypothesis needs to be verified in clinical trials. Such a treatment approach might be independent of the molecular cause of PAP, as it does not primarily involve impaired GM-CSF–dependent accumulation of CE in lipid droplets. In line with this, in all of the investigated forms of PAP, we identified quantitatively similar changes in the BAL fluids, i.e., an increase in the ratio of cholesterol to phospholipids by 3. The large, 60-fold increase in FC mass is consistent with the notion that the removal of cholesterol is preferentially impaired, and, together with the less-than-half expansion of the CE mass compared with FC, suggests a limitation of the esterification capacity. Of interest, there were no huge changes in the CE composition. In line with an increased abundance of the surfactant typical fatty acids palmitic (16:0) and oleic acid (18:1) in the alveolar space in PAP, CE 16: 0 and CE 18:1 were the most abundant molecular species (63% of all CE in control subjects and 51% in patients with PAP). The small decrease was due to an increased relative contribution of the other CE molecular species.

A hallmark of PAP on cytology smears is the presence of large amounts of amorphous globules and finely granular material in the background along with scattered foamy alveolar macrophages, neutrophils, and a mild or missing lymphocytic or eosinophilic infiltration. Focal clusters of alveolar macrophages with foamy or vacuolated cytoplasm can be noted (40). The emergence of this characteristic cytological pattern of PAP is likely related to an alveolar environment that is dominated by strong apoptotic signals. Here we demonstrate, as indicated by the more than 130-fold increases in Cer and

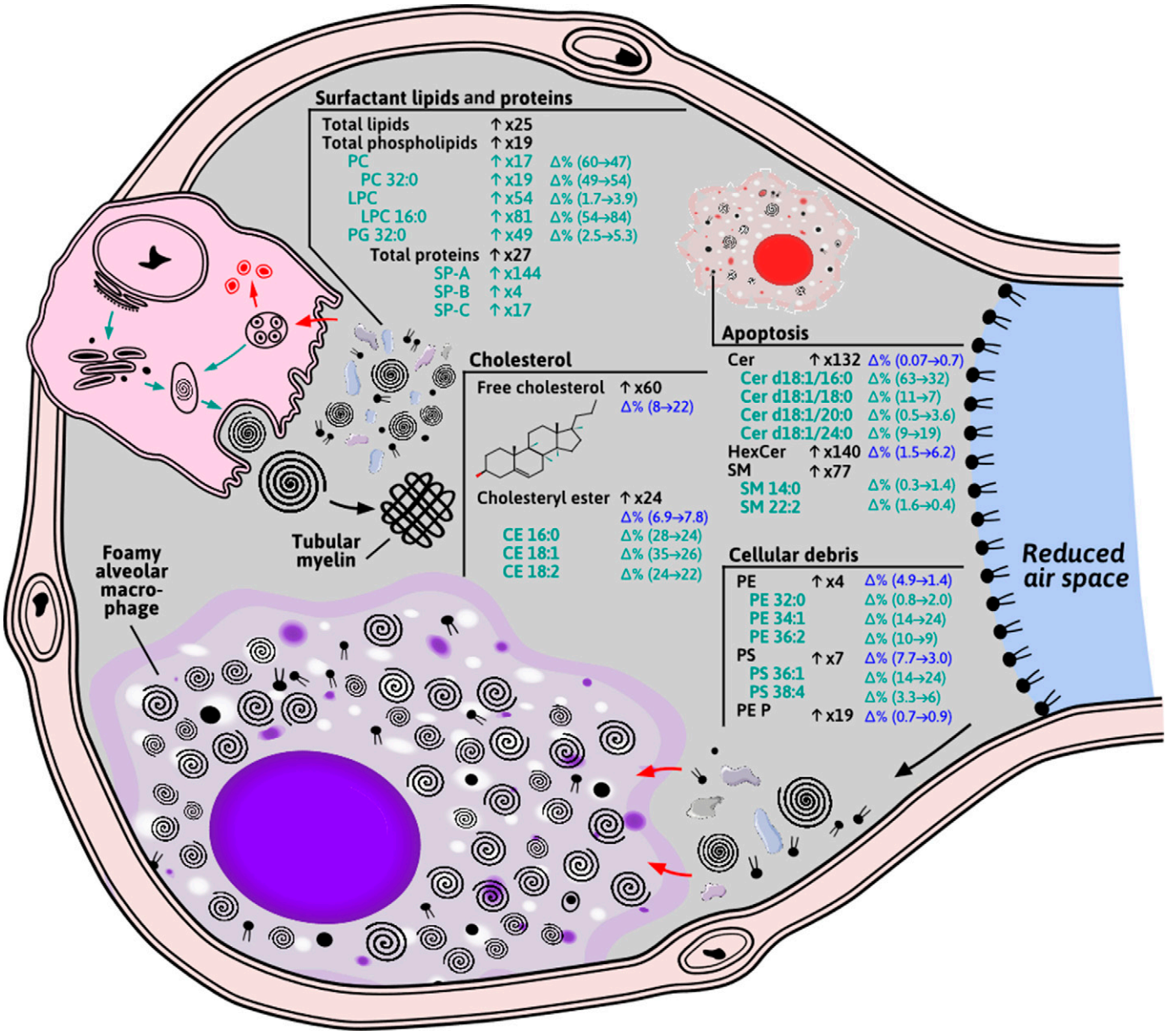

Figure 2. Schematic representation of the alveolar space in pulmonary alveolar proteinosis (PAP). Note the expanded lipid pool at the expense of the gas exchange area. The alveolar space is filled with enlarged macrophages, which are foamy from storage of ingested lipids, apoptotic cells, cellular debris, and huge amounts of cholesterol and surfactant lipids and proteins. The absolute changes of the concentrations are expressed as mean fold (x)-changes over control subjects. The level of autoimmune PAP, the most frequent form of PAP, is indicated. Changes for the other forms of PAP are listed in Table E1, and individual data and means are shown in Figures 1 and E1–E4. Changes in the relative distribution of lipid components are expressed as changes in the percentage (Δ%) within the lipid classes or for selected molecular species. CE = cholesteryl ester; Cer = ceramide; HexCer = hexosylceramide; LPC = lysophosphatidylcholine; PC = phosphatidylcholine; PE = phosphatidylethanolmine; PE P = phosphatidylethanolmine-based plasmalogens; PG = phosphatidylglycerol; PS = phosphatidylserine; SM = sphingomyelin; SP = surfactant protein.

other sphingolipids, that such an environment may exist, and hypothesize that the alveolar lipids themselves may affect their neighborhood strongly. Sphingolipids are of central importance for cellular senescence, a pseudopermanent proliferative arrest in cells by which they adapt to environmental stress (41). In this multistep process when cells stop dividing and tissue regeneration is organized, Cer is in the center of a complex network of molecules (41). Cer can self-assemble into channels, which are extensively regulated by dihydroceramides and Bcl-2 family proteins (42). Such Cer channels may initiate the intrinsic apoptotic pathway, which has been demonstrated for alveolar macrophages (43, 44).

An important regulatory factor is not merely the abundance of Cer, but their fatty acid chain length. The presence of long-chain Cers, e.g., Cer d18:1/20:0 and Cer d18:1/24:0, which were increased in their absolute and relative abundance,

reduces the membrane permeabilization potency of Cer d18:1/16:0 (42, 45). This may contribute to the modulated proapoptotic environment that we hypothesize to be present in PAP. Together with the observed accumulation of HexCer and SM, Cer-induced senescence has beneficial antifibrotic activity and may help prevent lung fibrosis, which is only very rarely seen in PAP (46–48), and foster recovery of the lungs (41). The special alveolar conditions in proteinosis are further revealed by our data, which show that the lipids that are typically derived from injured cells were increased only very moderately. PE and PS were enriched by four- to sevenfold, and the polyunsaturated PS 38:4, which is the major PS species in monocytes (49), increased by 9.4-fold and its relative contribution doubled from 3.3% in control subjects to 6% of PS in subjects with PAP. In contrast, the total amount of PE P, lipids that are involved in protecting cells as scavengers under conditions of oxidative stress and in the regulation of endo- and exocytosis (48), increased approximately 19-fold, and their relative distribution did not differ from that observed in control subjects. Although sphingolipids are attractive considering their biological properties in regulating tissue functions, their role in the pathogenesis of PAP needs to be proven in experimental and interventional studies.

Although we found large differences between healthy control subjects and patients with PAP, the different underlying diseases had very similar lipidome fingerprints. However, there are some considerations regarding the PAP sample collection investigated in this work that should be noted. First, a relatively large number of samples were available for only two diseases: autoimmune PAP and PAP due to MARS mutations. Fortunately, these two diseases had completely different molecular causes. The results obtained from the other rare PAP entities studied were clearly within the range of these two, except for NPC2, which had very low CE due to its genetic inability to esterify FC (11). Second, we used two independent control cohorts: one with normal BAL differential cell counts, and one with neutrophil-dominated airway inflammation. Reassuringly, we rarely noted any differences between these two groups, supporting the conclusion that inflammatory processes in the airway compartment contributed little to the lipid composition of the lavage material analyzed. The third consideration relates to the preanalytical processing of the material recovered in PAP, as it is a complex mixture of surfactant components, cellular debris, and other soluble and insoluble debris that cannot be separated easily (15, 21, 50). In accordance with Doyle and colleagues, who first lyophilized the fluid and then directly extracted lipids to ensure that no loss occurred, we also analyzed unfractionated lavage fluid (20). Prospective studies using freshly obtained fluid could define the composition of the different compartments in the lavage fluid.

In conclusion, in this work we not only quantitatively detailed the alveolar surfactant system with its surfactant-specific lipids and proteins, we also revealed the alveolar lipidomics in this large collection of samples from subjects with PAP. FC was identified as a major component that was enriched to more than 20% of all lipids and 60-fold by mass, making it an attractive target for therapeutic interventions. We newly defined the alveolar lipid environment as proapoptotic based on the huge accumulation of sphingolipids and in particular Cer. Lipidome analysis of pulmonary alveolar proteinosis can help investigators identify and validate new drug targets and therapies. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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